Original Article
J Vet Sci 2015, 16(4), 447-457 · http://dx.doi.org/10.4142/jvs.2015.16.4.447

Characterisation of genotype VII Newcastle disease virus (NDV) isolated from NDV vaccinated chickens, and the efficacy of LaSota and recombinant genotype VII vaccines against challenge with velogenic NDV

Kiarash Roohani[1], Sheau Wei Tan[1,*], Swee Keong Yeap[1], Aini Ideris[1,2], Mohd Hair Bejo[1,2], Abdul Rahman Omar[1,2]

[1]*Institute of Bioscience, and* [2]*Faculty of Veterinary Medicine, Universiti Putra Malaysia, Serdang 43400, Malaysia*

A Newcastle disease virus (NDV) isolate designated IBS002 was isolated from a commercial broiler farm in Malaysia. The virus was characterised as a virulent strain based on the multiple basic amino acid motif of the fusion (F) cleavage site ^{112}RRRKGF^{117} and length of the C-terminus extension of the hemagglutinin-neuraminidase (HN) gene. Furthermore, IBS002 was classified as a velogenic NDV with mean death time (MDT) of 51.2 h and intracerebral pathogenicity index (ICPI) of 1.76. A genetic distance analysis based on the full-length F and HN genes showed that both velogenic viruses used in this study, genotype VII NDV isolate IBS002 and genotype VIII NDV isolate AF2240-I, had high genetic variations with genotype II LaSota vaccine. In this study, the protection efficacy of the recombinant genotype VII NDV inactivated vaccine was also evaluated when added to an existing commercial vaccination program against challenge with velogenic NDV IBS002 and NDV AF2240-I in commercial broilers. The results indicated that both LaSota and recombinant genotype VII vaccines offered full protection against challenge with AF2240-I. However, the LaSota vaccine only conferred partial protection against IBS002. In addition, significantly reduced viral shedding was observed in the recombinant genotype VII-vaccinated chickens compared to LaSota-vaccinated chickens.

Keywords: genotype VII Newcastle disease virus, recombinant genotype VII Newcastle disease virus vaccine, vaccine efficacy, viral shedding

Introduction

Despite the introduction of vaccines for controlling Newcastle disease (ND) more than 60 years ago, ND is still one of the most significant avian diseases affecting major poultry farms in various countries. In addition, ND is regarded throughout the world as one of the two most important avian diseases aside from the highly pathogenic avian influenza. Strains of NDV are generally grouped as highly (velogenic), moderately (mesogenic), and weakly pathogenic (lentogenic) pathotypes [3]. The velogenic NDV strains carry two basic amino acids, either lysine (K) or arginine (R), at the fusion (F) cleavage site at residues 112 to 113 and 115 to 116 as well as a phenylalanine at residue 117 that is cleaved by ubiquitous intracellular proteases. On the other hand, lentogenic strains have a monobasic cleavage site that is cleaved by extracellular proteases; thus, replication of these strains is restricted to specific tissues [30]. The NDV envelope contains two surface glycoproteins: the hemagglutinin-neuraminidase (HN) protein, which is responsible for attachment of the virus to the host cell, and the F protein that is required for fusion of the virus to the host cell membrane [25]. The F and HN proteins are also the main targets of the immune response against NDV that provides protection against challenge with virulent NDV strains [34].

Aside from pathotype classification, NDV strains can also be grouped into different genotypes based on the sequence and phylogenetic analysis of the F gene. The majority of NDV vaccines are of genotypes I and II whilst virulent NDVs are grouped in genotypes III to X. In 1990s, two novel NDV genotypes, VII and VIII, were reported in Asia, South Africa, and several European countries [1,14,20,24]. Genotype VIII viruses have originated from South Africa since the 1960s and

Received 29 Sep. 2014, Revised 24 Dec. 2014, Accepted 30 Dec. 2014
*Corresponding author: Tel: +603-8947-2151; Fax: +603-8947-2153; E-mail: tansheau@upm.edu.my
pISSN 1229-845X
eISSN 1976-555X

continue to circulate in Southeast Asia [1,35]. The most well-characterised genotype VIII NDV isolate in Malaysia is AF2240, which was first isolated in the 1960s from Ipoh, Perak [21]. This isolate has been used as the challenge virus for studying vaccine efficacy using both specific pathogen-free (SPF) chickens and commercial flocks in Malaysia [2,31]. However, genotypes VII NDV strains were frequently reported since the 1990s in numerous geographical regions such as Europe [14], China, the Middle East, and South Africa [6,36]. In addition, molecular epidemiology studies have indicated that genotype VII NDV is the predominant virus currently circulating in Asian nations including Malaysia [5,36].

Although intensive vaccination programs have been implemented in many countries, genotype VII NDV outbreaks and sporadic cases occasionally occur, even in vaccinated farms in South America [12] and Asian countries such as China [32], Korea [17], and Malaysia [5]. In Malaysia, the index for reported genotype VII NDV outbreaks recorded by the Department of Veterinary Services, Ministry of Agriculture and Malaysia (unpublished data) in 2009 and 2010 were 5 and 75, respectively. In 2011, the outbreaks became more severe with an index of 153.

Current NDV vaccines consist of live and inactivated genotype I and/or II NDV. Repeated outbreaks of virulent NDV among vaccinated chickens indicate the need to revise the NDV vaccination strategy. Furthermore, several underlying factors may have contributed to vaccination failure such as the presence of immunosuppressive diseases [19] as well as poor cross-immunity between the vaccines and field challenge virus strains [40]. Through recombinant technology, newer NDV vaccines have been used in selected countries. These novel vaccines are based on recombinant herpesvirus vectored [30] and reverse genetic LaSota NDV vaccines expressing velogenic F and/or HN genes [7]. In addition, recent studies have shown that genotype-matched vaccines provide better protection against challenge with the virulent genotype VII NDV and significantly reduce virus shedding compared to the LaSota vaccine [16,40]. However, the efficacy of these recombinant genotype VII NDV vaccines against other velogenic genotypes NDV is not well-characterised since the genotype VII virus was used as challenge virus in the previous studies [7,16]. Thus, the present study was conducted to characterise the molecular properties of NDV isolates from vaccinated broiler farms in 2011 during ND outbreaks in Malaysia. For the vaccine efficacy trial, NDV field-vaccinated commercial broilers were challenged with velogenic genotype VII and VIII NDV. Introduction of the recombinant genotype VII NDV inactivated vaccine into the current vaccination program in the field was evaluated. Efficacy of the conventional genotype II LaSota vaccine and recombinant genotype VII NDV inactivated vaccine for conferring protection against challenge with velogenic genotype VII and VIII NDV was also characterised.

Materials and Methods

Reverse transcription (RT)-PCR detection of NDV and virus isolation

In 2011, the Laboratory of Vaccine and Immunotherapeutics, Institute of Bioscience (IBS) received samples from suspected cases of NDV outbreaks in different states of peninsular Malaysia. All the suspected cases came from commercial broiler farms at which at least two rounds of LaSota vaccination had been performed. The samples that included brain, lung, trachea, cecal tonsil, and intestine tissues were processed according to an OIE protocol [37]. RT-PCR was performed to detect NDV as previously described by Berhanu *et al*. [5]. All NDV-positive samples were then subjected to virus isolation using SPF embryonated eggs.

Mean death time (MDT) calculation

Procedures to measure the MDT were carried out according to Alexander [3]. Briefly, a ten-fold serial dilution of NDV isolate IBS002 was prepared in 1× PBS and injected into the allantoic cavity of 9 to 11 days SPF eggs (n = 5 for each dilution) in the morning. The same process was repeated 8 h later in the afternoon. All inoculated eggs were incubated at 37°C for 7 days. The lowest concentration of virus that killed all embryos was considered the minimal lethal dose, and the average death time for the minimum lethal dose was reported as the MDT. MDTs for the NDV strains were categorised as follows: velogenic (less than 60 h), mesogenic (60–90 h), and lentogenic (greater than 120 h) pathotypes.

Intracerebral pathogenicity index (ICPI)

The ICPI was evaluated using 1-day-old SPF chicks (n = 10) given an intracerebral injection of 0.05 mL with a 2^6 HA titer and diluted 10-fold in 1× PBS according to the OIE protocol [37]. The chicks were observed every 24 h for 1 week. Each healthy chicken was given a score of 0, a sick chicken was assigned a score of 1, and a deceased chicken was given a score of 2. The ICPI index was calculated based on the geometrical mean of the scores. Typically, velogenic strains had a value close to 2.0 while the lentogenic strains produced values close or equal to 0.

Amplification of full-length F and HN genes

First strand cDNA was synthesised using a SuperScript III RT kit (Invitrogen, USA) according to the manufacturer's instructions. The RT mixture was incubated at 50°C for 60 min for cDNA synthesis followed by 15 min at 70°C to inactivate the RT enzyme. PCR amplification was performed using previously described primers 5'-AGGCACCCAACGTGCTGTCG-3' (sense) and 5'-ACGGAGACTCAAGGGCCACC-3' (antisense) for the full-length F gene, and 5'- AGTCTCGAGACGGGTAGAAC GGTCG-3' (sense) and 5'- ACGGATCCTTTTCTTAATTAA

GTGGCTATTGACAAG- 3' (antisense) for the full-length HN gene [28]. The F and HN genes were amplified using a KOD Hot Start DNA Polymerase System (Merck, USA). The reactions were incubated at 95°C for 2 min for the initial denaturation followed by 35 cycles of 30 sec at 95°C for denaturation, 1 min of annealing, and 45 sec at 72°C for extension. The final extension was carried out at 72°C for 10 min. Primer annealing temperatures were set at 58°C for the F gene and 59°C for the HN gene.

Sequence and phylogenetic analyses

Sequences for the full-length F and HN genes were aligned with the ClustalW multiple alignment method using BioEdit 7.2.0 software (Ibis Biosciences, USA). Evolutionary and phylogenetic analyses were conducted based on the full-length F gene using MEGA 5.2 software (The Biodesign Institute, USA). A phylogenetic tree was constructed using the maximum-likelihood method with the general time reversible (GTR) model and 1,000 bootstrap replicates. The sequences of different NDV genotypes from GenBank (National Center for Biotechnology Information, USA) that were used for the phylogenetic analysis are as follows: Ulster (AY562991), LMV42 (DQ097394), I-2 progenitor (AY935500), I-2 thermostable (AY935499), V4 (JX524203), Avinew (HI587850.1), 98-1249 (AY935492), 02-1334 (AY935490), 01-1108 (AY935489), 99-1435i (AY935498), 99-0868hi (AY935495), VGGA clone 5 (EU289029), VGGA (EU289028), B1-Takaaki (AF375823), B1 (AF309418), HB92-V4 (AY225110), Clone 30 (Y18898), LaSota (AF077761), Mukteswar (JF950509; EF201805), JS7 (FJ430159), JS9 (FJ430160), Italien (EU293914.1), Herts-33 (AY741404), Namakkal (GU187941), (CA)211472 (AY562987), rAnhinga (EF065682), (Fl)44083 (AY562986), Fontana (AY562988), Sweden-95 (HQ839733), 2736 (AY562989), IT227 (AJ880277), SRZ03 (EU167540), ZJ1 (AF431744), SF02 (AF473851), GM (DQ486859), Guangxi7 (DQ485229), Guangxi9 (DQ485230), Cockatoo (AY562985), MM19 (JX532092), Banjarmasin (HQ697254), Astr-2755 (AY865652), AKO18 (JX390609), 1918-03 (JN800306), KBNP-4152 (DQ839397), KBNP- C4152R2L (EU140955), QH4 (FJ751919.1), QH1 (FJ751918), AF2240-I (JX012096) and DE-R4999 (DQ097393).

ND vaccines and challenge viruses

Two commercial NDV vaccines were used for this study: the genotype II LaSota vaccine along with a recombinant LaSota NDV vaccine strain containing the F and HN genes of genotype VII NDV (strain KBNP-4152) from Korea [7]. The LaSota is a widely used commercial vaccine produced by various manufacturers. The virus is classified as genotype II. Two velogenic NDV strains characterised in this study, AF2240-I of genotype VIII and IBS002 of genotype VII, were used as challenge viruses. AF2240 was first isolated by the Veterinary Research Institute (VRI) in the 1960s [21] and has been used as the standard virus for vaccine challenge studies [2,31]. AF2240 has been characterised as velogenic genotype VIII with an MDT of 48 h [22] and ICPI of 1.90 [21].

Chickens

Commercial broilers (Cobb) 28 days old were received from a commercial poultry farm in Perak, Malaysia. The birds were raised according to standard procedures in an open house system before transfer to the Universiti Putra Malaysia (UPM). For the NDV challenge study, the chickens were transferred to an animal experimental facility at the Institute of Bioscience, Faculty of Veterinary Medicine, UPM. Handling of the chickens was conducted in accordance with laboratory animal care guidelines, and the study protocol was approved by the Institutional Animal Care and Use Committee at the Faculty of Veterinary Medicine, UPM (reference no. UPM/IACUC/AUP-R028/2013).

Vaccine efficacy study in commercial broilers

The importance of including the recombinant genotype VII NDV inactivated vaccine in the current NDV vaccination program was assessed in this experiment. The commercial broilers were divided and kept in two separate houses. Broilers in House 1 received the following NDV vaccination regime administered by the farmer: vaccinations on the day of hatching with live NDV B1 vaccine by spraying, recombinant genotype VII NDV inactivated vaccine and IBDV live vaccine delivered subcutaneously, IB nephropathogenic strain vaccine administered by spraying, IB nephropathogenic strain at 7 days of age by spraying, and live LaSota vaccine at 14 days of age delivered via drinking water. Broilers in House 2 were subjected to the same vaccination schedule as described above except that the recombinant genotype VII NDV inactivated vaccine was replaced with a commercially available genotype II LaSota NDV inactivated vaccine on the day of hatching. A group of newly hatched commercial broiler chicks were kept at the experimental facility as the non-vaccinated control group. Pre-vaccination sera samples were taken from ten broilers in each group and tested using a hemagglutination inhibition (HI) assay to measure the maternal-derived antibody level.

Broilers from Houses 1 and 2 were transferred to the experimental facility at 28 days of age and the birds from each house were further divided into two sub-groups. All chickens were maintained for another 7 days before the challenge study. A total of four vaccinated broiler groups (House 1a, 1b, 2a and 2b) were challenged with 10^6 EID_{50}/0.1 mL of velogenic NDV strains at 35 days of age. Broilers in Houses 1a (n = 20) and 2a (n = 20) were challenged intraocularly with genotype VIII isolate AF2240-I whilst broilers in Houses 1b (n = 20) and 2b (n = 20) were challenged intraocularly with genotype VII isolate IBS002. Non-vaccinated broilers were also divided into two

groups and the virus challenge was carried out as described above. The birds were kept for 14 days, and monitored daily for clinical signs and mortality while serum and swab samples were collected for further analysis. At the end of the trial, all surviving chickens were euthanized.

Serological tests

Hemagglutination (HA) and HI assays were performed using the standard microtiter plate method as recommended by the OIE [37]. The HI tests were carried out with 4 HA units of LaSota and recombinant genotype VII vaccine strains per well.

Measurement of viral shedding using RT-qPCR

Oropharyngeal and cloacal swabs were randomly taken from six birds in each group. Viral RNA was extracted using an RNeasyPlus Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. Specific probes and primers were designed based on the F protein gene to detect viral RNA using RT-qPCR. The primers, probes, and PCR conditions for the assay were previously described in the literature [31]. The amount of viral shedding was expressed as the number of viral RNA copies. Absolute quantification of the number of viral RNA copies in the swabs was based quantification cycle (Cq) values for each sample and the standard curve method for qPCR. The number of viral RNA copies was calculated with the following formula:

$$\frac{(\text{concentration in g}) \times (6.023 \times 10^{23})}{330 \times \text{total amplicon length}}$$

Statistical analysis

Statistically significant differences in the serological analysis data and level of viral shedding between different groups were evaluated with a one-way analysis of variance (ANOVA) using SPSS (ver. 21.0; IBM, USA).

Results

Detection of NDV and virus isolation

A total of five suspected cases had positive RT-PCR and NDV isolation results. These isolates were designated as IBS001 to IBS005. Both isolates possessed a multiple basic amino acid motif at F cleavage site ^{112}RRRKRF^{117} along with a predicted length of 571 amino acids at the carboxyl-terminus of the HN protein based on HN gene extension. Due to the close nucleotide similarities among the viruses, a representative NDV isolate (IBS002) was chosen for further pathogenicity analysis along with amplification of the full-length F and HN genes.

Pathogenicity assessment of NDV isolate IBS002

The pathogenicity of NDV isolate IBS002 was examined using two standard measurements: the MDT and ICPI. Results of the evaluation indicated that IBS002 is a velogenic NDV with an MDT of 51.2 h and ICPI of 1.76.

Sequence analysis of the full-length F and HN genes from NDV isolate IBS002

Full-length F and HN genes from isolate IBS002 were sequenced and submitted to GenBank under accession numbers KF026013 and KF188408, respectively. Distance matrix analysis results of the F and HN genes compared to other isolates, namely genotype VIII NDV isolate AF2240-I, genotype II LaSota vaccine, and recombinant genotype VII vaccine, are shown in Table 1. Both challenge viruses used in this study (IBS002 and AF2240-I) showed a higher genetic variation with genotype II LaSota vaccine compared to the recombinant genotype VII vaccine. A maximum distance of 17.71% nucleotide variation and 18.67% amino acid variation was observed between the F genes of IBS002 and LaSota. The same pattern was observed for the HN gene in which the maximum distance was detected between IBS002 and LaSota with 20.89% nucleotide variation and 23.37% amino acid variation. Similarly, substantial genetic distance was also found between AF2240-I and LaSota with 14.00% nucleotide variation and 16.53% amino acid variation for the F gene, and 16.83% nucleotide variation and 19.06% amino acid variation for the HN gene (Table 1). Among all the NDV isolates and vaccine strains analyzed, IBS002 displayed the highest

Table 1. Percentage of total nucleotide (lower triangle) and amino acid (upper triangle in bold) variations among F and HN proteins of isolate IBS002 compared to previously published genotype VIII NDV AF2240-I and two different NDV vaccines

		F protein				HN protein			
		1	2	3	4	1	2	3	4
1	IBS002	–	**18.67**	**9.77**	**9.12**	–	**23.37**	**11.60**	**15.34**
2	LaSota vaccine	17.71	–	**20.34**	**16.53**	20.89	–	**19.86**	**19.06**
3	Recombinant genotype VII vaccine	8.79	17.52	–	**11.46**	9.17	20.46	–	**14.18**
4	AF2240-I	10.68	14.00	11.04	–	13.19	16.83	12.82	–

F, fusion; HN, hemagglutinin-neuraminidase NDV, Newcastle disease virus.

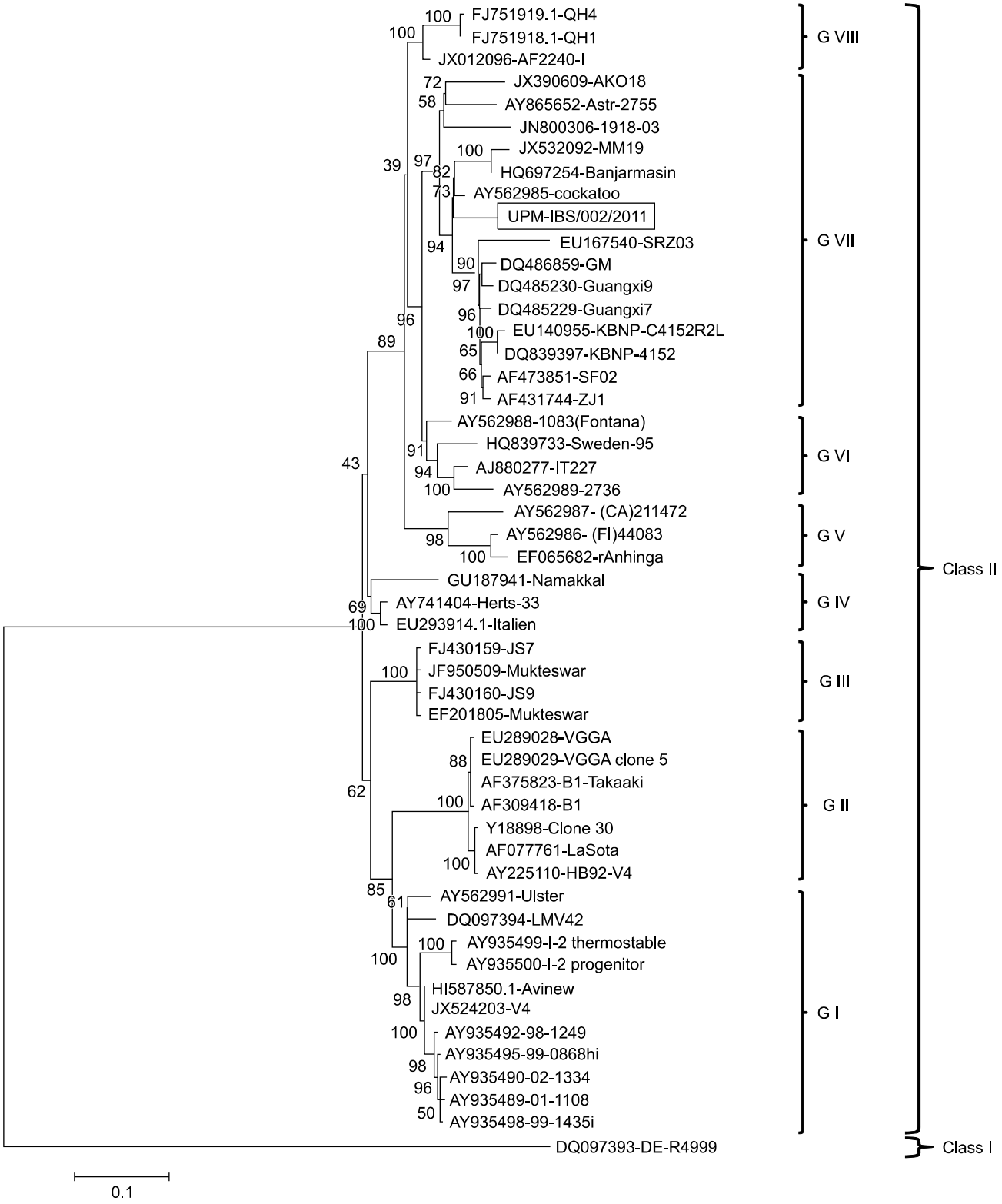

Fig. 1. Phylogenetic relationships among 49 published NDV isolates and isolate IBS002 based on the full F gene nucleotide sequences obtained from GenBank. The phylogenetic tree was constructed using the maximum likelihood method after 1,000 bootstrap replications. The isolate from the present study is boxed.

similarity with the recombinant genotype VII vaccine strain in both the F and HN genes with 90.83% to 91.21% nucleotide similarity and 88.40% to 90.23% amino acid similarity (Table 1).

Phylogenetic analysis

Using the maximum likelihood method, a phylogenetic tree was constructed based on the full-length F gene nucleotide sequences of isolate IBS002 and 49 previously published NDVs. Genotypes I to VIII of class II of NDV were used for the analysis in which all eight genotypes were distinguished based on branching and topology of the constructed tree. Results of the analysis revealed that IBS002 was grouped as genotype VII (Fig. 1) and was phylogenetically close to NDV isolates from Indonesia, China, and Korea.

Vaccine efficacy study in commercial broilers

Mean titers obtained for the commercial broilers pre- and post-vaccination with the recombinant genotype VII inactivated vaccine are showed in Table 2. From day 7 to day 35 post-vaccination, chickens in both House 1 and 2 exhibited positive HI titers for NDV, which increased throughout the vaccination course. For House 1, the antibody titers produced by LaSota and recombinant genotype VII antigens in the HI test were comparable to each other, ranging from 2.48 $\log_2$ to 5.60 $\log_2$ (Table 2). On the other hand, sera collected from the chickens in House 2 had significantly different HI titers when tested with homologous (LaSota) and heterologous (recombinant genotype VII NDV) antigens. The HI titers were low, ranging from 1.92 $\log_2$ to 3.05 $\log_2$, when the heterologous antigen was used (Table 2).

The non-vaccinated NDV-challenged broilers developed severe depression, respiratory problems, and neurological signs that began on day 5 post-challenge (p.c.). Mortality reached 100% on day 8 p.c. Neither clinical signs of disease nor mortality were observed for broilers from House 1. In contrast, about 20% (4/20) of birds in House 2 were depressed with reduced activity that started on day 5 p.c. with AF2240-I and IBS002. A mortality rate of 15% (3/20) was recorded on day 9 p.c. for the chickens in House 2 challenged with IBS002. As shown in Table 2, all the chickens vaccinated with recombinant genotype VII inactivated vaccine (House 1) were protected against challenge with both AF2240-I and IBS002 viruses. However, chickens for which the recombinant genotype VII inactivated vaccines were replaced with LaSota killed vaccine had partial protection with 17 out of 20 birds (85%) surviving the challenge with IBS002. In contrast, all these birds were protected against challenge with AF2240-I.

For the non-vaccinated group, viral RNA was only detected in the chickens challenged with AF2240-I at day 3 p.c. Swabs collected from vaccinated broilers on day 3 p.c. did not produce any positive results except for detection of viral RNA in a single oropharyngeal swab from a House 2 broiler challenged with IBS002 (Table 3). On days 5 and 7 p.c., the non-vaccinated broilers shed abundant quantities of virus, especially in cloacal swabs, and the detected viral RNA copy numbers were as high as 10^{11} (Table 3). Viral RNA was found in swabs collected from both Houses 1 and 2 on day 5 p.c. These results revealed that the chickens in House 2 vaccinated with LaSota alone shed significantly higher quantities of virus compared to the chickens in House 1. In addition, the number of chickens positive for viral shedding in House 2 was also higher than that for House 1. On day 7 p.c., all swabs collected from House 1 were negative for viral shedding whereas the chickens from House 2 continuously shed the virus. No viral RNA was detected in any swabs collected on day 14 p.c. (Table 3). Overall, the viral load detected in cloacal and oropharyngeal swabs did not vary significantly.

Discussion

Vaccination against ND has been practised for more than 60 years. Despite this fact, ND is still one of the most significant avian diseases affecting major poultry farms in various countries. Among the underlying factors that contribute to poor NDV vaccine-induced immunity are inappropriate vaccination dose and regime; presence of concurrent infection, especially immunosuppressive diseases such as chicken infectious anaemia (CAV); infectious bursal disease (IBD) or Marek's disease, and nutritional deficiencies as well as mycotoxins in feed [13,33]. There are probable reasons for decreased vaccine-induced immunity. Studies have also shown that commercial NDV vaccines provide various levels of protection against challenge with different genotypes of NDV [16,26], raising the importance of the relationship between vaccines and NDV field strains. In the present study, the causative agent of ND outbreaks in vaccinated broiler flocks from Malaysia during 2011 was isolated and characterised. Subsequently, levels of protection induced by the genotype II LaSota vaccines and recombinant genotype VII NDV vaccines were compared following challenge with the recently acquired genotype VII NDV isolate IBS002 and genotype VIII NDV isolate AF2240-I.

Sequencing of the F cleavage site of the isolated viruses showed the presence of the polybasic amino acid motif ^{112}RRRKRF^{117}, indicating that all NDV isolates analysed in this study (IBS001−IBS005) were classified as velogenic NDV. This was confirmed by the length of the HN protein, which was predicted to contain 571 amino acids, without an extension at the C-terminus. A previous study by Berhanu *et al*. [5] indicated that genotype VII viruses isolated from Malaysia between 2004 and 2007 also had similar motifs at the F cleavage site. Other groups reported that genotype VII isolates have the same highly pathogenic motif such as ^{112}RRQKRF^{117} in European isolates during the 1990s [1] as well as ^{112}RRQKRF^{117} and ^{112}RRRKRF^{117} isolates in China also acquired during the 1990s [23]. Analysis

Table 2. Serum HI antibody responses in broiler chickens following vaccination with a recombinant genotype VII NDV inactivated vaccine

Group	Challenge virus	Morbidity[‡] (%)	Mortality[§] (%)	HI test antigen[†]	HI GMT* (post-vaccination)					
					Day 0[b]	Day 7	Day 14	Day 21	Day 28	Day 35
Control	AF2240-I	100% (20/20)	100% (20/20)	LaSota	2.55 ± 0.51^{b,c,d}	ND^{a}	ND^{a}	ND^{a}	ND^{a}	ND^{a}
	IBS002	100% (20/20)	100% (20/20)	Recombinant genotype VII virus	2.48 ± 0.52^{b,c,d}	ND^{a}	ND^{a}	ND^{a}	ND^{a}	ND^{a}
House 1 with recombinant genotype VII NDV inactivated vaccine	AF2240-I	0% (0/20)	0% (0/20)	LaSota	2.48 ± 0.52^{b,c,d}	3.86 ± 0.80^{e,f}	3.93 ± 0.76^{e,f}	4.31 ± 0.91^{f,g}	5.23 ± 0.59^{h}	5.00 ± 1.84^{g,h}
	IBS002	0% (0/20)	0% (0/20)	Recombinant genotype VII virus	**2.55 ± 0.51**^{b,c,d}	**3.19 ± 0.70**^{d,e}	**3.24 ± 0.46**^{d,e}	**3.89 ± 0.59**^{e,f}	**5.17 ± 0.56**^{h}	**5.60 ± 2.09**^{h}
House 2 without recombinant genotype VII NDV inactivated vaccine	AF2240-I	20% (4/20)	0% (0/20)	LaSota	**2.55 ± 0.51**^{b,c,d}	**4.15 ± 0.68**^{f}	**5.11 ± 0.52**^{g,h}	**5.19 ± 0.41**^{h}	**5.29 ± 0.72**^{h}	**5.30 ± 1.08**^{h}
	IBS002	20% (4/20)	15% (3/20)	Recombinant genotype VII virus	2.48 ± 0.52^{b,c,d}	1.92 ± 0.53^{b}	1.98 ± 0.59^{b}	2.35 ± 0.51^{b,c}	2.87 ± 0.80^{c,d}	3.05 ± 1.96^{c,d}

*GMT expressed as reciprocal $\log_2$, The HI test was carried out with four HA NDV antigens. Different superscript letters denote significant differences ($p < 0.05$) between groups on different sampling days. [†]Homologous responses are noted in bold. [‡,§]Observation of clinical signs and death caused by challenge with NDV in broiler chickens. GMT, geometric mean titer; HI, hemagglutination inhibition; HA, hemagglutination; ND: not detected.

Table 3. Detection of viral shedding from commercial broilers on days 3, 5, 7, and 14 post-challenge with genotype VIII NDV isolate AF2240-I and genotype VII NDV isolate IBS002

Day post-challenge	Swab	AF2240-I			IBS002		
		Viral copy number, $\log_{10}$ (mean ± SD)*			Viral copy number, $\log_{10}$ (mean ± SD)*		
		Control	House 1	House 2	Control	House 1	House 2
3	Cloacal	9.55 ± 0.59^{d} (6/6)†	ND^{a} (0/6)	ND^{a} (0/6)	ND^{a} (0/6)	ND^{a} (0/6)	ND^{a} (0/6)
	Oropharyngeal	$7.59 \pm 0.61^{c,d}$ (6/6)	ND^{a} (0/6)	ND^{a} (0/6)	ND^{a} (0/6)	ND^{a} (0/6)	0.98 ± 2.39^{b} (1/6)
5	Cloacal	11.15 ± 0.54^{d} (6/6)	1.71 ± 4.18^{b} (1/6)	$4.72 \pm 5.19^{b,c}$ (3/6)	11.10 ± 0.61^{d} (6/6)	1.35 ± 3.31^{b} (1/6)	$7.64 \pm 4.18^{c,d}$ (5/6)
	Oropharyngeal	9.97 ± 0.73^{d} (6/6)	1.46 ± 3.26^{b} (1/6)	$4.52 \pm 4.96^{b,c,c}$ (3/6)	10.27 ± 0.50^{d} (6/6)	0.93 ± 2.28^{b} (1/6)	$7.47 \pm 3.98^{c,d}$ (5/6)
7	Cloacal	11.51 ± 0.30^{d} (6/6)	ND^{a} (0/6)	$4.01 \pm 4.40^{b,c}$ (3/6)	10.80 ± 0.79^{d} (6/6)	ND^{a} (0/6)	$3.94 \pm 4.33^{b,c}$ (3/6)
	Oropharyngeal	$9.54 \pm 0.67^{c,d}$ (6/6)	ND^{a} (0/6)	ND^{a} (0/6)	$7.48 \pm 0.46^{c,d}$ (6/6)	ND^{a} (0/6)	$3.81 \pm 4.22^{b,c}$ (3/6)
14	Cloacal	NS	ND^{a} (0/6)	ND^{a} (0/6)	NS	ND^{a} (0/6)	ND^{a} (0/6)
	Oropharyngeal	NS	ND^{a} (0/6)	ND^{a} (0/6)	NS	ND^{a} (0/6)	ND^{a} (0/6)

*Different superscript letters denote significant differences ($p < 0.05$) between groups on different sampling days. †Swabs were randomly taken from six birds in each group and evaluated to quantify virus shedding. Frequency of birds detected with challenge virus is shown in parenthesis and expressed as the number of positive swabs/total number of swabs tested. ND, not detected NS, no survival.

of the C-terminus extension length of the HN gene revealed that all five isolates had the predicted total length of 571 amino acids for the HN protein and no extension in amino acids sequence was observed. Previous study has shown that isolates with HN proteins containing 571 amino acids are exclusively viscerotropic velogenic NDVs [9].

A representative NDV, isolate IBS002, was selected for further analysis based on results of the *in vivo* pathotyping study. An MDT of 51.2 h and ICPI of 1.76 confirmed that the isolated virus was a velogenic NDV. Moreover, phylogenetic studies on the full F gene revealed that IBS002 is classified as a genotype VII NDV and clusters together with other genotype VII isolates from Indonesia [38], Cambodia [8], and China [39]. The distance matrix analysis of the full-length F and HN genes demonstrated that IBS002 had the highest amino acid variation compared to the genotype II LaSota vaccine (18.67%–23.37%) followed by genotype VIII NDV isolate AF2240-I (9.12%–15.34%). These findings concur with data from a previous full-length nucleotide sequence analysis of the F genes from 103 NDV strains carried out by Diel *et al*. [11] indicating that genotype VII NDV had an overall amino acid distance of 21.5% with genotype II and 12.8% with genotype VIII isolates. It was expected that the F and HN amino acid sequence of IBS002 would share a close similarity (9.77%–11.60%) with the recombinant genotype VII vaccine strain since the recombinant vaccine contains both F and HN genes originating from a contemporary genotype VII virus, KBNP-4152, from Korea [7,8].

SPF chickens have been used in most studies that evaluated the efficacy of genotype-matched NDV vaccines [16,18]. In the current investigation, the recombinant genotype VII NDV inactivated vaccine was introduced into the current commercial broiler vaccination program in the field and its efficacy against velogenic NDV challenge was assessed. Evaluation of the vaccination program showed that commercial broilers treated with genotype II vaccines (LaSota and B1) on the day of hatching and 14 days of age were susceptible to velogenic NDV challenge. These results confirmed that introduction of the recombinant genotype VII NDV inactivated vaccine provided better protection against morbidity and mortality.

HI antibody titres of all chickens vaccinated with the recombinant genotype VII inactivated vaccine (House 1) were not significantly different when tested with both LaSota and recombinant genotype VII antigens since the birds in House 1 received a combination of LaSota and recombinant genotype VII inactivated vaccines. However, chickens that were vaccinated with live and killed genotype II vaccines (House 2) presented significantly higher HI antibody titres when tested with the homologous LaSota antigen. These findings further confirmed that HI antibody titres can greatly vary depending on the homologous and heterologous antigens used for testing. Homologous antigens were found to identify higher HI antibody

titres compared to heterologous antigens [15]. In the current study, two different antigens (namely the LaSota virus and recombinant genotype VII virus) were used for the HI assay. Presence of the LaSota virus backbone of the recombinant vaccine had minimal influence on the antibody levels measured with the HI assay. The reason is the HI assay detected antibody specific for the HN surface antigen, which is known to correspond to antibody that provides protection from disease. The recombinant genotype VII vaccine expresses the HN gene of genotype VII NDV [7] that is different from the HN gene of LaSota (genotype II). Thus, birds that were vaccinated with recombinant genotype VII inactivated vaccine had higher antibody titers when tested with genotype VII antigen. In addition, a previous study by Miller *et al*. [27] indicated the importance of testing the same vaccinates against antigens of the likely challenge virus when measuring the HI antibody in order to obtain a better estimation of the immune response and protection level of vaccinated birds.

NDV is mostly transmitted from an infected chicken to a susceptible one via a faecal-oral route [4]. Feed and water sources could be contaminated by NDV shed from the upper respiratory tract of diseased chickens and transmitted to other birds. Aside from mortality and morbidity, levels of virus shedding in poultry houses are also an important for measuring disease control and prevention [17,29] since mass-vaccination can protect only about 60% of the flock [10]. Apart from antibody production, controlling virus shedding can therefore help prevent chickens with insufficient levels of immunity from being infected [18].

NDV strains have been divided into different genotypes (I–XI) based on the F gene sequence. However, these viruses are antigenically recognized as one single serotype. Currently, the most widely used LaSota and B1 vaccines that belong to genotype II offer substantial protection against morbidity and mortality caused by a virulent NDV. However, neither vaccine completely prevents infection or virus shedding in vaccinated birds. Previous studies have demonstrated that genotype-matched vaccinations reduce virus shedding following challenge with velogenic NDV isolates more efficiently compared to the LaSota strain [16,26]. Similar results were found in the current study in which the recombinant genotype VII inactivated vaccine provided better control and prevention of virus shedding after NDV infection. In the vaccine trial with commercial broiler chickens, the recombinant genotype VII inactivated vaccine reduced significant virus shedding (fewer birds shed the virus and at lower concentration) compared to the LaSota vaccine at day 5 p.c. with IBS002 and AF2240-I. No virus shedding from recombinant genotype VII vaccinated birds was detected by day 7 p.c. However, three out of six birds vaccinated with LaSota shed the virus following challenge with IBS002 and AF2240-I at day 7 p.c.

In conclusion, the causative agent of recent ND outbreaks in vaccinated broiler flocks from Malaysia was found to belong to velogenic genotype VII. This strain was genetically close to other Malaysian genotype VII isolates obtained during the last decade. Results of the vaccine efficacy trial indicated that the recombinant genotype VII NDV inactivated vaccine was able to provide full protection against challenge with genotype VII NDV isolate IBS002 and genotype VIII isolate AF2240-I. It was notable that LaSota vaccinated commercial broilers were protected from AF2240-I challenge; however, this vaccine conferred only partial protection against IBS002 challenge. In addition, the recombinant genotype VII vaccine significantly reduced virus shedding upon challenge with genotype VII and genotype VIII NDV compared to the LaSota vaccine. Overall, the recombinant genotype VII vaccine may provide better ND control among commercial broilers.

Acknowledgments

We thank Dr. David Kho (Nutri Pharmax Sdn. Bhd, Malaysia) for providing the recombinant genotype VII NDV vaccine and commercial broiler chickens. The study was supported by an Institute of Bioscience, Higher Institution Centre of Excellence (IBS HICoE) grant from the Ministry of Higher Education, Malaysia.

Conflict of Interest

There is no conflict of interest.

References

1. **Abolnik C, Horner RF, Bisschop SP, Parker ME, Romito M, Viljoen GJ.** A phylogenetic study of South African *Newcastle disease virus* strains isolated between 1990 and 2002 suggests epidemiological origins in the Far East. Arch Virol 2004, **149**, 603-619.
2. **Aini I, Ibrahim AL, Spradbrow PB.** Field trials of a food-based vaccine to protect village chickens against Newcastle disease. Res Vet Sci 1990, **49**, 216-219.
3. **Alexander DJ.** Newcastle disease diagnosis. In: Alexander DJ (ed.). Newcastle Disease. pp. 147-160, Kluwer Academic Publishers, Boston, 1988.
4. **Alexander DJ.** Newcastle disease: methods of spread. In: Alexander DJ (ed.). Newcastle Disease. pp. 256–272, Kluwer Academic Publishers, Boston, 1988.
5. **Berhanu A, Ideris A, Omar AR, Bejo MH.** Molecular characterization of partial fusion gene and C-terminus extension length of haemagglutinin-neuraminidase gene of recently isolated Newcastle disease virus isolates in Malaysia. Virol J 2010, **7**, 183.
6. **Bogoyavlenskiy A, Berezin V, Prilipov A, Usachev E, Lyapina O, Korotetskiy I, Zaitceva I, Asanova S, Kydyrmanov A, Daulbaeva K, Shakhvorostova L, Sayatov M, King D.** Newcastle disease outbreaks in Kazakhstan and Kyrgyzstan during 1998, 2000, 2001, 2003, 2004, and 2005 were caused

by viruses of the genotypes VIIb and VIId. Virus Genes 2009, **39**, 94-101.
7. **Cho SH, Kwon HJ, Kim TE, Kim JH, Yoo HS, Park MH, Park YH, Kim SJ.** Characterization of a recombinant Newcastle disease virus vaccine strain. Clin Vaccine Immunol 2008, **15**, 1572-1579.
8. **Choi KS, Kye SJ, Kim JY, Damasco VR, Sorn S, Lee YJ, Choi JG, Kang HM, Kim KI, Song BM, Lee HS.** Molecular epidemiological investigation of velogenic Newcastle disease viruses from village chickens in Cambodia. Virus Genes 2013, **47**, 244-249.
9. **Czeglédi A, Ujvári D, Somogyi E, Wehmann E, Werner O, Lomniczi B.** Third genome size category of avian paramyxovirus serotype 1 (Newcastle disease virus) and evolutionary implications. Virus Res 2006, **120**, 36-48.
10. **Degefa T, Dadi L, Yami A, Gmariam K, Nassir M.** Technical and economic evaluation of different methods of Newcastle disease vaccine administration. J Vet Med A Physiol Pathol Clin Med 2004, **51**, 365-369.
11. **Diel DG, da Silva LH, Liu H, Wang Z, Miller PJ, Afonso CL.** Genetic diversity of avian paramyxovirus type 1: proposal for a unified nomenclature and classification system of Newcastle disease virus genotypes. Infect Genet Evol 2012, **12**, 1770-1779.
12. **Diel DG, Susta L, Cardenas Garcia S, Killian ML, Brown CC, Miller PJ, Afonso CL.** Complete genome and clinicopathological characterization of a virulent Newcastle disease virus isolate from South America. J Clin Microbiol 2012, **50**, 378-387.
13. **Habibian M, Ghazi S, Moeini MM, Abdolmohammadi A.** Effects of dietary selenium and vitamin E on immune response and biological blood parameters of broilers reared under thermoneutral or heat stress conditions. Int J Biometeorol 2014, **58**, 741-752.
14. **Herczeg J, Wehmann E, Bragg R, Travassos Dias PM, Hadjiev G, Werner O, Lomniczi B.** Two novel genetic groups (VIIb and VIII) responsible for recent Newcastle disease outbreaks in Southern Africa, one (VIIb) of which reached Southern Europe. Arch Virol 1999, **144**, 2087-2099.
15. **Hu Z, Hu S, Meng C, Wang X, Zhu J, Liu X.** Generation of a genotype VII Newcastle disease virus vaccine candidate with high yield in embryonated chicken eggs. Avian Dis 2011, **55**, 391-397.
16. **Hu S, Ma H, Wu Y, Liu W, Wang X, Liu Y, Liu X.** A vaccine candidate of attenuated genotype VII Newcastle disease virus generated by reverse genetics. Vaccine 2009, **27**, 904-910.
17. **Jeon WJ, Lee EK, Lee YJ, Jeong OM, Kim YJ, Kwon JH, Choi KS.** Protective efficacy of commercial inactivated Newcastle disease virus vaccines in chickens against a recent Korean epizootic strain. J Vet Sci 2008, **9**, 295-300.
18. **Kapczynski DR, Afonso CL, Miller PJ.** Immune responses of poultry to Newcastle disease virus. Dev Comp Immunol 2013, **41**, 447-453.
19. **Kattenbelt JA, Stevens MP, Selleck PW, Gould AR.** Analysis of Newcastle disease virus quasispecies and factors affecting the emergence of virulent virus. Arch Virol 2010, **155**, 1607-1615.
20. **Ke GM, Liu HJ, Lin MY, Chen JH, Tsai SS, Chang PC.** Molecular characterization of Newcastle disease viruses isolated from recent outbreaks in Taiwan. J Virol Methods 2001, **97**, 1-11.
21. **Lai MC, Ibrahim A.** Velogenic viscerotropic Newcastle disease virus. In: Copland JW (ed.). Newcastle Disease in Poultry: A New Food Pellet Vaccine. 5th ed. pp. 33-34, ACIAR, Canberra, 1987.
22. **Lee TC, Yusoff K, Nathan S, Tan WS.** Detection of virulent Newcastle disease virus using a phage-capturing dot blot assay. J Virol Methods 2006, **136**, 224-229.
23. **Liu XF, Wan HQ, Ni XX, Wu YT, Liu WB.** Pathotypical and genotypical characterization of strains of *Newcastle disease virus* isolated from outbreaks in chicken and goose flocks in some regions of China during 1985−2001. Arch Virol 2003, **148**, 1387-1403.
24. **Liu H, Wang Z, Wu Y, Zheng D, Sun C, Bi D, Zuo Y, Xu T.** Molecular epidemiological analysis of Newcastle disease virus isolated in China in 2005. J Virol Methods 2007, **140**, 206-211.
25. **Mahon PJ, Mirza AM, Iorio RM.** Role of the two sialic acid binding sites on the Newcastle disease virus HN protein in triggering the interaction with the F protein required for the promotion of fusion. J Virol 2011, **85**, 12079-12082.
26. **Miller PJ, Estevez C, Yu Q, Suarez DL, King DJ.** Comparison of viral shedding following vaccination with inactivated and live Newcastle disease vaccines formulated with wild-type and recombinant viruses. Avian Dis 2009, **53**, 39-49.
27. **Miller PJ, King DJ, Afonso CL, Suarez DL.** Antigenic differences among Newcastle disease virus strains of different genotypes used in vaccine formulation affect viral shedding after a virulent challenge. Vaccine 2007, **25**, 7238-7246.
28. **Murulitharan K, Yusoff K, Omar AR, Molouki A.** Characterization of Malaysian velogenic NDV strain AF2240-I genomic sequence: a comparative study. Virus Genes 2013, **46**, 431-440.
29. **Palya V, Kiss I, Tatár-Kis T, Mató T, Felföldi B, Gardin Y.** Advancement in vaccination against Newcastle disease: recombinant HVT NDV provides high clinical protection and reduces challenge virus shedding with the absence of vaccine reactions. Avian Dis 2012, **56**, 282-287.
30. **Pedersen JC, Senne DA, Woolcock PR, Kinde H, King DJ, Wise MG, Panigrahy B, Seal BS.** Phylogenetic relationships among virulent Newcastle disease virus isolates from the 2002-2003 outbreak in California and other recent outbreaks in North America. J Clin Microbiol 2004, **42**, 2329-2334.
31. **Rasoli M, Yeap SK, Tan SW, Moeini H, Ideris A, Bejo MH, Alitheen NBM, Kaiser P, Omar AR.** Alteration in lymphocyte responses, cytokine and chemokine profiles in chickens infected with genotype VII and VIII velogenic Newcastle disease virus. Comp Immunol Microbiol Infect Dis 2014, **37**, 11-21.
32. **Rui Z, Juan P, Jingliang S, Jixun Z, Xiaoting W, Shouping Z, Xiaojiao L, Guozhong Z.** Phylogenetic characterization of Newcastle disease virus isolated in the mainland of China during 2001−2009. Vet Microbiol 2010, **141**, 246-257.
33. **Saif YM.** Immunosuppression induced by infectious bursal disease virus. Vet Immunol Immunopathol 1991, **30**, 45-50.

34. **Seal BS, King DJ, Sellers HS.** The avian response to Newcastle disease virus. Dev Comp Immunol 2000, **24**, 257-268.
35. **Tan SW, Ideris A, Omar AR, Yusoff K, Hair-Bejo M.** Sequence and phylogenetic analysis of Newcastle disease virus genotypes isolated in Malaysia between 2004 and 2005. Arch Virol 2010, **155**, 63-70.
36. **Wang Z, Liu H, Xu J, Bao J, Zheng D, Sun C, Wei R, Song C, Chen J.** Genotyping of Newcastle disease viruses isolated from 2002 to 2004 in China. Ann N Y Acad Sci 2006, **1081**, 228-239.
37. **World Organisation for Animal Health.** Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. Vol. 1. Chap. 2.3.14. Newcastle disease, OIE, Paris, 2012.
38. **Xiao S, Paldurai A, Nayak B, Samuel A, Bharoto EE, Prajitno TY, Collins PL, Samal SK.** Complete genome sequences of Newcastle disease virus strains circulating in chicken populations of Indonesia. J Virol 2012, **86**, 5969-5970.
39. **Xie Z, Xie L, Chen A, Liu J, Pang Y, Deng X, Xie Z, Fan Q.** Complete genome sequence analysis of a Newcastle disease virus isolated from a wild egret. J Virol 2012, **86**, 13854-13855.
40. **Yi J, Liu C, Chen B, Wu S.** Molecular characterization of a virulent genotype VIId strain of Newcastle disease virus from farmed chickens in Shanghai. Avian Dis 2011, **55**, 279-284.