2025, Scienceline Publication
World's Veterinary Journal
World Vet J, 15(3): 678-687.
ISSN 2322-4568

DOI: https://dx.doi.org/10.54203/scil.2025.wvj68
PII: S232245682500068-15

Assessment of Active LaSota and Inactivated Newcastle Vaccines and Their Nucleotide Sequence Homology on the Virulence of Wild-type Newcastle Disease Genotype VIIi Virus

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ORIGINAL ARTICLE
Received: June 17, 2025
Revised: July 23, 2025
Accepted: August 27, 2025
Published: September 25, 2025

ABSTRACT

Many vaccines are commercially available to prevent Newcastle Disease (ND) in chickens, but the disease is still endemic in many countries. The present study aimed to evaluate the efficacy of ND vaccines *in vivo* and to analyze the nucleotide sequence similarity between the vaccines and the wild-type virus encountered in the field, as well as the underlying reasons for vaccination failures against ND in chickens. One hundred laying Isabrown hens, aged 22 weeks with an average weight of 1.75 kg, from two farms (Farm A and B) in Tabanan, Bali, Indonesia, served as experimental animals. All laying hens had previously been vaccinated four times orally with LaSota vaccines (Farm A) and alternately with LaSota and inactive ND vaccines (Farm B). They were then given a booster vaccination orally with live LaSota (Farm A) and intramuscularly with an inactive vaccine (Farm B). Sera samples were collected every week for four weeks in Farm A and for five weeks in Farm B, and the antibody titers were examined by hemagglutination inhibition (HI) test. The genotypes of the field isolates were determined through sequencing and bioinformatics analysis using MEGA 7. The mean $\log_2$ antibody titers for hens vaccinated with the LaSota vaccine were 7.2 HI units before vaccination, 7.3 HI units at one week post-vaccination, 8.1 HI units at two weeks post-vaccination, and 7.9 HI units at three weeks post-vaccination. In contrast, the mean $\log_2$ antibody titers for those vaccinated with the inactive NDV vaccine were 6.4 HI units at pre-vaccination, 7.3 HI units at one week, 5.5 HI units at two weeks, 5.0 HI units at three weeks, and 5.8 HI units at four weeks post-vaccination. The NDV-LaSota vaccine illustrated 80-81% similarity to a recent ND virus isolate from chickens in Bali (ND/chicken/GAYK01/Penebel Bali/2023), while inactive vaccines exhibited 96-98% similarity. Thus, vaccines were closely related to the Bali virus, whereas the active vaccine was less similar. Based on antibody responses and homology levels, the inactive NDV vaccine induced stronger protective immunity against NDV field isolates than the Active LaSota vaccine.

Keywords: Antibody titer, Field isolate, Neutralization, Newcastle disease vaccine, Sequencing

INTRODUCTION

Newcastle disease (ND) is a significant viral disease in poultry caused by Avian Paramyxovirus type 1, a genus within the *Avulavirus* family (ICTV, 2019; Rima et al., 2019). The Newcastle disease virus (NDV) infects the respiratory tract, digestive tract, and nervous system, causing clinical signs such as anorexia, diarrhea, respiratory and neurological disorders with high morbidity and mortality rates (Samad et al., 2022). Although vaccinations to prevent the disease in poultry have been carried out regularly, the disease remains endemic in Indonesia, resulting in significant economic losses for the poultry industry. The ND cases continue to persist in poultry, including vaccinated chickens (Kencana et al., 2018), indicating that such vaccination cannot fully protect chickens from ND. Several factors, including genetic and antigenic differences between the vaccines and field isolates, as well as improper vaccination procedures, may contribute to vaccination failure (Hu et al., 2022).

The NDV is an enveloped virus with negative-sense ssRNA consisting of six open reading frames (ORF) encoding for nucleocapsid protein (NP), phosphoprotein (P), matrix (M) protein, fusion (F) protein, hemagglutinin/neuraminidase (HN), and large (L) RNA-directed RNA polymerase (Adi et al., 2010; Zhao et al., 2018; Song et al., 2019; Nath et al., 2020). The F protein plays an essential role in the host's immunity against the virus, as it contains the neutralizing epitope of NDV (Zhao et al., 2019). Additionally, the amino acid sequence of the F protein at its cleavage site plays a crucial role in determining the pathogenicity of the virus in hosts (Kim et al., 2013; Wang et al., 2017). A vaccine with a high homology level to the virulent field NDV is likely to induce a highly protective immune response against the field virus.

To cite this paper: Kencana G.A.Y., Astawa I.N.M., Kendran A.A.S., and Pratomo A.A. (2025). Assessment of Active LaSota and Inactivated Vaccines and Their Nucleotide Sequence Homology on the Virulence of Wild-type Newcastle Disease Genotype VIIi Virus. *World Vet. J.,* 15(3): 678-687. DOI: https://dx.doi.org/10.54203/scil.2025.wvj68

Based on their virulence, NDV is classified into three pathotypes, including lentogenic, mesogenic, and velogenic NDV (Ashraf et al., 2014). Field NDV, which causes significant economic losses to breeders, is the virulent ND virus, known as very virulent Newcastle disease (VVND), resulting in high mortality rates. Currently, active NDV vaccines are generally created using low- to moderate-virulence NDV, referred to as lentogenic and mesogenic NDV, respectively. Nevertheless, the inactive NDV vaccine made from vvNDV offers a higher similarity to field vvNDV strains. Infection with NDV in chickens causes diseases with clinical signs that range from subclinical to severe clinical syndromes. Infection with lentogenic strains typically results in mild clinical signs, including sneezing and slight nasal discharge. Moderate clinical signs, such as respiratory and neurological disorders, can occur in chickens infected with mesogenic NDV. Meanwhile, velogenic NDV causes severe clinical signs with high morbidity and mortality rates (Ghiamirad et al., 2010; Samad et al., 2022).

Different vaccines are commercially available to prevent NDV infection in chickens and other avian species, with several vaccination programs. In Indonesia, active NDV vaccines prepared using low-virulent viruses, such as La Sota and B1, have been proven safe and capable of inducing humoral, cellular, and mucosal immunity in chickens. However, antigenic mismatch between the vaccine and virulent NDV is often found in the field and is believed to be the cause of outbreaks. Therefore, an inactive vaccine using homologous NDV is also prepared to improve the efficacy of the NDV vaccine in the field (Damayanti et al., 2023). The NDV vaccines can be administered as either single active or inactive vaccines, or as combined active and inactive vaccines (Kencana et al., 2016). Although different vaccination regimes have been implemented to prevent ND in poultry, cases of NDV remain prevalent among chickens in Indonesia. The presence of antibodies against NDV among unvaccinated chickens indicated that NDV is still circulating among the chicken population in the field (Kencana et al., 2018). In highly susceptible chickens, infection with this virus can cause severe disease with a high mortality rate (Dharmayanti et al., 2024). Additionally, changes in the antigenicity and pathogenicity of low virulent NDV, such as the La Sota strain, can lead to ND outbreaks (Xiao et al., 2009; Ke et al., 2010; Wibowo et al., 2017). The level of nucleotide sequence homology between neutralization epitopes and the cleavage site of the F gene, as well as between the vaccine and the field virus, is crucial for the success of vaccination in preventing outbreaks of the disease (Ke et al., 2010). Therefore, the present study aimed to determine the ability of antibodies against the vaccine to neutralize field NDV isolates and to investigate the nucleotide sequence homology between the vaccine and field isolates, and identifying the cause of NDV vaccination failure in chickens.

MATERIALS AND METHODS

Ethical approval

The present study was approved by the Animal Ethics Committees of the Faculty of Veterinary Medicine, Udayana University, Indonesia, with the Animal Ethics Approval Certificate number: B/284/UN14.2.9/PT.01.04/2023

Experimental animals

A total of 100 laying hens from two poultry farms in Tabanan regency, consisting of 50 animals from farm A and 50 animals from farm B, were used. Farm A, with a population of 5500 laying hens, was raised in an open house pen setting in an environment with 65% humidity and an average daily temperature of 28 °C. Meanwhile, Farm B, with a population of 8000 laying hens, was raised in an open house pen setting in an environment with 60% humidity and an average daily temperature of 22 °C. The Isa brown laying hens aged 22 weeks with an average body weight of 1.7 kg were obtained from PT Charoen Pokphand, Indonesia. Laying hens in Farm A were previously vaccinated four times monthly with active LaSota vaccine, whereas those in Farm B were vaccinated four times monthly with alternately with LaSota and inactive vaccines. Laying hens in farm A were then booster vaccinated once orally with active La Sota vaccine (Sanavac LS/H120, Indonesia), whereas those in farm B were booster vaccinated once intramuscularly with inactive vaccine (Medivac ND-IB, Indonesia). All laying hens from selected farms used in field trials of the active Sanavac LS/H120 and the inactive Medivac ND-IB vaccines were fed *ad libitum* with feed concentrate for laying chickens (Charoen Pokphand Indonesia). Sera samples were collected every week for four weeks in Farm A and for five weeks in Farm B. Two mL of blood was collected from each animal via brachialis vein, and sera were then collected by centrifugation at 1000xg for five minutes (Abdi et al, 2016).

Examination of the antibody against Newcastle disease virus

The antibody against NDV in serum samples was tested by hemagglutination inhibition (HI) test, following the procedure described by the OIE (2012). A serial two-fold dilution of sera samples was performed in U-bottomed 96-well microtitration plates. Each sera dilution was mixed with 4 hemagglutination (HA) units of NDV and incubated at room temperature for 60 minutes. After adding 1% chicken red blood cells in phosphate-buffered saline, the samples were incubated for 30 minutes at room temperature, and the dilution endpoint of each serum sample was determined. The antibody titer was expressed as the antilog of the highest dilution of the serum sample that completely inhibited NDV from hemagglutinating 1% red blood cells. Each serum sample was tested in triplicate.

Isolation of wild-type Newcastle disease virus

Two wild-type NDV isolates were obtained from field cases, which were isolated and identified using a method similar to that described by Alazawy and Al Ajeeli (2020). The two NDV isolates were collected from infected chickens in the area's proximity to the vaccine trials. The virus was inoculated into chicken embryonated eggs, and they were subjected to sequencing analysis to determine their genotypes at PT Sanbio Laboratories, Indonesia. The virus isolates were inoculated into 10-day-old chicken embryos and incubated for three days at 37℃. Chorioallantoic fluid was collected, and the HA test confirmed the presence of NDV.

Adaptation and titration of wild-type Newcastle disease virus in BHK-21 cells

One wild-type NDV isolate was adapted to replicate in BHK-21 cell culture. The virus (0.1 mL) at the titer of 2^7 HA units was initially inoculated into chicken embryos as described by Alazawy and Ajeeli (2020). The virus was then inoculated into BHK-21 cell culture at a multiplicity of infection of one. After three passages in BHK cells, the media were collected in aliquots and stored at -80 °C until further use. A ten-fold dilution of the virus was prepared in a 96-well tissue culture plate. Each dilution of the virus was added to five wells of BHK-21 cells, which were grown in 96-well TC plates. The presence of cytopathic effects (CPE) was observed in each well. The presence of CPE was characterized by the formation of syncytia and detachment from the surface of the flasks or plates where the cells were grown. The tissue culture infective dose ($TCID_{50}$) of the virus was determined using the method of Reed and Muench (1938).

Polymerase chain reaction and nucleotide sequencing of wild-type Newcastle disease virus isolates

Total RNA was extracted from the infected chorioallantoic fluid of chicken embryonated eggs using TRIzol LS (Thermo Fisher Scientific, USA) methods, as described by the manufacturer's instructions. The extracted RNA samples were subjected to a reverse-transcription polymerase chain reaction (RT-PCR) assay. A 5 µL RNA-extracted sample was reverse-transcribed into cDNA using random primers and amplified using primers specific to the NDV *F1* gene. Primers used for RT-PCR were forward 5′-ATCCAAGCAGGTACCCAACG-3′ and reverse 5′-AAGTCGGAGGATGTTGGCAG-3′, with an expected amplicon of 732 bp (Putri et al., 2021). The RT-PCR procedures were conducted as follows. Firstly, viral RNA was reverse-transcribed into cDNA at 50°C for 30 minutes, followed by the first denaturation at 94°C. Forty amplification cycles were performed, consisting of denaturation at 94°C for 15 seconds, annealing at 56°C for 30 seconds, and elongation at 68°C for 60 seconds. Finally, the cycles were terminated by incubation at 68°C for five minutes. The PCR products were analyzed using 1% agarose gel and stained with ethidium bromide (Putri et al., 2021).

Phylogenetic analysis

Nucleotide sequencing was performed using the PCR product isolated from the electrophoresis gel. Sequencing was performed at PT Sanbio Laboratory, Bogor, Indonesia. Based on the nucleotide sequence of the *F1* gene, phylogenetic analysis was conducted using the MEGA7 software (Kumar et al., 2016). The *F1* gene coding sequence of NDV Bali isolates was compared with the sequence of the *F1* gene of NDV isolated from other genotypes available in GenBank, as listed in Table 1.

Table 1. Newcastle disease virus isolates from the Gene Bank as references for alignment of NDV Bali-Penebel isolates

No	Accession number of ND isolates	No	Accession number of ND isolates
1.	AB465607.1|Japan/Ishii/62	21.	JX854452.1|Pheasant/MM20/Pakistan/2011
2.	AF079323.1|DB5	22.	KC906188.1|VG/GA-AVINEW
3.	AF534997.1|ZJ/2000	23.	KF026013.1|UPM-IBS/002/2011
4.	AY861659.1|SP13	24.	KF792018.1|chiken/Israel/2011/1115_818
5.	D00243.1|NDVFHN	25.	KF792019.1|chicken/KY-Israel/2013/50_826
6.	DQ195265.1|Lasota	26.	KF792020.1|parrot/Israel/2012/841_824
7.	EF464163.1|JL01	27.	KM056356.1|ndv60/Avinew
8.	EU239663.1|JL-1	28.	M24693.1|NDVFPB
9.	EU258665.1|NDV027344	29.	M24696.1|NDVFPE
10.	EU289028.1|VG/GA	30	JN872151.1_Hitchner
11.	EU330230.1|D58	31	PP695101.1_MHW-G-VII-i/19-A
12.	FJ600542.1|HN0801	32.	PP695102.1_MHW-G-VII-i/19-C
13.	FJ608340.1|MQ/Liaoning/05	33.	MT988380.1_NDV/Duck/M147/19
14.	HQ697255.1_chicken/Sukorejo/019/10	34.	MT988381.1_NDV/Duck/A74/19
15.	HQ697256.1|chicken/Makassar/003/09	35.	MZ488460.1_Broiler-Indonesia-GunungKidul-ISW19-2019
16.	HQ697257.1|strain_chicken/Gianyar/013/10	36.	MZ488462.1_Chicken-Indonesia-BandarLampung-P032001007-2020
17.	HQ697258.1|strain_chicken/Sragen/014/10	37.	MZ488463.1_Chicken-Indonesia-LampungTengah-BRS20-2020
18.	HQ697259.1|strain_chicken/Kudus/017/10	38.	MZ488464.1_Chicken-Indonesia-MuaraEnim-A031909005-2019
19.	HQ697260.1|strain_chicken/Kudus/018/10	39.	MZ488467.1_Layer-Indonesia-Yogyakarta-DOCHTN20-2020
20.	HQ697261.1|chicken/bali/020/10	40.	MZ488468.1_Peacock-Indonesia-Palembang-P032105010-2021

Cross-neutralization assay

The antibody raised against NDV vaccines was examined for neutralizing activity against wild-type NDV. Wild NDV at 100 $TCID_{50}$ was added to each 2-fold dilution of serum samples and incubated for 60 minutes at 37°C. The virus-serum mixture was inoculated into five wells of BHK-21 monolayers grown in 96-well tissue culture plates (Lu et al., 2018). The cells were then incubated at 37°C, and the presence of CPE in each well was observed. The neutralizing titers of the antibody were expressed as the protective dose (PD_{50}), calculated using the method of Reed and Muench (1938).

Statistical analysis

The data on antibody titers of chickens following booster vaccinations with LaSota and inactivated ND vaccines were presented descriptively. Phylogenetic analysis was conducted using Mega7 software.

RESULTS

Antibody titers following booster vaccinations

Prior to the present study, laying hens on Farm A had been vaccinated 4 times orally with both active La Sota, and those in Farm B were vaccinated 4 times alternately with LaSota and inactive vaccines. Following booster vaccinations once with active LaSota and inactive vaccines respectively for Farm A and Farm B, the log_2 mean antibody titers of laying hens after booster vaccinations in Farm A were 6.4 HI units at pre-booter vaccination, 7.3 HI units at the first week, 8.1 HI units at the second week, and 7.9 HI units at the third week post-vaccination. The log_2 mean antibody titers for those following booster vaccination with the inactive NDV vaccine in Farm B were 7.2 HI units at pre-vaccination, 7.3 HI units at the first week, 5.5 HI units at the second week, 5.0 HI units at the third week, and 5.8 HI units at the fourth week post-vaccination (Figure 1).

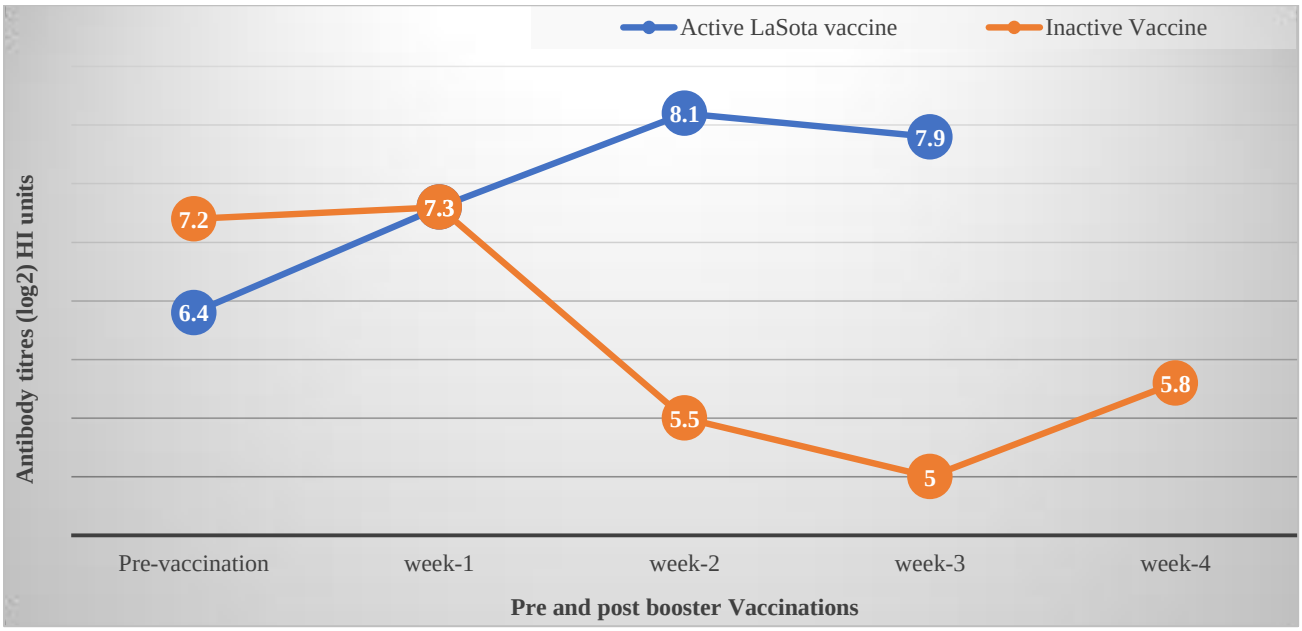

Figure 1. Serum antibody titers of Isabrown chickens following booster vaccination with active LaSota and inactive Newcastle disease virus vaccines

Adaptability of Bali NDV isolates in BHK-21 cell culture

Two Bali NDV isolates were adaptable in BHK-21 cell culture, characterized by the presence of CPE. Initially, the infected cells appeared as small syncytia, which were visible at 24 hours post-infection. At 48 hours post-infection, the syncytia enlarged, and the cells began to detach from the surface of the flask where they were grown (Figure 2).

Genotype characteristics of field wild-type NDV isolates

Using primers specific to the F1 gene, the RT-PCR assay yielded a single 732 bp amplicon in both NDV isolates, corresponding to the *F1* gene. Two NDV Bali isolates, B1 (ND/chicken/GAYK01/PenebelBali/2023 and B2 (ND/Chicken/GAYK02/Penebel Bali/2023), were subjected to RT-PCR and both resulted in 732 bp DNA bands (Figure 3).

Sequencing analysis demonstrated that two wild-type NDV of Bali isolates, B1 (ND/chicken/GAYK01/PenebelBali/2023 and B2 (ND/Chicken/GAYK02/PenebelBali/2023), were both grouped into genotype VIIi. Compared with those of genotype VII, genotype VIIi of two NDV Bali isolates has two amino acid changes, R78K (Neutralizing epitope) and R114Q (Cleavage site; Figures 4 and 5).

Sequencing analysis revealed that the identity level of the amino acid sequence for the F1 gene among field NDV Bali isolates and the inactive vaccine used in this booster vaccination was 96-98%. These identity levels were 19-20% higher compared to those among field NDV Bali isolates and LaSota, which was 80-81%, used for booster vaccination (Table 2).

Phylogenetic tree analysis indicated that two virulent wild-type NDV of Bali isolates belong to genotype VIIi. Both isolates were closely related to virulent NDV circulating among chickens in Indonesia, specifically those identified as NDV/ck/West Java/S081/2023 and NDV/ck/East Java/S102/2023 (Figure 5). Nucleotide sequence analysis indicated a homology level of active LaSota and inactive with two field ND isolates, ND/chicken/GAYK01/Penebel Bali/2023 were 80-81% and 96-98%, respectively.

Sequencing analysis demonstrated that the identity level of the amino acid sequence for the *F1* gene among field NDV Bali isolates and the inactive vaccine used in this booster vaccination was 96-98%. These identity levels were 19-20 % higher than those among the field NDV of Bali isolates and LaSota (Table 2), which was used for booster vaccination. Amino acid sequence of 112RRQKR↓F117 and 112RRRKR↓F117 at the cleavage site of the F protein showed that the NDV isolates used in the present study were velogenic NDV belonging to VIIh.

Phylogenetic tree analysis demonstrated that two virulent wild-type NDV Bali isolates belong to genotype VIIi. Both isolates were closely related to virulent NDVs circulating among chickens in Indonesia, specifically those identified as NDV/ck/West Java/S081/2023, NDV/ck/East Java/S102/2023 (Figure 6). Nucleotide sequence analysis indicated a homology level of active LaSota and inactive with two field ND isolates, ND/chicken/GAYK01/Penebel Bali/2023 were 80-81% and 96-98%, respectively.

Neutralizing activities of the antibody against the field wild-type NDV were examined using BHK-21 cell culture. Only the antibody against the active LaSota vaccine was tested against one field NDV isolate. The collected antibodies were pooled and tested using the HI test. The Geometric mean titer of antibody obtained from pooled serum was 7.4 (log_2) HI units. The neutralizing titer of the antibody obtained was 9.4 (log_2) PD_{50}. The evidence for the presence of an escape mutant was observed in some wells at dilutions of 6 and 7 (log_2), showing the presence of CPE (Figure 1), which was not expected to occur in the neutralization assay (Table 3).

The antibody against field wild-type NDV was examined using BHK-21 cell culture. Only the antibody against the active LaSota vaccine was tested against one field NDV isolate. The collected antibodies were pooled and tested using the HI test. The Geometric mean titer of antibody obtained from pooled serum was 7.4 (log_2) HI units. The neutralizing titer of the antibody obtained was 9.4 (log_2) PD_{50}. Evidence of an escape mutant was observed, as some wells at dilutions of 6 and 7 (log_2) showed the presence of CPE (Figure 6), which was not expected to occur in the neutralization assay (Table 3). The presence of such an escape mutant requires further investigation.

Table 2. Amino Acid and nucleotide sequence homology of *F1* gene between Newcastle disease virus of Genotype VII and Genotype II

	Amino Acid sequence				**Nucleotide sequence**			
	Seed GVIIi	Seed GVIIh	Seed GII	Bali	Seed GVIIi	Seed GVIIh	Seed GII	Bali
Bali	96-98	89-90	80-81	97-100	97-99	87	79-80	97-100

Table 3. Schematic representation of neutralization assay at various dilutions of serum against LaSota vaccine and field Newcastle disease virus isolate in BHK-21 cells

Assay replicates / **Serum dilution**	**1**	**2**	**3**	**4**	**5**
2^{-6}	-	+	-	+	-
2^{-7}	-	-	-	-	+
2^{-8}	-	-	-	+	-
2^{-9}	-	-	+	+	-
2^{-10}	+	+	+	+	+
2^{-11}	+	+	+	+	+
2^{-12}	+	+	+	+	+
Uninfected BHK-21	-	-	-	-	-

Legend (-): No cytopathic effect was observed; (+): Cytopathic effect was observed

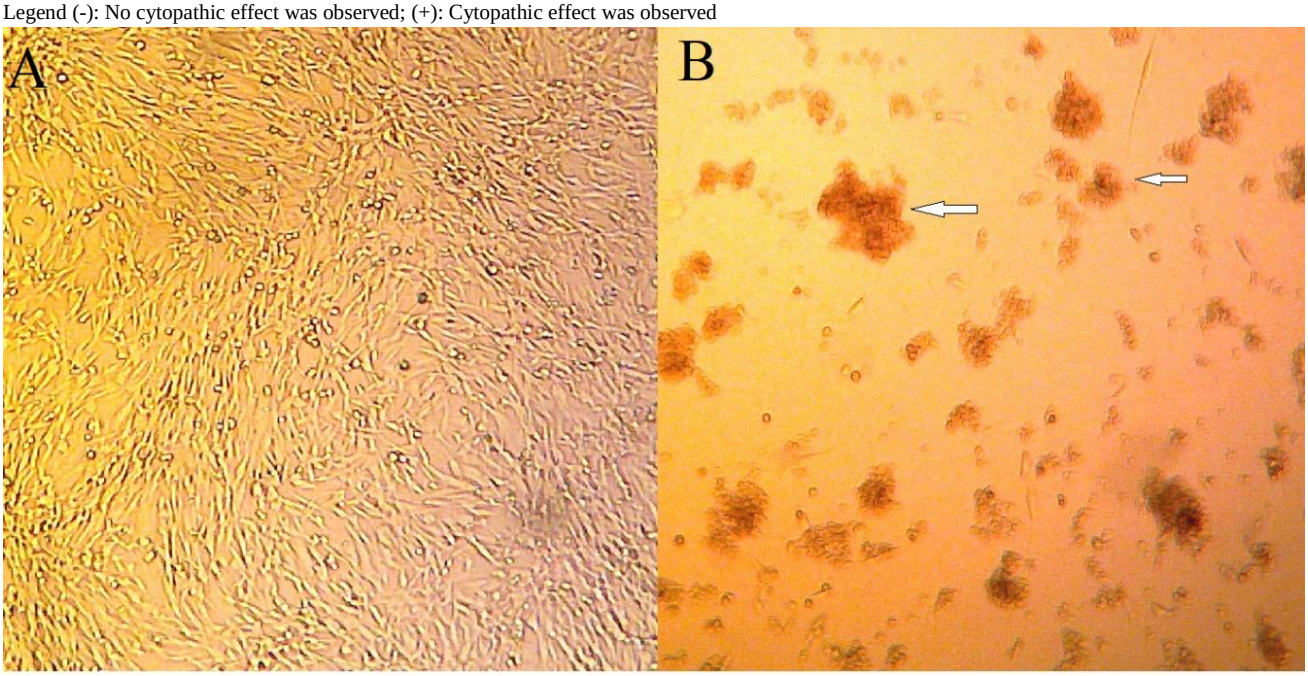

Figure 2. Cytopathic effect induced by Newcastle disease virus in BHK-21 cells. No cytopathic effect was observed in normal uninfected cells (**A**). Syncytium and detachment of cells were observed in the infected cells (**B**)

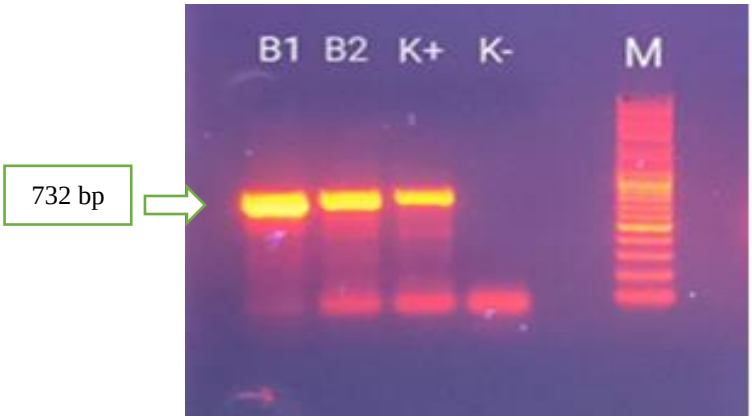

Figure 3. Profiles of F1 amplicons of virulent Newcastle disease virus isolates analyzed by polymerase chain reaction assay. Lanes **B1**: ND/chicken/GAYK01/PenebelBali/2023, **B2**: ND/Chicken/GAYK02/PenebelBali/2023, **K+**: Positive control, **K-**: Negative control, **M**: Markers

Figure 4. Mutation sites of Newcastle disease virus at K78R (neutralizing epitope) and Q114R (cleavage site) as markers of virulent Newcastle disease virus. The mutations were determined based on alignment and comparison of Genotype II with those of Genotype VII.

	58	89	91	113	138	189	195	228	239	259	278	287	307	308	309	401	411
ND/WS/G7H	S	R	V	T	P	R	I	F	M	A	I	R	S	T	S	G	R	I
ND/WS/G7I	P	.	I	V	L	W	.	Y	L	T	V	K	A	A	P	R	Q	V
NDV/Chicken/GAYK01/PenebelBali/2023	P	.	I	V	L	W	.	Y	L	T	V	K	A	.	.	R	Q	V
NDV/Chicken/GAYK02/PenebelBali/2023	P	K	T	V	L	W	V	Y	L	T	V	K	A	.	.	R	Q	V

Figure 5. Amino acid polymorphisms at the neutralizing epitope and cleavage sites of the F gene among Newcastle disease virus of genotype VII.

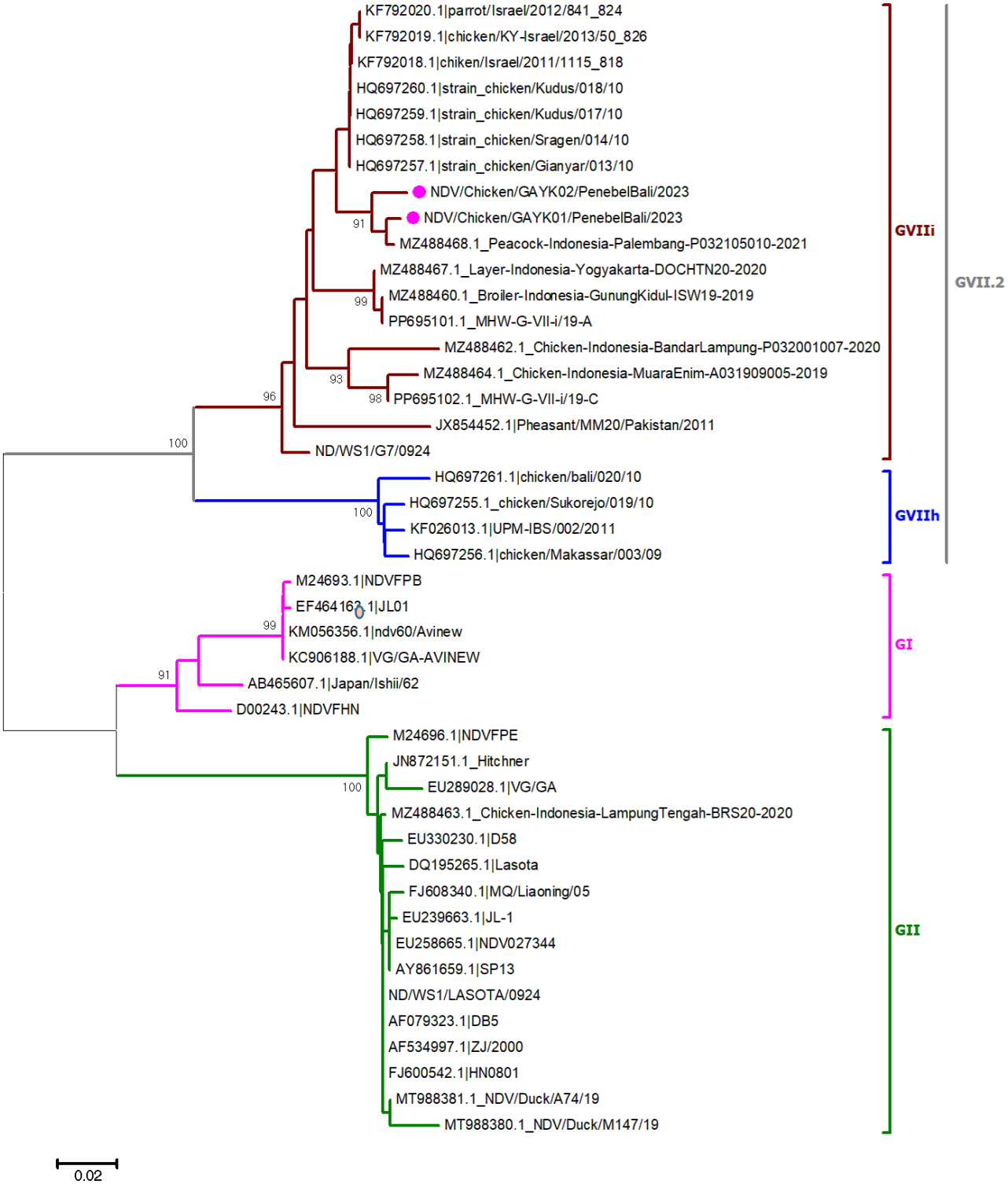

Figure 6. Phylogenetic relationships of the F1 gene of two virulent Newcastle disease virus Bali isolates with those previously published GenBank sequences for reference. The virulent Newcastle disease virus isolates used in this study were marked with a purple circle (●).

DISCUSSION

Booster vaccination in Layer chickens with active LaSota vaccine appeared to induce a higher antibody response as compared to inactive vaccine (Figure 1). Live NDV vaccines, such as LaSota, are prepared using a virus strain with low virulence (lentogenic) and have been widely used for chicken vaccination (Li et al., 2020). However, compared to the inactive vaccine prepared with local virulent NDV, it was expected to exhibit a higher level of homology to the field wild-type NDV. Therefore, it was anticipated that the antibody response against the inactive NDV vaccine would provide better protection of chickens against NDV infection in the field. Nucleotide and amino acid sequences were then analyzed to determine the genetic distance of the vaccine strain and field wild-type NDV isolates.

To explore the homology levels of both active LaSota and inactive vaccine against field NDV isolates, a portion of the *F1* gene, approximately 732 bps long, from wild-type NDV Bali isolates, B1 (ND/chicken/GAYK01/PenebelBali/2023) and B2 (ND/Chicken/GAYK02/Penebel/Bali/2023), was sequenced. As expected, compared to the LaSota vaccine, the *F1* gene of the inactive vaccine used in the present study exhibited higher homology with field wild-type NDV isolates. The F1 region is a part of the *F* gene that includes the neutralizing epitope K78R and the cleavage site Q114R (Liu et al., 2008; Samal et al., 2011). These elements are vital for the host's immune response to NDV infection. Changes in amino acids within this neutralizing epitope can result in a new escape mutant variant, enabling the virus to evade the neutralizing activities of antibodies induced by vaccines. Additionally, changes in the amino acid sequence at the cleavage site of the F protein can alter the pathogenicity of the virus, as it serves as a determinant of virulence and membrane fusion activity. As shown in Figure 3, a change in amino acid sequence Q114R was found at the cleavage site, which can reduce the replication ability and pathogenicity of the virus (Samal et al., 2011). Kim et al. (2013) reported that while the current NDV vaccines remain effective in preventing the disease, they are not entirely protective against infection, as the vaccines are made from viruses with different genotypes than those of field wild-type NDV. Only vaccines prepared using closely related genotypes will be able to prevent infection and effectively stop the spread of the virus. The use of a chimeric LaSota virus, which involves replacing its *F* and *H*/*N* genes with those from genotype VII, has been shown to effectively prevent NDV infection (Qiao et al., 2021).

The role of the F protein in immunity and protection of the host against NDV infection has been reported (Umali et al., 2013). A LaSota vaccine containing F protein cleavage sites (FPCS) of APMV-8 (Genotype VII) was shown to induce high neutralizing antibodies and protect chickens from challenge with wild-type genotype VII. The cleavage site of the F protein of NDV is vital in vaccine preparation, and the vaccine containing FPCS of NDV genotype VII is expected to be more effective in preventing NDV infection in chickens. As the two wild-type NDVs of Bali isolates used in the present study were grouped into genotype VIIi (Figure 4) and exhibited higher homology levels to the inactive vaccine than to the active LaSota vaccine, the inactive NDV vaccine was expected to induce a better protective immune response.

In Indonesia, it was found that the genetic distances among circulating NDV isolates, based on their *F* gene, have been reported to range from 0.4 to 9.6%, with a homology level of 90.4-99.5% (Wibowo, 2017). All virulent NDV strains have the F protein cleavage site motif of R-R-Q-R/K-R-F and are generally associated with high pathogenicity indexes, ranging from 1.05 to 1.87 in day-old chicks (Bogoyavlenskiy et al., 2009).

The antibody raised against LaSota was shown to be able to neutralize the wild-type NDV isolates belonging to serotype VII. This demonstrated that the antibody against LaSota remained capable of inducing protective immunity against the field NDV isolate. In Indonesia, virulent NDV typically belongs to genotypes VIIh and VIIi. The primary differences in the *F1* gene among VII (VIIh and VIIi) and genotype II (LaSota) occur at the neutralizing epitope K78R and at Q114R (Cleavage site; Liu et al., 2008; Samal et al., 2011). As shown in the neutralization test, a new escape mutant may exist, as the raised antibody against LaSota was not able to completely neutralize the wild-type NDV isolates. Escape mutants can arise from amino acid alterations in the neutralizing epitope and the cleavage site of the *F1* gene.

Kim et al. (2013) reported that although the current NDV vaccines are still effective in preventing ND, they are not fully protective against infection, as the vaccines are prepared using viruses with different genotypes than those of wild-type NDVs found in the field. Only a vaccine prepared with closely related genotypes can prevent infection and the spread of the virus. The approach of using chimeric LaSota by replacing its *F* and *H*/*N* genes with those of Genotype VII has been proven effective in preventing NDV infection (Qiao et al., 2021).

CONCLUSION

The active LaSota NDV vaccine was able to induce a protective immune response in laying hens after booster vaccination, with steadily increasing titers following the first, second, and third immunizations. However, with the

booster vaccination using the inactive vaccine, the antibody titers declined until the third vaccination and only increased after the fourth vaccination. Antibodies induced by the active LaSota vaccine were capable of neutralizing field virulent NDV isolates. The presence of escape mutants was detected in association with the CPE observed in several wells exhibiting low antibody levels. Sequencing analysis of the *F1* gene was conducted on two NDV isolates collected from the field, identified as ND/chicken/GAYK01/PenebelBali/2023 and ND/chicken/GAYK02/PenebelBali/2023. The results indicated that both isolates belong to the NDGVIIIi (Newcastle Disease Virus Genotype VIIi) and demonstrated homology levels of 80-81% with the active LaSota vaccine and 96-98% with the inactive NDV vaccine. Further studies are required to explore the presence of the escape NDV mutant.

DECLARATIONS

Funding

The present study was funded by the Institute of Research and Community Service, Udayana University, Indonesia (Hibah Unggulan Udayana Scheme) under the grant number B/1.486/UN14.4.A/PT.01.03/2023.

Authors' contributions

Gusti Ayu Yuniati Kencana conceived and designed the experiment, collected samples, and performed data analysis. I Nyoman Mantik Astawa and Anak Agung Sagung Kendran helped in conceiving and designing the experiment. All authors conducted laboratory testing. All authors read and approved the final edition of the manuscript for publication.

Competing Interests

The authors declared that they have no competing interests.

Ethical considerations

The authors declare that this manuscript is original and is not being considered elsewhere for publication. Other ethical issues, including consent to publish, misconduct, data fabrication, and redundancy, have been reviewed by the authors.

Availability of data and materials

All data from this study are available upon reasonable requests from the authors.

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