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Efficacy of genotype-matched Newcastle disease virus vaccine formulated in carboxymethyl sago starch acid hydrogel in chickens vaccinated via different routes

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ABSTRACT

Background: The commercially available Newcastle disease (ND) vaccines were developed based on Newcastle disease virus (NDV) isolates genetically divergent from field strains that can only prevent clinical disease, not shedding of virulent heterologous virus, highlighting the need to develop genotype-matched vaccines
Objectives: This study examined the efficacy of the NDV genotype-matched vaccine, mIBS025 strain formulated in standard vaccine stabilizer, and in carboxymethyl sago starch-acid hydrogel (CMSS-AH) following vaccination via an eye drop (ED) and drinking water (DW).
Methods: A challenge virus was prepared from a recent NDV isolated from ND vaccinated flock. Groups of specific-pathogen-free chickens were vaccinated with mIBS025 vaccine strain prepared in a standard vaccine stabilizer and CMSS-AH via ED and DW and then challenged with the UPM/NDV/IBS362/2016 strain.
Results: Chickens vaccinated with CMSS-AH mIBS025 ED (group 2) developed the earliest and highest Hemagglutination Inhibition (HI) NDV antibody titer ($8\log_2$) followed by standard mIBS025 ED (group 3) ($7\log_2$) both conferred complete protection and drastically reduced virus shedding. By contrast, chickens vaccinated with standard mIBS025 DW (group 5) and CMSS-AH mIBS025 DW (group 4) developed low HI NDV antibody titers of $4\log_2$ and $3\log_2$, respectively, which correspondingly conferred only 50% and 60% protection and continuously shed the virulent virus via the oropharyngeal and cloacal routes until the end of the study at 14 dpc.
Conclusions: The efficacy of mIBS025 vaccines prepared in a standard vaccine stabilizer or CMSS-AH was affected by the vaccination routes. The groups vaccinated via ED had better protective immunity than those vaccinated via DW.

Keywords: Genotype matched NDV vaccine; carboxymethyl sago starch-acid hydrogel; vaccination routes; genotype VII NDV

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Conflict of Interest
The authors declare no conflicts of interest.

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INTRODUCTION

Newcastle Disease (ND) is a highly contagious economically important avian viral disease that was categorized as a list A disease of poultry by the Office International des Epizooties [1]. Since its first emergence in Java Island, Indonesia [2] and Newcastle-upon-Tyne, England in 1926 [3,4], outbreaks of the disease have continued to spread globally, leading to substantial economic losses to the global poultry industry [5-7]. In Malaysia, ND was first reported in poultry flocks in Parit Buntar, Perak, in 1934 [8] and has continued to cause major disease outbreaks in commercial and backyard poultry flocks throughout the country. The disease is caused by the Newcastle disease virus (NDV), an avian orthoavulavirus-1 belonging to the genus *Orthoavulavirus,* subfamily *Avulavirinae* and family *Paramyxoviridae* [9]. The virus is classified into 3 pathotypes (velogenic, mesogenic, and lentogenic strains) based on World Organisation for Animal Health (OIE) recommended pathogenicity indices in 1-day-old specific-pathogen-free (SPF) chicks (intracerebral pathogenicity index [ICPI]) and in 9–11 day-old SPF embryonated chicken eggs (ECE) [10]. The viruses can also be classified based on the amino acid composition of the F protein cleavage site, where the virulent NDV has polybasic cleavage sites while the avirulent NDV has a monobasic F cleavage site [11].

The continuous evolution of NDV is a major driver of the enormous genetic diversity of the virus. Thus far, NDV has been classified into 21 distinct genotypes (I–XXI) based on the most objective criteria recently proposed by Dimitrov et al. [12]. Interestingly, all commercially available NDV vaccines are classified as either genotype I or II. On the other hand, the viral isolates responsible for disease outbreaks in various parts of the world belong to other genotypes. In particular, genotype VII NDV has been shown to cause substantial disruption of poultry production over the last 2 decades, particularly in Asia [13-19]. Genotype VII NDV isolates are responsible for the ongoing fifth ND panzootic and have been isolated even among vaccinated farms in different parts of the world. Thus, genotype VII NDV is a considerable threat that must be addressed to ensure the maximum productivity of the global poultry industry.

Effective control of ND requires tight biosecurity measures and an efficient vaccination program. Vaccines against ND have been available for more than 6 decades. Nevertheless, ND outbreaks still occur in commercial and backyard poultry farms [20] due to various factors that interfere with the induction of complete immunity after vaccination under field conditions. Genotype mismatch between the vaccine strains and field NDV isolates is possibly the main reason for the suboptimal efficacy of the currently used ND vaccines in the field. Phylogenetically, all commercially available ND vaccines, such as B1, LaSota, and V4, are classified as genotype I or II. They share at least 10% genetic divergence with genotype VII isolates based on a pairwise comparison of their F and HN nucleotide sequences [21-23]. In addition, several studies have reported that vaccines developed based on circulating strains, the so-called genotype-matched vaccines, provide superior efficacy than genotype I and II vaccines, especially in reducing the load and duration of virus shedding post-challenge [24-26]. Recently, the authors developed a genotype matched ND vaccine (mIBS025) with lentogenic properties from a virulent naturally detected recombinant NDV isolate, IBS025/13, using reverse genetic technology [22]. A single vaccination of SPF chickens with mIBS025 via the eye drop (ED) route induced high Hemagglutination Inhibition (HI) antibody titers, conferred 100% protection against clinical disease, and reduced virus shedding substantially compared to LaSota vaccine, following a challenge with another velogenic genotype VII NDV isolate [22]. This provides further evidence on the

improved efficacy of the genotype matched vaccine against the prevailing field NDV isolates compared to currently used vaccines.

Formulation of live NDV vaccines requires stabilizers that help preserve the vaccine antigen against a loss of antigenicity or infectivity during the production, storage, and transportation phases [27-29]. While several stabilizers, such as liposomes, lipoplexes, magnesium chloride (MgCl2), sorbitol-gelatin, and hydrogel, are widely used, recent studies have unlocked the prospects of polysaccharide hydrogels, such as gelatin, chitosan, starch, and methylcellulose, in preserving the vaccine potency during storage [30,31]. A previous study reported that carboxymethyl sago starch-acid hydrogel (CMSS-AH) powder could preserve the infectivity and antigenicity of the NDV LaSota strain (genotype II) for up to 30 d at room temperature [32]. The present study examined the protective efficacy of the NDV mIBS025 vaccine formulated in CMSS-AH and administered via different routes in SPF chickens.

MATERIALS AND METHODS

Ethical statement

The animal trials were carried out under the supervision of the institution veterinarian according to the guidelines stated in the Code of Practice for Care and Use of Animals for Scientific Purposes as stipulated by Universiti Putra Malaysia (UPM). The study complied with the current guidelines for the care and use of animals, as approved by the Institutional Animal Care and Use Committee (IACUC) under authorized usage policy (AUP) number: UPM/IACUC/AUP-R010/2018 dated June 5, 2018.

Vaccine virus preparation

The vaccine virus, mIBS025, was derived from a naturally detected recombinant genotype II and genotype VII NDV strain IBS025/2013 [18] modified genetically using reverse genetic technology to produce a live-attenuated virus with a lentogenic pathotype [22]. The mIBS025 virus was propagated in SPF ECE and then prepared into 2 different formulations in a standard stabilizer and in CMSS-AH.

Standard vaccine stabilizer

The standard vaccine stabilizer was prepared according to the Malaysian Vaccines and Pharmaceuticals (MVP) protocol, Malaysia. The standard vaccine stabilizer was prepared by adding 0.68 g of sodium dihydrogen phosphate (Sigma, USA), 0.125 g of potassium phosphate (Sigma), 74.6 g of sucrose (Sigma), and 10 g of bovine serum albumin (Sigma) to 10 mL sterile distilled water (Millipore, USA). The pH was then adjusted to 6.8 and filtered through a 0.22 μm syringe filter (Sartorius, Germany). Briefly, 1 mL of mIBS025 vaccine virus in the form of an allantoic fluid was mixed with 0.5 mL of standard stabilizer (MVP, Malaysia). The mixture was vortexed for 3 sec then freeze-dried for 24 h. The vaccines were resuspended in 1.5 mL phosphate-buffered saline (PBS) and subjected to an 50% of the embryo infectious dose (EID_{50}) titration to determine the required virus titer for vaccination at 10^6 EID_{50}/0.1 mL. The standard mIBS025 vaccine was then filtered through a 0.45 μm syringe filter (Sartorius) before the vaccination of the experimental chicks.

CMSS-AH

CMSS-AH is a polysaccharide hydrogel developed from sago starch by Tuan Mohamood et al. [33]. CMSS-AH was prepared using a series of chemical and heat modifications to maximize

the water absorption and water retention properties in the form of CMSS-AH powder. Briefly, 1 mL of mIBS025 vaccine virus in the form of allantoic fluid was mixed with 95 mg of CMSS-AH powder to form a mixture of gel. The gel was then vortexed for 3 min and centrifuged at 13,000 × g and 10°C for 45 min. The gel was then freeze-dried for 24 h. The vaccine virus was then resuspended in 1.5 mL PBS, vortexed, and centrifuged at 1,000 × g and 10°C for 5 min. The supernatant was subjected to titration to determine the required virus titer for vaccination, which is 10^6 EID_{50}/0.1 mL. The vaccine virus was then filtered using a 0.45 µm syringe filter before vaccinating the experimental chicks.

Virus vaccine titration

The infectivity titer of the virus before and after formulation with the standard and CMSS-AH vaccine stabilizer were determined to be EID_{50} in SPF ECE [1]. Briefly, a ten-fold serial dilution (10^{-1} to 10^{-10}) of each virus vaccine was prepared. Subsequently, 200 µL of allantoic fluid was diluted into 800 µL of PBS to prepare a 10-fold dilution. The diluted virus was filtered through a 0.45 µM syringe filter before being inoculated into SPF ECE and calculated using the Reed Muench table [1].

Immunization and challenge experiment

Experimental design

Both prepared vaccines, standard mIBS025 and mIBS025 CMSS-AH, were administered via 2 different vaccination routes: ED and drinking water (DW). Five-day-old chicks (n = 50) were divided equally into 5 treatment groups containing 10 chicks per group. Group 1, 2, 3, 4, and 5 were inoculated with PBS via EDs as the negative control, mIBS025 CMSS-AH ED, standard mIBS025 ED, mIBS025 CMSS-AH DW, and standard mIBS025 DW, respectively, with a vaccination dose of 10^6 EID_{50}/0.1 mL.

Serological profiling

Sera samples were collected from each treatment group (n = 10) at 3 day-old for pre-vaccination screening, followed by 7, 14, and 21 days post-vaccination (dpv). The harvested sera were subjected to a HI test to determine the NDV specific HI antibody titer. The HI assay was performed according to the standard procedures with 4 HAU virus/antigen in 0.025 mL by OIE [1]. This assay was performed using the genotype II NDV LaSota strain as the antigen, and the titers were calculated as the highest reciprocal serum dilution providing complete HI. The serum titers with the HI titer of 8 (2^3) or lower were considered negative for the antibodies against the NDV [1].

Characterization of challenge virus

At 21 dpv, all treatment groups were challenged with UPM/NDV/IBS362/2016 strain at a dose of 10^5 EID_{50}/0.1 mL via bilateral EDs inoculation. The challenge virus was isolated from a vaccinated commercial broiler flock at the Avian Diagnostic Unit, Laboratory of Vaccines & Biomolecules (VacBio), Institute of Bioscience (IBS), UPM. The virus was characterized genetically by F gene sequence analysis and biologically by the mean death time (MDT) and ICPI according to OIE [1]. The pairwise evolutionary distances of the nucleotide and amino acid sequences between the challenge virus and the representative strains of the different subgenotypes NDV were calculated using the *p*-distance method included in MEGA v7.0.

Post-challenge clinical signs

Chicks from all treatment groups were monitored daily, starting from 1-day post-challenge (dpc) to 14 dpc. The daily clinical signs and morbidity scores were recorded as 0 for normal, 1 for mild depression, 2 for severe depression, and 3 for death [34].

Viral shedding assay

The amount of virus from the oropharyngeal and cloacal swabs was determined by measuring the DNA copy number of the NDV using the standard curve method in a quantitative real-time polymerase chain reaction (qPCR). The oropharyngeal and cloacal swabs collected on 3, 5, 7, 10, 12, and 14 dpc were subjected to viral RNA extraction and proceeded with qPCR. The qPCR was performed using QRT-PCR Probe One Step Kit LRox (Biotechrabbit™, Germany) together with the forward primer 5′-TCCGCAAGATCCAAGGGTCT-3′; reverse primer 5′-CGCTGTTGCAACCCCAAG-3′ and probe 5′-(6FAM)-AAGCGTTTCTGTCTCCTTCCTCCA-(BHQ1)-3′ as described by Rasoli et al. [35]. The PCR mix was comprised of 10 µL of QPCR Probe Mix (2×), 1 µL of RTase with RNase Inhibitor (20×), 1 µL of the forward primer (10 µM), 1 µL of the reverse primer (10 µM), 1 µL of the specific probe (5 µM), and 4 µL of the RNA template. The cycling program was started at 48°C for 10 min for reverse transcription, then 95°C for 3 min as the initial activation, followed by 40 cycles of 95°C for 20 sec as denaturation, 58°C for 30 sec for annealing, 72°C for 20 sec for extension, and 72°C for 5 min as a final extension.

Statistical analysis

Statistical analysis was performed using SPSS Statistics 22 (IBM, USA). The mean comparison of the parameter between the experimental groups was analyzed by repetitive analysis of variance with a Tukey *post hoc* test (SPSS v22.0). Statistical significance was inferred when the p value was less than 0.05 ($p < 0.05$). The results are expressed as the mean and standard error.

RESULTS

Molecular and biological characterization of the challenge virus

An analysis of the partial F gene sequence alignment showed that UPM/NDV/IBS362/2016 has > 96% identity with the previously characterized genotype VII NDV. Phylogenetic analysis based on gene segment corresponding to nucleotide position 47 to 535 compared to reference genotype VII strains [12,36] (**Table 1**) also grouped the virus in the same cluster with other subgenotype VII.2/VIIi isolates (**Fig. 1**). Furthermore, the virus possesses multibasic amino acid composition (^{112}RRQKRF^{117}) at the F cleavage site, ICPI of 1.7, and MDT of 58.4 h, suggesting its virulence. Based on pairwise similarity matrix, UPM/NDV/IBS362/2016 strain was shown to share the highest nucleotides and amino acids similarity with IBS025/13 (Malaysia) and Ck/Banjarmasin/010/10 (Indonesia) at 97.1% and 98.5%, and with Ck/Kulonprogo/14171317/2017 at 95.8% and 97.8%, respectively (**Table 2**). On the other hand, UPM/NDV/IBS362/2016 shared the lowest amino acids (94.1%) and nucleotide (89.7% to 90.2%) similarities with members of the subgenotype VII.2/VIIh previously isolated from Malaysia (IBS002/11 and IBS005/11) and Indonesia (Ck/Makassar/003/09).

Serological profiling

The sera were collected at 0, 7, 14, and 21 dpv to determine the HI NDV antibody titer. Pre-vaccination screening confirmed that the 1-day-old chicks were free of NDV HI antibodies. As shown in **Fig. 2**, the chicks from the group 1 inoculated with PBS remained negative throughout the study. At the same time, treatment group 2 (CMSS-AH mIBS025 ED) developed the highest HI antibody titer, followed by group 3 (standard mIBS025 ED), group 4 (standard mIBS025 DW), and finally group 5 (CMSS-AH mIBS025 DW) by 21 dpv. Generally, chicks vaccinated via ED showed both earlier detection and higher titer of HI antibodies. For

Table 1. NDV reference strains representing subgenotype VII NDV

Diel et al. (2012) [36]	Dimitrov et al. (2019) [12]	Isolates/Strain	Accession number	Origin
VIIb	VII.1.1	Ck/SD-01-12-Ch	KJ184594.1	China
		Ck/JS-17-11-Ch	JQ013871.1	China
		Ck/SD704/12	JX840454	China
VIId	VII.1.1	Ck/MB016/07	GQ901894.1	Malaysia
		Ck/MB064/05	GQ901893.1	Malaysia
VIIe	VII.1.1	Go/GD/1/98	AF456437.1	China
		Ck/Ibaraki/SG106/1999	AB853927.2	Japan
VIIf	VII.1.2	ND/03/018	GQ338309.1	China
		Ck/ND/03/044	GQ338310.1	China
VIIh	VII.2	Ck/Makassar/003/09	HQ697256.1	Indonesia
		Ck/IBS005/11	KR074405.1	Malaysia
		Ck/IBS002/11	KR074404.1	Malaysia
VIIi	VII.2	Ck/Banjarmasin/010/10	HQ697254.1	Indonesia
		Ck/Kulonprogo/04171317/2017	MK069429.1	Indonesia
		Ck/IBS025/13	KT355595.1	Malaysia
VIIj	VII.1.1	Dk/JLQG/2013	KJ136259.1	China
		Ck/JLJT/2012	KJ136258.1	China
VIIl	VII.1.1	Ck/IR/MAM81/2018	MH481363.1	Iran
		Ck/IR/MAM68/2017	MH481361.1	Iran
		Ck/IR/MAM55/2017	MH247187.1	Iran

NDV, Newcastle disease virus.

example, by 7 dpv, up to 5 birds were found positive for HI antibodies in group 2, whereas none of the Group 3 chicks tested positive for HI antibodies at that time point. Similarly, the mean $\log_2$ HI NDV antibody titer of groups of chicks vaccinated via EDs increased steadily from 3.46 ± 0.53 to 8.18 ± 0.63 for group 2 and from 3.0 ± 0.0 to 7.96 ± 0.82 for group 3 at 21 dpv, respectively. On the other hand, the increase in HI titer was similar in all the groups. The groups of chicks vaccinated via DW from groups 4 and 5 showed an increasing HI antibody

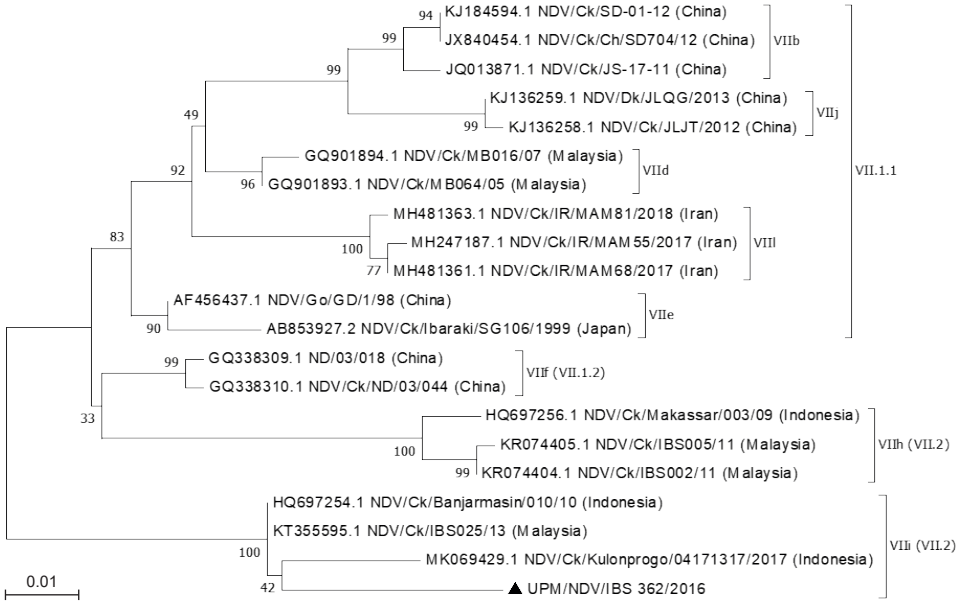

Fig. 1. Phylogenetic analysis of the challenge NDV strain UPM/NDV/IBS362/2016 and 20 previously characterized NDV isolates. The tree was inferred using the Maximum Likelihood method based on the Kimura-2 parameter model (1,000 bootstrap replicates) using MEGA v7.0 software.
NDV, Newcastle disease virus; UPM, Universiti Putra Malaysia; IBS, Institute of Bioscience.

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Table 2. Pairwise similarity matrix of amino acids sequences (lower diagonal) and nucleotides sequences (upper diagonal) between challenge virus (UPM/NDV/IBS362/2016) with reference strains representing subgenotypes VII NDV

	Isolates	Subgenotypes	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	UPM/NDV/IBS362/2016	VII.2/VIIi		89.7	89.2	91.4	91.9	92.7	91.9	92.4	92.4	89.7	89.7	90.2	**97.1**	**97.1**	**95.8**	90.2	90.2	91.0	90.7
2	Ck/SD-01-12-Ch	VII.1.1/VIIb	95.6		90.0	95.8	96.3	95.6	94.9	94.6	94.1	91.9	91.7	91.9	91.9	91.9	91.2	97.1	97.1	94.9	94.6
3	Ck/JS-17-11-Ch	VII.1.1/VIIb	94.8	99.3		95.8	96.3	95.6	94.9	94.6	94.1	91.9	93.4	91.9	91.9	91.9	91.0	97.1	97.1	94.4	94.1
4	Ck/MB016/07	VII.1.1/VIId	97.0	97.0	96.3		99.5	97.3	96.1	95.8	95.8	93.6	93.4	93.2	93.2	93.2	91.7	95.8	95.8	96.3	96.1
5	Ck/MB064/05	VII.1.1/VIId	97.8	97.8	97.0	99.3		97.8	96.6	96.3	96.3	93.6	94.1	93.2	93.6	93.6	92.2	95.8	95.8	96.8	96.6
6	Go/GD/1/98	VII.1.1/VIIe	97.8	97.8	97.0	97.8	98.5		98.8	97.6	97.6	94.4	93.4	94.4	94.9	94.9	93.4	95.1	95.1	96.3	96.1
7	Ck/Ibaraki/SG106/1999	VII.1.1/VIIe	97.0	98.5	97.8	97.0	97.8	99.3		96.3	96.3	93.6	94.1	93.6	94.1	94.1	92.2	94.4	94.4	95.1	94.9
8	ND/03/018	VII.1.2/VIIf	96.3	96.3	95.6	96.3	97.0	98.5	97.8		99.5	94.4	94.1	94.4	94.6	94.6	93.2	93.6	93.6	94.9	94.6
9	Ck/ND/03/044	VII.1.2/VIIf	96.3	96.3	95.6	96.3	97.0	98.5	98.0	100		94.4	98.3	94.4	94.6	94.6	93.2	93.6	93.6	94.9	94.6
10	Ck/Makassar/003/09	VII.2/VIIh	94.1	95.6	94.8	94.1	94.8	96.3	97.0	96.3	96.3			98.5	92.2	92.2	91.2	91.4	91.4	92.2	91.9
11	Ck/IBS005/11	VII.2/VIIh	94.1	95.6	94.8	94.1	94.8	96.3	97.0	96.3	96.3	100		99.8	91.9	91.9	90.5	91.7	91.7	91.9	91.7
12	Ck/IBS002/11	VII.2/VIIh	94.1	95.6	94.8	94.1	94.8	96.3	97.0	96.3	96.3	100	100		92.2	92.2	90.7	91.9	91.9	92.2	91.9
13	Ck/Banjarmasin/010/10	VII.2/VIIi	**98.5**	97.0	96.3	97.0	97.8	99.3	98.5	97.8	97.8	95.6	95.6	95.6		100	98.0	91.9	91.9	92.7	92.4
14	Ck/IBS025/13	VII.2/VIIi	**98.5**	97.0	96.3	97.0	97.8	99.3	98.5	97.8	97.8	95.6	95.6	95.6	100		98.0	91.9	91.9	92.7	92.4
15	Ck/Kulonprogo/04171317/2017	VII.2/VIIi	**97.8**	96.3	95.6	96.3	97.0	98.5	97.8	97.0	97.0	94.8	94.8	94.8	99.3	99.3		91.0	91.0	91.4	91.2
16	Dk/JLQG/2013	VII1.1/VIIj	94.8	99.3	98.5	97.8	97.0	97.0	97.8	95.6	95.6	94.8	94.8	94.8	96.3	96.3	95.6		99.8	93.9	93.6
17	Ck/JLJT/2012	VII1.1/VIIj	94.8	99.3	98.5	97.8	97.0	97.0	97.8	95.6	95.6	94.8	94.8	94.8	96.3	96.3	95.6	100		93.9	93.6
18	Ck/IR/MAM81/2018	VII1.1/VIIl	93.3	94.8	94.1	94.8	95.6	95.6	94.8	94.1	94.1	93.3	93.3	93.3	94.8	94.8	94.1	94.1	94.1		99.3
19	Ck/IR/MAM68/2017	VII1.1/VIIl	93.3	94.8	94.1	94.8	95.6	95.6	94.8	94.1	94.1	93.3	93.3	93.3	94.8	94.8	94.1	94.1	94.1	98.5	

Percentage similarity of studied isolate with reference subgenotype VII.2/VIIi isolates were bolded.
NDV, Newcastle disease virus; UPM, Universiti Putra Malaysia; IBS, Institute of Bioscience.

titer but to a much lesser extent than the groups vaccinated by ED (**Table 3**). In both groups, the mean $\log_2$ titer increased gradually from 2.45 ± 0.53 to 4.40 ± 0.97 for group 5 and from 2.08 ± 0.32 to 3.69 ± 1.03 for group 4 at 21 dpv (**Table 3**). Similarly, the increase in HI titer was similar in the 2 groups. Overall, regardless of the vaccine stabilizer used, groups vaccinated via ED (standard mIBS025 and CMSS-AH mIBS025) have a significantly higher antibody titer than the groups vaccinated via DW (standard mIBS025 and CMSS-AH mIBS025) at $p < 0.05$.

Post-challenged clinical signs

Chickens from all the challenged groups showed varying clinical signs ranging from normal to severe, depending on their treatment. On the first 2 dpc, the morbidity scores were similar

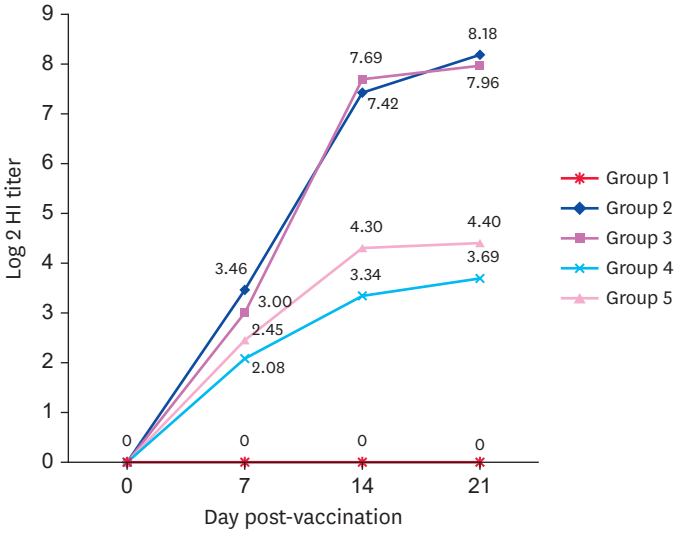

Fig. 2. NDV HI antibody titer of chickens from different treatment groups following vaccination with mIBS025.
NDV, Newcastle disease virus; HI, Hemagglutination Inhibition.

Table 3. Total number of ND seropositive chickens and geometric mean NDV HI antibody titer of chicken post-vaccination

Treatment groups	No. of HI NDV antibody positive chicks/Total No. of chicks				Antibody titer, log_2 (geometric mean ± SD)				Grand mean
	DOC	7 dpv	14 dpv	21 dpv	DOC	7 dpv	14 dpv	21 dpv	
Group 1	0/10	0/10	0/10	0/10	0	0	0	0	0
Group 2	0/10	5/10	10/10	10/10	Nd	3.46 ± 0.53	7.42 ± 1.18	8.18 ± 0.63	4.77^{*cd}
Group 3	0/10	0/10	10/10	10/10	Nd	3.00 ± 0.00	7.69 ± 0.48	7.96 ± 0.82	4.67^{*cd}
Group 4	0/10	0/10	3/10	5/10	Nd	2.08 ± 0.32	3.34 ± 0.70	3.69 ± 1.03	2.28^{*ab}
Group 5	0/10	0/10	8/10	8/10	Nd	2.45 ± 0.53	4.30 ± 0.97	4.40 ± 0.97	2.79^{*ab}

Chicks with HI antibody titer of 16 and above (≥ 2^4) were considered positive.
DOC, day-old-chick; Nd, not detected; ED, eye drop; DW, drinking water; ND, Newcastle disease; NDV, Newcastle disease virus; HI, Hemagglutination Inhibition; dpv, day post-vaccination; CMSS-AH, carboxymethyl sago starch-acid hydrogel.
The values with '*' are significantly different at ($p < 0.05$) when compared to the Control group at the same time point.
The values with 'a' are significantly different at ($p < 0.05$) when compared to the CMSS-AH mIBS025 ED group at the same time point.
The values with 'b' are significantly different at ($p < 0.05$) when compared to the standard mIBS025 ED group at the same time point.
The values with 'c' are significantly different at ($p < 0.05$) when compared to the CMSS-AH mIBS025 DW group at the same time point.
The values with 'd' are significantly different at ($p < 0.05$) when compared to the standard mIBS025 DW group at the same time point.

Table 4. Chicken's daily morbidity score post-challenged in all treatment groups

dpc	Group 1	Group 2	Group 3	Group 4	Group 5
1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
3	1.1 ± 0.83	0.0 ± 0.0*	0.0 ± 0.0*	0.4 ± 0.49*	0.4 ± 0.49*
4	1.7 ± 0.45	0.0 ± 0.0*	0.0 ± 0.0*	0.4 ± 0.49*	0.5 ± 0.67*
5	2.2 ± 0.4	0.0 ± 0.0*	0.0 ± 0.0*	1.0 ± 0.78	1.1 ± 0.94
6	Nil	0.0 ± 0.0	0.0 ± 0.0	1.5 ± 0.92^{ab}	2.4 ± 1.2^{ab}
7	Nil	0.0 ± 0.0	0.0 ± 0.0	1.4 ± 1.11^{ab}	0.9 ± 0.7^{ab}
8	Nil	0.0 ± 0.0	0.0 ± 0.0	0.5 ± 0.81^{ab}	0.9 ± 0.7^{ab}
9	Nil	0.0 ± 0.0	0.0 ± 0.0	0.8 ± 1.08^{ab}	0.4 ± 0.66^{ab}
10	Nil	0.0 ± 0.0	0.0 ± 0.0	0.4 ± 0.66^{ab}	0.9 ± 0.83^{ab}
11	Nil	0.0 ± 0.0	0.0 ± 0.0	0.5 ± 0.92^{ab}	1.1 ± 1.04^{ab}
12	Nil	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.64^{ab}	0.3 ± 0.64^{ab}
13	Nil	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.64^{ab}	0.3 ± 0.64^{ab}
14	Nil	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.64^{ab}	0.3 ± 0.64^{ab}

dpc, day post-challenge; Nil, chickens start to die from 6 dpc to 14 dpc; ED, eye drop; CMSS-AH, carboxymethyl sago starch-acid hydrogel.
The values with '*' differ significantly with the control group at the same time point.
The values with 'a' differed significantly with the CMSS-AH mIBS025 ED group at the same time point.
The values with 'b' differed significantly with the CMSS-AH mIBS025 ED group at the same time point.

among the groups (**Table 4**) because none of the challenged chickens showed clinical signs. Later, group 1 showed clinical signs at 3 dpc where 70% of chickens had swollen eyes with discharges, watery diarrhea, and inappetence. The disease worsened on 4 dpc with 100% morbidity characterized by dyspnea and recumbency. By 5 dpc, the mortality was 20% and reached 100% by 6 dpc. The morbidity scores of the control group were significantly higher than the other vaccinated groups ($p < 0.05$).

Generally, similar clinical signs were observed in challenged chickens from group 5. On the other hand, the clinical signs were less severe than the control groups, where 40% of the birds at 3 dpc had watery diarrhea, swollen eyes with discharges, and inappetence. The morbidity increased to 70% at 6 dpc, and the mortality reached 40% at 11 dpc (**Table 4**). A similar trend was also observed in group 4, where the chickens started to show clinical signs at 3 dpc with 40% morbidity and increased to 70% morbidity at 7 dpc. The mortality began with 30% at 7 dpc and reached 50% at 11 dpc. On the other hand, the morbidity scores between these 2 groups are not significant. Interestingly, no clinical signs were observed in groups 2 and 3 throughout the experiment. In addition, there were no significant differences in the morbidity scores between these 2 groups at all time points (**Table 4**).

Table 5. Oropharyngeal virus shedding from all treatment groups at different time points

Time points (dpc)	Group 1		Group 2		Group 3		Group 4		Group 5	
	No. of positive/ Total	VCN	No. of positive/ Total	VCN	No. of positive/ Total	VCN	No. of positive/ Total	VCN	No. of positive/ Total	VCN
3	5/5	8.70 ± 0.47	2/5	7.68 ± 0.12*	5/5	8.70 ± 0.47	4/5	8.13 ± 0.63	4/5	8.39 ± 1.11
5	3/3	11.65 ± 0.32	2/5	7.13 ± 0.13*	3/5	11.65 ± 0.32*a	5/5	9.06 ± 0.83*	5/5	8.96 ± 1.06*b
7	NS	-	2/5	6.98 ± 0.00*	2/5	8.37 ± 0.51*a	5/5	8.12 ± 0.85*	5/5	7.44 ± 0.15*b
10	NS	-	0/5	No virus shedding detected	2/5	7.37 ± 0.24*	3/5	7.58 ± 0.17*	3/5	7.28 ± 0.09*b
12	NS	-	0/5	No virus shedding detected	0/5	No virus shedding detected	2/5	7.49 ± 0.19*	2/5	7.14 ± 0.02*b
14	NS	-	0/5	No virus shedding detected	0/5	No virus shedding detected	2/5	7.27 ± 0.05*	1/5	6.52 ± 0.00*b

dpc, day post-challenge; NS, no survived chickens; VCN, virus copy number $\log_{10}$ (mean ± SD).
Values with '*' differ significantly at ($p < 0.05$) within the same time point while those with 'a' differ significantly with group 2, value 'b' differ significantly group 5.

All chickens in group 1 succumbed to the infection (100% mortality), followed by group 4 with 50% mortality and group 5 with 40% mortality. Group 1 started to show mortality with 20% at 5 dpc, reaching 100% at 6 dpc. Group 4 started with 30% at 6 dpc and 40% at 8 dpc, reaching 50% mortality at 9 dpc, while group 5 started with 10% mortality at 5 dpc, 30% at 6 dpc, reaching 40% mortality at 9 dpc. By contrast, groups 2 and 3 showed no mortality.

Viral shedding assay

Oropharyngeal viral shedding

The oropharyngeal swabs collected at 3 and 5 dpc from all challenged groups were positive for virus shedding. On the other hand, the chickens from group 1 showed the highest mean virus copy number with all 5 swab samples testing positive on 3 dpc and 5 dpc compared to the other 4 challenged groups ($p < 0.05$). In addition, at 6 dpc, all the control chicks were dead (**Table 5**).

Two out of 5 chickens from group 2 tested positive at 3, 5, and 7 dpc, but the mean virus copy number at different time points decreased as viral shedding decreased gradually and finally stopped at 10 dpc. In addition, the virus copy number of group 2 was significantly lower ($p < 0.05$) than group 3 starting on 5 dpc to 7 dpc. The chicks from group 3 showed one positive sample detected at 3 dpc, which then increased to 3 samples at 5 dpc, and later decreased to 2 positive samples at 7 dpc and 10 dpc, respectively. Viral shedding was stopped at 12 dpc (**Table 5**).

On the other hand, the chickens from group 4 showed 4 positive samples detected on 3 dpc, which increased to 5 positive samples each on 5 and 7 dpc and later decreased to 2 positive samples on 14 dpc. In addition, the virus copy number of group 4 was significantly higher ($p < 0.05$) than group 5 starting on 5 to 14 dpc. The chickens from group 5 showed 4 positive samples detected at 3 dpc, which then increased to 5 samples at 5 and 7 dpc. They later decreased to 3 positive samples at 10 dpc and one at 14 dpc, respectively. Groups 4 and 5 continuously shed viruses via the oropharyngeal route until the study ended at 14 dpc (**Table 5**).

Cloacal viral shedding

The cloacal viral shedding patterns of all the challenged groups were similar to oropharyngeal viral shedding at 3 and 5 dpc. In group 2, 2 out of 5 samples were positive, and the mean virus copy number at the different time points showed declining trends. By 12 dpc, no cloacal viral shedding was detected. In addition, the virus copy number of the Group 2 was significantly lower ($p < 0.05$) than group 3, starting from 7 dpc to 10 dpc (**Table 6**). Group 3 showed one

Table 6. Cloacal viral shedding from all treatment groups at different time points

Time points (dpc)	Group 1		Group 2		Group 3		Group 4		Group 5	
	No. of positive/ Total	VCN	No. of positive/ Total	VCN	No. of positive/ Total	VCN	No. of positive/ Total	VCN	No. of positive/ Total	VCN
3	5/5	8.04 ± 0.56	2/5	7.84 ± 0.39*	1/5	7.10 ± 0.00*	0/5	No virus shedding detected	2/5	7.39 ± 0.32*b
5	3/3	11.40 ± 0.42	2/5	7.42 ± 0.30*	3/5	7.22 ± 0.11*	4/5	9.60 ± 0.40*	4/5	8.32 ± 1.19*b
7	NS	-	1/5	6.94 ± 0.10*	2/5	7.07 ± 0.05*a	3/5	8.47 ± 0.32*	3/5	7.54 ± 0.47*b
10	NS	-	1/5	6.22 ± 0.07*	2/5	6.88 ± 0.04*a	3/5	7.63 ± 0.27*	2/5	7.13 ± 0.24*b
12	NS	-	0/5	No virus shedding detected	0/5	No virus shedding detected	3/5	7.31 ± 0.09*	2/5	6.85 ± 0.01*b
14	NS	-	0/5	No virus shedding detected	0/5	No virus shedding detected	3/5	7.25 ± 0.07*	1/5	6.52 ± 0.00*b

dpc, day post-challenge; NS, no survived chickens; VCN, virus copy number $\log_{10}$ (mean ± SD).
Values with '*' differ significantly at ($p < 0.05$) within the same time point while those with 'a' differ significantly with group 2, value 'b' differ significantly group 4.

positive detection at 3 dpc, which then increased to 3 positives at 5 dpc, and later decreased to 2 at 10 dpc.

On the other hand, group 4 showed the highest detection of 4 out of 5 samples on 5 dpc, which then decreased to 3 positive samples at 7 dpc to 14 dpc. The virus copy number of the group 4 are significantly higher ($p < 0.05$) than group 5 at 5 dpc to 14 dpc (**Table 6**). Group 5 showed 2 positive samples at 3 dpc, which increased to 4 at 5 dpc and later decreased to one positive sample at 14 dpc. Hence, groups 4 and 5 continuously shed viruses via the cloacal routes until the study ended at 14 dpc.

DISCUSSION

Poultry is the main animal industry that contributes to high-quality protein supply for the growing global population. Unfortunately, this important livestock subsector is seriously threatened by several infectious diseases, particularly those caused by avian respiratory viruses. ND is an economically important poultry disease, rating second to avian influenza in causing disease mortality. The disease affects all age groups of different species of birds, with particular severity in chickens, where an entire flock can be wiped out. Therefore, disease control is a serious priority to ensure optimal poultry production. Vaccines for the control of ND have been available since the 1950s. Nevertheless, outbreaks of ND still occur both in commercial and backyard poultry. Although the 60-year-old ND vaccines are still effective in preventing clinical disease and death from ND [37], they cannot block virus shedding and prevent infection post-challenge because of the genotype mismatch between the field strains and vaccine strains. Studies have shown that when chickens vaccinated with the LaSota (genotype II) vaccine are challenged with genotype VII NDV, they shed a large amount of challenged virus over a long duration of time, thereby increasing the persistence of NDV in the field and subsequent spread to naive chickens [22,24]. On the other hand, when genotype-matched vaccines are used, such virus shedding is reduced substantially in terms of quantity and duration of shedding. Consequently, the use of a genotype matched vaccine is strongly advocated for improved control of ND outbreaks, particularly those caused by Genotype VII NDV.

Several approaches are available for generating genotype-matched ND vaccines. In some laboratories, the NDV LaSota strain was engineered to express the F or HN genes (or both)

of velogenic NDV homologous to the circulating NDV genotype [21,22]. In such cases, the F cleavage site was often modified to be composed of monobasic instead of polybasic amino acids found in all lentogenic NDV isolates. In another approach, the field virus itself can be used as the backbone virus so that only its F cleavage site is modified to resemble the LaSota vaccine [11,24]. This approach was recently applied in the authors' laboratory to develop a genotype-matched ND vaccine called mIBS025 [22] using reverse genetic manipulation of a naturally detected recombinant NDV (IBS025/13) isolated by Satharasinghe et al. [18]. Compared to the LaSota vaccine, a single vaccination with mIBS025 vaccine via the ED route in SPF chickens leads to complete protection against genotype VII.2/VIIh NDV (IBS002/11 strain) challenge, and drastically reduces the duration and quantity of oropharyngeal and cloacal viral shedding [22].

The present study evaluated the efficacy of the mIBS025 vaccine in vaccinated SPF chickens after being challenged with another subgenotype, VII.2/VIIi, UPM/NDV/IBS362/2016. Molecular and biological characterization of UPM/NDV/IBS362/2016 confirmed this strain to be the velogenic NDV subgenotype VII.2/VIIi clustered together with other subgenotypes VII.2/VIIi, including the IBS025/13 strain. The pairwise similarity matrix showed that the challenge virus (UPM/NDV/IBS362/2016) shared the highest nucleotide and amino acid similarities with the NDV subgenotype VII.2/VIIi IBS025/13 strain and Ck/Banjarmasin/010/10 (Indonesia) at 97.1% and 98.5%. Among the different subgenotypes of VII, the subgenotype VII.2/VIIi UPM/NDV/IBS362/2016 strain shared the lowest amino acid (94.1%) and nucleotide (89.7% to 90.2%) similarities with the NDV subgenotype VII.2/VIIh viruses, including virus strains isolated previously from Malaysia (IBS002/11 and IBS005/11) and Indonesia (Ck/Makassar/003/09) (**Table 2**). SPF chickens vaccinated with mIBS025 vaccines (standard mIBS025 and CMSS-AH mIBS025) via the ED route were completely protected against a challenge with genotype VII.2/VIIi, UPM/NDV/IBS362/2016, with a drastic decrease in the duration and quantity of oropharyngeal and cloacal viral shedding compared to chickens vaccinated via the DW route. Hence, mIBS025 vaccines can protect against challenges with velogenic subgenotype VII.2/VIIi (**Tables 4**-**6**) and VII.2/VIIh [22] provided the vaccine is administered via the ED route.

Similar to other live attenuated vaccines, the mIBS025 vaccine needs to be in a cold chain to preserve the vaccine potency throughout the vaccination process. In recent years, a handful of polysaccharides hydrogels from biopolymers were shown to be capable of serving as vaccine stabilizers. Examples include gelatin, chitosan, starch, and methylcellulose in preserving vaccine potency during the preparation and storage phase [30-31]. Tuan Mohamood [32] previously showed, using *in vitro* studies, that CMSS-AH is a potent vaccine stabilizer. Therefore, in the present study, the mIBS025 vaccine was prepared in CMSS-AH, and a standard vaccine stabilizer used in commercial vaccine production by MVP Malaysia and vaccinated various groups of chickens via the ED or DW routes. The findings showed that both vaccines preparations remained potent at $10^{8.56}EID_{50}$/0.1 mL before and after the vaccine stabilizers had been added. On the other hand, using the ED route, chickens vaccinated with CMSS-AH mIBS025 (group 2) developed the highest HI antibody titer ($8log_2$) at 21 dpv compared to those vaccinated with mIBS025 formulated in the standard vaccine stabilizer (group 3) via the same route (**Fig. 2**, **Table 3**). Although both groups were 100% protected against a lethal challenge with velogenic UPM/NDV/IBS/362/2016 strain (**Table 4**), the chickens in group 2 were more efficient in reducing the duration and load of both oropharyngeal and cloacal viral shedding. Compared to chickens in group 3, the chickens in group 2 could induce an earlier HI titer with 5 chickens testing positive at 7 dpv and inhibit oropharyngeal

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virus shedding by 10 dpc (**Tables 3** and **5**). The performances of both vaccine stabilizers were comparable when the vaccines were delivered via the DW route, where a slight decrease in the HI antibody titer and protection against clinical disease were observed in group 4 compared to group 5, but the difference was not statistically significant. Nevertheless, the virus shedding of these 2 groups was significantly different. These results show that the efficacy of the CMSS-AH formulated mIBS025 vaccine depends on the vaccine administration route chosen.

The most common vaccination routes practiced in the industry are via intranasal aerosol spray (IN), DW, and intraocular or ED because these vaccination routes mimic a natural infection of ND [1,38]. Several studies have shown that the ED vaccination route produces the best highest antibody titer ($8\log_2$–$9\log_2$), followed by intranasal spray ($7\log_2$) and the DW and oral routes ($3\log_2$–$6\log_2$) [38-40]. Furthermore, vaccination with EDs and spray offers better protection than DW for both genotype-matched and LaSota vaccines [24,26,38-40]. Hence, these results and other studies indicate that the vaccination route is a critical determinant of vaccine-induced immunity. The low ND vaccine efficacy following DW administration is probably associated with the destruction of the vaccine viruses by gastric secretions or some of the birds underdosing by drinking less [38-40], resulting in poor protection against challenges with velogenic NDV.

This study confirmed the possible use of natural biopolymers, such as sago starch, as a vaccine stabilizer. Furthermore, sago is available abundantly in Malaysia, which can be developed as an inexpensive vaccine stabilizer. The mass vaccination using NDV vaccines via intranasal aerosol spray is far more effective in inducing protection than DW [38-40]. Therefore, it will be interesting to evaluate the efficacy of mIBS025 following an aerosol spray vaccination in a control experiment involving SPF chickens and in a field setting involving commercial chickens.

In conclusion, mIBS025, a genotype matched NDV vaccine, offered protection against the recently emerged NDV subgenotype VII.2/VIIi strain UPM/NDV/IBS362/2016. In addition, CMSS-AH is a more promising vaccine stabilizer than the standard vaccine stabilizer in providing early protection against virus shedding. On the other hand, vaccination routes affect the vaccination effectiveness, where vaccination via the ED route was far more efficacious than via the DW route in both types of vaccine stabilizers.

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