Characterization of Malaysian velogenic NDV strain AF2240-I genomic sequence: a comparative study

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Abstract Newcastle disease virus (NDV) strain AF2240 is a viscerotropic velogenic strain that is used as a vaccine challenge virus in Malaysia. The identification of the full length genome will be a crucial platform for further studies of this isolate. In this study, we fully sequenced the genome of a derivative of this strain named AF2240-I. The 15,192 nt long genome contains a 55-nt leader sequence at the 3' whereas the trailer region consists of 114 nt at the 5'. The intergenic sequences between the NP-P, P-M, M-F, F-HN, and HN-L genes comprise 1, 1, 1, 31, and 47 nt, respectively. The acknowledged cleavage site of fusion protein showed amino acid sequence of 112-R-R-Q-K-R-F-117, which corresponds to those of virulent NDV strains. Phylogenetic analysis of the whole virus genome shows that the strain AF2240-I belongs to genotype VIII and is more closely related to velogenic strains QH1, QH4, Fontana, Largo, and Italianas compared to other strains of NDV. Differences are noticed in the hemagglutinin-neuraminidase (HN) and matrix (M) gene between AF2240 and its derivative AF2240-I. This is the first report of a complete genome sequence of an NDV strain isolated in Malaysia.

Keywords Newcastle disease virus · Strain AF2240-I · Complete genome sequence

Abbreviations

UTR Untranslated region

IGS Intergenic sequences
RACE Rapid amplification of cDNA ends
ORF Open reading frame

Introduction

Newcastle disease (ND) is a fatal avian disease that affects most species of birds and currently remains as a constant major threat to commercial poultry production [1]. The severity of the infection varies with different species, and it has led to severe economic losses in the poultry industry worldwide. First detected in Java, Indonesia in 1926 and in Newcastle-on-Tyne, England in 1927 [2, 3], ND is now recognized as a highly contagious disease. The disease is caused by Newcastle disease virus (NDV) or avian paramyxovirus 1 which is a member of the genus *Avulavirus* of the family *Paramyxoviridae* [4]. It has various degrees of severity depending on the strain of virus and is classified into three pathotypes: the lentogenic strains are non-virulent, the mesogenic strains are intermediate virulent, and the velogenic (velogenic neurotropic or velogenic viscerotropic) strains are highly virulent and cause high mortality [5].

The genome of NDV contains a non-segmented single-stranded negative-sense RNA of approximately 15.2 kb [6–8]. Its structure is in the order of 3'–5' and contains six genes which encode the nucleocapsid (NP) protein, phosphoprotein (P), matrix (M) protein, fusion (F) protein, hemagglutinin-neuraminidase (HN) protein, and large (L) protein. During replication, two additional proteins known as V and W proteins [9, 10] are also produced by RNA editing during P gene transcription. The anti-genomic RNA serves as a template for the synthesis of full-length

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genomic RNA [11]. The replication of NDV strictly follows “the rule of six” where efficient replication only takes place if the genome size is a multiple of six nucleotides (nt) [8]. The gene is flanked by a leader and a trailer sequence at its 3′ and 5′ ends, respectively [12]. Meanwhile, each gene contains an untranslated region (UTR) which is a conserved transcriptional control sequence at the beginning and end of the gene, known as the gene start and gene end, respectively [13, 14]. UTR is known to play a role in viral genome transcription, translation, and virulence effect [15]. Located between the gene boundaries are non-coding intergenic sequences (IGS). The length within this region varies from 1 to 47 nt [6, 16].

Classification of NDV is commonly conducted by categorizing NDV into two major divisions, Class I and Class II. Class I consists of strains of genome size 15,198 bp, and Class II contains strains of genome size 15,186 and 15,192 bp. In addition, Class I comprises only avirulent strains of NDV [17]. Class II can be further divided into ten genotypes I to X [17–21]. The first panzootic in the 1920s was caused by genotypes II, III, and IV, and the second and third panzootics were caused by genotypes V–VI [22–24]. Genotype VII which is further subdivided into five sub-genotypes (VII a–e) is responsible for the fourth panzootic which is currently on-going [18, 20, 25–28]. Finally, genotypes VIII, IX, and X have been found in South Africa, China, and Taiwan, respectively [18, 19, 21, 29]. The most recently proposed classification system of NDV genotypes was based on complete F gene sequences, and it revealed that Class I viruses contain a single genotype while Class II NDV isolates contain 15 genotypes [30].

AF2240 is a Malaysian viscerotropic velogenic strain that was isolated during an outbreak in the country in the 1960s [31]. There have been several *in vivo* and *in vitro* studies conducted on this strain on its oncolytic effect on brain tumor cells, MCF-7 breast cancer cells, HeLa cervical cancer cells and also on its cytolytic effects against leukemic cell lines [32–36]. Currently, it is used as the challenge virus in vaccine trials in Malaysia [37]. The sequences of six genes of this strain were submitted to NCBI from various authors over a period of time ranging from 1995 to 2005. HN gene was sequenced in 1995 [38], M and F gene in 1998, P and NP genes were sequenced in 2000, and L gene in 2003 (GenBank: AF060563, AY262106, AF048763, AF284647, AF284646, and X79092). We decided to complete the remaining genome sequence of this strain (UTR, promoters, 3′ leader, and 5′ trailer) and re-amplify the genes for verification. However, due to the findings of some differences with the previously reported gene sequence of AF2240, we have renamed this strain as AF2240-I, a derivative of AF2240. We report here the complete genome sequence of this derivative and its relationship with other strains.

Results and discussion

Complete genome analysis

The amplification of HN gene of the strain given (initially presumed AF2240) has indicated the presence of Arg 403 residue in the HN gene, which was reported to be absent in the HN gene of strain AF2240 [38]. A frameshift was also observed between the amplified M gene of AF2240-I and the published sequence of AF2240. AF2240-I notes a distinguishable frameshift with a deletion of an adenosine at position 585 and an addition of thymine at position 933 of the ORF in the M gene. The frameshift may be due to the interference of quasispecies contributed by mutations that occur through errors made in the process of copying existing RNA genome sequences [39]. Previously recorded frameshifts were noted in the L gene of NDV strain Clone30, a vaccine strain derived from the lentogenic NDV strain La Sota [40]. With the exception of the HN and M gene differences noted in the sequencing results of AF2240, the remaining four genes were highly similar and differed by only 7 nt at most, located in the ORFs of the genes. The dissimilarities in the HN and M gene had led to the decision for the strain to be renamed AF2240-I, and its complete genome sequence was deposited into GenBank [GenBank: JX012096]. The possibility of these differences may lie in the numerous passaging history of the strain AF2240. The presence of Arg403 at the exact same position that it was reported missing [38] may also indicate a possibility that the strains were derived independently from a common progenitor.

The genome of AF2240-I is 15,192 nt in length, which follows the rule of six for a competent viral replication. The difference in genome lengths is contributed by the additional 6 nt in the 3′ UTR of NP gene or 12 nt in the ORF of P gene [22, 41]. AF2240-I contains the 6 nt insertion confirming the genome length is 15,192 nt.

Whole genome comparison was performed on AF2240-I and 63 other NDV strains. Table 1 shows that strains QH1, QH4, Fontana, Largo, and Italien shared the highest identity of nt (>90 %) with strain AF2240-I, in contrast to the strains of Class I which showed the least nucleotide identity. QH1 and QH4 are of genotype VIII while Fontana, Largo, and Italien belong to genotypes VI, V, and IV, respectively.

As previously recorded, there were considerable variations observed in the non-coding regions of NP and P genes among virulent and non-virulent strains [6]. The velogenic strains containing 15,192 nt genome length share ≥ 88 % sequence homology at nt level. QH1 and QH4 are highly similar with 93 % homology. Strains of 15,186 nt genome lengths differ in nt identity based on the strain virulence. Italien and Herts/33 (genotype IV) show a similarity of

Table 1 Comparison of whole genome sequence of various NDV strains with AF2240-I

Strains	Years	Origin	Pathogenicity	Genotype	Accession no.	Genome similarity
QH1	1979	Western China	Velogenic	VIII	FJ751919	93
QH4	1985	Western China	Velogenic	VIII	FJ751918	93
Fontana	1972	USA	Velogenic	VI	AY562988	92
Italien	1944	Italy	Velogenic	IV	EU293914	92
Largo	1971	USA	Velogenic	V	AY562990	92
Mexico P05	2005	Mexico	Velogenic	V	HM117720	91
ZhJ-3	1997	China	Velogenic	VI	FJ766529	91
Herts/33	1933	UK	Velogenic	IV	AY74140	91
Sweden/95	1995	Sweden	Velogenic	VI	HQ839733	90
Cockatoo	1990	Indonesia	Velogenic	VII	AY562985	90
NDV-2/chicken/Namakkal	1987	India	Velogenic	IV	GU187941	90
XJ-2/97	1997	China	Velogenic	VII	JN618348	89
Fujian/FP1	2002	China	Velogenic	VII	FJ872531	89
Mukteswar	2010	China	Mesogenic	III	JF950509	89
Guangxi14	2002	China	Velogenic	VII	JX193075	89
JS/07/16/Pi	2007	China	Velogenic	VI	FJ766527	89
NA-1	n/a	China	Velogenic	VII	DQ659677	89
Dove/Italy	2000	Italy	Velogenic	VI	AY562989	89
Sweden/97	1997	Sweden	Velogenic	VII	GU585905	89
ZJ1	2000	China	Velogenic	VII	AF431744	89
Pi/Rus/Vladimir/687	2005	Russia	Velogenic	VI	JF827026	89
PX2/03	2003	China	Velogenic	VII	JN653340	89
Guangxi7	2002	China	Velogenic	VII	DQ485229	89
China/SDLC	2011	China	n/a	VI	JQ979176	89
F48E9	1946	China	Lentogenic	IX	FJ436302	89
Guangxi11	2003	China	Velogenic	VII	DQ485231	89
GM	n/a	China	Velogenic	VII	DQ486859	89
Rus/Kemerovo/0267	2009	Russia	Velogenic	VI	JF827027	89
SRZ03	2003	China	Velogenic	II	EU167540	88
2K3 Chennai	2000	India	Velogenic	IV	FJ986192	88
Ulster/67	1967	UK	Lentogenic	I	M24694	88
YZCQ/Liaoning	2008	China	Velogenic	VII	JX867334	88
Go/CH/HLJ/LL01	2008	China	Velogenic	VII	GU143550	88
Egret/China/Guangxi	2011	China	Velogenic	VII	JX193074	88
Banjarmasin	2011	Indonesia	Velogenic	VII	HQ697254	88
Sukorejo	2011	Indonesia	Velogenic	VII	HQ697255	88
V4	1966	Australia	Lentogenic	I	JX524203	88
NDV4-C	1996	China	Lentogenic	I	JX443519	88
TS09-C	2009	China	n/a	I	JX110635	88
Peru/1918-03	2008	Peru	Velogenic	VII	JN800306	88
China/SD04	2011	China	Velogenic	VII	JQ015296	88
China/SDWF07	2011	China	n/a	VII	JQ015295	88
Togo/ako	2009	Togo	Velogenic	VII	JX390609	88
CP/Pakistan	2010	Pakistan	Velogenic	VII	JN682211	88
MM19	2012	Pakistan	Velogenic	VII	JX532092	87
WDK/JX/7793	2004	China	Lentogenic	I	HM125898	87
M4	2007	China	n/a	I	JN653339	87
Dominican Rep.	2008	Dominican Rep.	Velogenic	n/a	JX119193	87

Table 1 continued

Strains	Years	Origin	Pathogenicity	Genotype	Accession no.	Genome similarity
CBU2374	2007	South Korea	n/a	I	JX401405	87
Guangxi16	2008	China	Lentogenic	I	JX193077	87
D3	2007	China	Lentogenic	I	HM063422	87
BHG/Sweden	1994	Sweden	Lentogenic	I	GQ918280	86
Egypt/1	2005	Egypt	Velogenic	II	FJ939313	86
NDV2K17/Quail/Chennai	1998	India	Velogenic	II	HQ902590	86
HX01	2009	China	Lentogenic	II	JF795531	86
Clone30	n/a	USA	Lentogenic	II	Y18898	86
La Sota	1946	USA	Lentogenic	II	JF950510	86
B1	1947	USA	Lentogenic	II	AF077761	86
Guangxi21	2010	China	Lentogenic	II	JX193082	86
Teal/France	2010	France	Lentogenic	Class I	JQ013039	72
DE-R49	1949	Germany	Lentogenic	Class I	DQ097393	72
D-AH-10-02	2010	China	Lentogenic	Class I	JN688863	72
Ch-Zj1	1986	China	Lentogenic	Class I	JN688863	72

Genome similarity of the nucleotides is shown in percentage. QH1, QH4, Fontana, and Italien show the highest percentage of nucleotide identity (based on pairwise distances calculated using Mega5)

Table 2 Total gene, coding and protein length of NDV strain AF2240-I

Gene/region	Leader	NP	P	M	F	HN	L	Trailer
Length (nt)	55	1,753	1,451	1,241	1,792	2,002	6,703	114
ORF (nt)	–	1,470	1,188	1,095	1,662	1,749	6,615	–
Protein length (nt)	–	489	395	364	553	582	2,204	–
Intergenic (nt)	–	1	1	1	31	47	–	–
Nucleotide/amino acid identity (%) ^a								
QH1	97	97	91	95	97	95	96	91
QH4	97	97	92	95	97	95	96	91
Fontana	91	98	92	98	96	95	96	87
Largo	93	97	89	98	95	94	96	86
Italien	92	97	90	97	95	86	96	87

Intergenic sequences are also provided. The five highest strains of nucleotide or protein similarities are shown

^a Percentage of similarity for leader and trailer was based on nucleotide similarity, and the percentage of similarity for NP, P, M, F, HN, and L proteins was based on amino acid

91 %; meanwhile, Clone 30, La Sota, and B1 (genotype II) are only 86 % similar. Strains of Class I showed a similarity of 72 %.

Similar to other paramyxoviruses, AF2240-I consists of six genes encoding six proteins (Table 2). The start which is the ribosomal binding site of NP, P, M, F, and HN genes was found to be ACGGGTAGAA, and for L, it was found to be ACGGGTAGGA. The gene terminal was TTAGA_{6/7} for NP, M, and L genes, whereas it was TAAGA₆ for P, F, and HN genes.

The 3'-leader and 5'-trailer regions of AF2240-I consist of 55 nt and 114 nt, respectively (Table 2). The 55 nt of the leader sequence was shown to be close to QH1 and QH4

with an identity of 97 % followed by strain Mexico P05 (genotype V) at 94 %. The leader sequence of strain AF2240-I also showed complete similarity to the first 12 nt of 62 strains except strains CP/Pakistan and Namakkal/Tamil. Meanwhile, the trailer sequence of AF2240-I shows a highly conserved region of 20 nt at the 5' region with most strains. (Figure not included). The highly conserved region of the trailer among most strains of varied virulence may not play a role in NDV virulence [6].

The first three IGSs of AF2240-I, the NP-P, P-M, and M-F gene junctions each had only 1 nt. The remaining two IGSs, F-HN and HN-L, contain 31 and 47 nt. There were studies on the unsuccessful attempts to recover viable NDV

through replacing the IGS at the F-HN and HN-L gene junctions. Moreover, changes in the number of nt within these IGS seemed to affect the transcription of the downstream genes and viral pathogenicity [12].

When compared with Class I strains, AF2240-I displayed frameshift mutations in the trailer region, HN-L IGS region, and in all genes except for HN. These insertions and deletions (indels) are also noticed in all other Class II strains at the same nt position. Within the NP gene, indels are noticed at nt positions 1,596, 1,618, 1,681, 1,759, and 1,785 comprising 2 nt insertion, 1 nt deletion, 1 nt insertion, 1 nt insertion, and 3 nt deletion, respectively, compared to those of Class I strains. The highest occurrences of indels are observed in the P gene. Meanwhile, the F gene recorded only two indels; an insertion of adenosine and a deletion of thymine at nucleotide positions 1,718 and 1,748, respectively. These indels may cause low nucleotide similarity between AF2240-I and Class I strains, and this observation may clarify the relationship between Class I and Class II NDVs.

Protein analysis

The complete nucleotide sequence of the NP gene of strain AF2240-I contains 1,753 nt with an open reading frame (ORF) of 1,470. This ORF contains a coding region of 489 amino acid [42] residues. Comparison of the NP protein with those from selected strains of NDV shows that the NP gene is quite conserved among all strains with a conservation value of more than 90 % (Table 3). Fontana and QH1 displayed the highest amino acid similarity of 98 %, while QH4 came second at 97 %. As previously reported, the conservation of amino acids is observed to be high in the first 400 aa and becomes more divergent from 401 aa till the end of the gene [6]. Two unique substitutions of Asn 80 with His and Thr 100 with Ala were recorded in the NP protein of AF2240-I.

The P gene has a length of 1,451 nt with an ORF of 1,188 nt, and it is edited at the editing site (476-CU-AAAAAGGGCCCA-489) to be transcribed into three proteins, P, V, and W [9, 10]. Just as observed in the P gene, there is least variability between Fontana, QH1, QH4, and AF2240-I with a difference below 10 %. Meanwhile, strains of Class I show a similarity of only 27 % indicating a high divergence of the P gene between AF2240-I and Class I strains mainly contributed by the additional 12 nt which are present in all Class I strains. The M protein is involved in the assembly of NDV [37]. AF2240-I translates the ORF of M gene into 364 aa. This frameshift of AF2240-I from its derivative AF2240 has further increased the homology of amino acid of AF2240-I when compared with 63 other strains. The identity value of amino acid was at an average of 94 %; the lowest being

strain Mexico P05 (genotype V) and the highest being strain Fontana (genotype VI). Strains of Class I recorded an identity value of 91 %.

The pathogenicity of NDV strains is mainly determined by the F protein cleavage site [43]. The F gene of 1,792 nt translates the ORF into 1,662 aa. The cleavage site of AF2240-I is 112-R-R-Q-K-R-F-117 which corresponds to those of other virulent strains [41, 44]. In general, when compared with selected strains, the F protein of AF2240-I shows a high conservation value from 85 to 97 %. All strains of Class II show a conservation value of above 90 % of amino acids. There is a high divergence observed at the beginning of the sequence from 2 to 32 aa. There is only one unique substitution of Thr 367 with Ala of AF2240-I.

The HN protein is reported to be responsible for virus–host cell interaction [14, 45]. There have also been reports on the role of HN in virulence determination [46]. A variation in the size of the HN gene of NDV strains has also been reported due to the position of the stop codon resulting in amino acid lengths of 571, 577, 578, 580, 585, and 616 aa [47, 48]. In this paper, we report that the complete nucleotide sequence of the HN gene of the strain AF2240-I is 2,002 nt long with an ORF of 1,749 nt encoding 582 aa.

It has been shown that the length of HN protein differs regardless of strain virulence [22, 47]; meanwhile, genotypes have shown some form of relationship in terms of HN length [49]. Although most strains of genotype VIII possess an HN length of 571 aa, our result shows that the HN of AF2240-I is made up of 582 aa. This is the first report of such length. This indicates that HN protein length varies in spite of coming from the same genotype. Irrespective of different lengths of the HN protein, the identity recorded is still considerably high with strain Fontana at 95 % as compared to that of AF2240-I. In this comparison, five amino acids; Val 50, Phe 56, Val 117, Leu 175, and Met 378 of AF2240-I recorded unique substitutions. The highly conserved amino acid regions that are involved in the activity of sialic acid-binding Arg 174, Tyr 526, Glu 401, and Arg 416 [50] in the HN protein are also present in AF2240-I. L is the largest structural protein of NDV comprising 2,204 aa that are involved in the synthesis of viral RNA [51]. This protein of AF2240-I shows a high conservation value at 96 % with QH4, QH1, Italien, and Fontana. Although it has been reported that the L gene was the most conserved among other genes, in our current study, NP gene showed the highest percentage of amino acid homology [49].

Phylogenetic analysis

Based on the classification system of two major divisions, Class I and Class II [22, 52, 53], it was shown that the F

Table 3 Estimates of evolutionary divergence of each gene between strain AF2240-I and selected strains

Strains	Distance					
	NP	P	M	F	HN	L
B1	92.64	83.80	89.84	90.24	89.58	74.11
Banjarmasin	96.52	84.56	95.33	95.30	92.12	78.53
BHG/Sweden	93.46	84.81	93.68	91.86	90.52	73.91
CBU2374	94.48	86.08	93.68	93.49	91.72	73.54
2K3 Chennai	95.91	87.34	91.21	91.86	89.49	77.89
Sweden/95	96.32	89.37	97.25	96.02	94.57	80.84
China/SDLC	95.09	86.58	95.88	94.21	91.77	79.50
China/SDWF07	96.11	85.82	94.23	93.49	91.77	77.08
Ch-Zj1	91.41	27.00	90.66	84.99	81.72	48.52
Clone30	93.05	86.08	90.38	90.96	89.76	73.91
Cockatoo	94.68	88.10	95.88	96.02	92.99	80.78
CP/Pakistan	94.68	84.56	96.15	94.58	92.12	77.67
D3	94.27	86.84	93.13	93.49	90.97	73.32
D-AH-10-02	90.80	27.00	90.66	84.09	81.90	48.47
DE-R49	90.80	27.00	90.93	86.80	83.62	48.85
Dominican Rep.	95.09	86.84	95.33	91.50	91.24	74.83
Dove/Italy	94.07	86.33	93.41	95.12	92.36	80.35
Egret/China/Guangxi	96.52	86.08	95.60	93.85	93.17	77.78
Egypt/1	93.46	86.08	91.48	91.32	88.54	74.50
F48E9	95.71	86.33	92.03	93.85	91.42	79.01
Fontana	97.96	92.15	97.80	96.38	95.45	84.49
Fujian/FP1	96.11	87.34	95.88	93.85	93.70	79.60
GM	97.14	87.59	95.60	93.31	93.35	78.85
Go/CH/HLJ/LL01	95.71	86.58	95.33	93.31	93.35	77.40
Guangxi7	95.71	87.34	94.78	93.31	93.35	79.07
Guangxi11	96.73	86.84	94.78	93.67	92.64	78.53
Guangxi14	96.52	87.85	95.88	93.85	93.17	78.80
Guangxi16	93.87	85.82	93.96	92.41	91.03	73.70
Guangxi21	92.84	86.08	90.38	90.96	89.24	73.75
Herts/33	96.73	90.63	95.60	95.30	92.47	82.34
HX01	93.05	86.33	90.11	90.78	89.06	74.02
Italien	97.14	90.63	95.88	95.12	92.99	82.98
JS/07/16/Pi	94.89	86.84	95.88	94.94	92.99	80.09
Rus/Kemerovo/0267	95.50	85.32	95.88	92.59	92.29	78.37
Largo	97.14	88.86	97.53	94.58	94.05	85.78
La Sota	91.82	86.33	90.11	90.78	89.76	73.79
M4	95.09	84.56	94.23	93.67	91.21	73.81
Mexico P05	96.52	86.84	85.99	96.02	93.87	83.79
MM19	94.89	84.56	86.81	93.31	88.79	77.28
Mukteswar	94.68	86.08	92.31	93.13	91.42	80.62
NA-1	95.91	87.34	95.88	94.76	93.17	78.96
NDV-2/chicken/Namakkal	95.71	87.34	92.03	92.22	88.79	82.98
NDV2K17/Quail/Chennai	92.64	86.33	91.21	91.50	88.19	74.13
NDV4-C	94.89	86.08	94.23	93.13	91.21	76.70
Peru/1918-03	95.91	84.81	95.33	93.85	91.24	77.67
Pi/Rus/Vladimir/687	96.52	85.57	95.05	93.85	92.29	79.07
PX2/03	96.11	87.09	95.60	93.84	93.35	78.90

Table 3 continued

Strains	Distance					
	NP	P	M	F	HN	L
QH1	97.55	91.39	95.60	97.29	95.27	86.53
QH4	97.34	91.90	95.60	96.93	94.75	86.63
China/SD04	96.11	86.33	94.78	93.49	92.29	77.19
SRZ03	97.14	86.84	95.60	91.68	93.17	78.31
Sukorejo	96.93	86.33	95.88	95.48	91.77	77.40
Sweden/97	96.11	86.58	96.70	94.58	92.64	80.03
Teal/France	91.21	26.00	92.31	87.52	84.28	48.95
Togo/ako	94.27	87.85	95.05	93.49	92.12	76.69
TS09-C	94.89	86.08	93.96	92.95	90.86	76.70
Ulster/67	94.68	86.08	91.48	92.59	91.55	77.08
V4	94.89	86.08	94.23	93.13	91.38	76.76
WDK/JX/7793	95.30	86.08	94.51	93.67	91.90	73.81
XJ-2/97	96.93	86.84	96.15	94.94	94.22	79.96
YZCQ/Liaoning	96.52	84.30	95.05	93.67	93.35	77.78
ZhJ-3	97.34	91.14	97.80	96.20	95.45	82.02
ZJ1	96.52	86.84	95.60	93.31	93.35	78.96

The number of amino acid differences per site between AF2240-I and 63 strains in all six gene sequences is shown. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA5

gene of strain AF2240 belongs to genotype VIII of class II [28]. Meanwhile, under the new unified classification of NDV genotypes of F gene [30], AF2240 is still classified as genotype VIII. Using the previous classification method based on two major divisions, a phylogenetic tree of the F gene and full length of AF2240-I show similar result to that of AF2240 and suggest a phylogenetic clustering with strains QH1 and QH4 (genotype VIII). The sequence homology among the two isolates of genotype VIII, QH1 and QH4, was 93 % (Fig. 1).

The high degree of nucleotide identity identified with certain velogenic strains would serve as a reference for future studies on AF2240-I. Availability of the complete genome sequence of strain AF2240-I for the first time in Malaysia is crucial in engineering its full length virus for various applications. The most profound application of genetically engineered virus is for vaccine development and as a therapeutic agent.

Methods and materials

Preparation of virus and RT-PCR

Newcastle disease virus strain AF2240 was available from virus stock at Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia. It was grown in 9-day-old embryonated SPF chicken eggs and purified as

previously described [54]. Viral genomic RNA was isolated with Trizol reagent (Invitrogen, USA). The cDNA for UTR region was made with RevertAid Premium first strand cDNA synthesis kit (Thermo, USA) using gene-specific primers.

Amplification of the untranslated regions

PCR primer sets were designed from the available sequences of strain AF2240 [GenBank: AF060563, AY262106, AF048763, AF284647, AF284646, and X79092]. Primers used to amplify all UTRs are as follows: primers 5'-GCACAGAGCACCAACCCACCC-3' and 5'-TCGGGGTTGTCCTGGCTGGT-3' for NP-P, primers 5'-GCGGGCGGACCTGATATGGG-3' and 5'-GCTGCTGGAGGGAAGGGCAG-3' for P-M, primers 5'-AGGCACCAACGTGCTGTCG-3' and 5'-CTGCAAGTGGCCTGCCGTCT-3' for M-F, primers 5'-TCCGGTGTAGTTAGCCTGGT-3' and 5'-ACGGA GACTCAAGGGCCACC-3' for F-HN, and primers 5'-AAGGCAGCATACACGACATCGACA-3' and 5'-TGGAGCGGCCGACTCAAGTA-3'. All PCR products were amplified using Platinum *Pfx* polymerase (Invitrogen, USA) followed by gel purification with QIAquick Gel Extraction Kit (Qiagen, USA) and cloned into pJET 1.2/blunt cloning vector using CloneJET PCR Cloning Kit (Thermo, USA). Several independent clones were sequenced from both directions to verify the plasmid insertion.

Fig. 1 The phylogenetic tree inferred using the neighbor-joining method. **a** Phylogenetic tree of the full length sequence. **b** Phylogenetic tree of the complete open reading frame of the F gene. The bootstrap consensus tree inferred from 1,000 replicates. Branches corresponding to partitions reproduced in <50 % bootstrap replicates are collapsed. The % of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown

next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method and are in the units of the number of base differences per site. Evolutionary analyses were conducted in MEGA5. Both *trees* indicate AF2240-I is from genotype VIII

Amplification of the 3'- and 5'-terminal ends of the viral RNA

The leader and trailer sequences at the 3'- and 5'-terminal ends of the viral RNA, respectively, were determined by rapid amplification of cDNA ends (RACE) as described previously [8]. To determine the sequence of the 5' end, primer L-cDNA (5'-GGTGATGTAATTGGCTTAGTAC TC-3'), which was derived from the published sequence of the L-gene of NDV strain AF2240, was used to generate single-stranded cDNA with reverse transcriptase (RT) as described above. The single-stranded cDNA was ligated to anchor-primer anchor as previously published in a study on strain La Sota [8] using T4 RNA ligase [55]. One microliter of the ligation mixture was used in a PCR with Phusion Hot Start High-Fidelity DNA Polymerase (Thermo, USA) and primers used were anchor-comp (5'-GAAGGATCCAGAA TCGATAG-3'), complementary to anchor primer), and L-PCR (5'-CAGCCAAGGGATATTACAGTAACT-3'). The PCR consisted of 35 cycles of 1 min at 98 °C, 30 s at 55 °C, and 20 s at 72 °C. The PCR products were cloned into pJET 1.2 blunt/cloning. Three independent clones were sequenced from both directions. To determine the sequence of the 3'-terminal end, primer anchor was ligated to the 3' end of genomic RNA with T4 RNA ligase as described by [56]. The mixture was incubated overnight at room temperature, and 5 µl of the ligation reaction was used as template in a reverse transcription reaction with anchor-comp primer. One microliter of the RT reaction was used in a PCR reaction with primers anchor-comp and NP-PCR (5'-GGAGCTGCTCGT ATTCATC-3'). The latter primer was derived from the published sequence of the NP gene of AF2240 strain. The RT-PCR and cloning conditions were as described above for the 5' RACE. Three independent clones were sequenced from both directions. The genome was then sequenced at different positions to be crosschecked with the published sequence of AF2240.

Amplification of the genes

PCR primer sets for each gene were designed from the sequenced UTRs. All PCR products were amplified using Platinum *Pfx* polymerase (Invitrogen, USA) followed by gel purification with QIAquick Gel Extraction Kit (Qiagen, USA) and cloned into pJET 1.2/blunt cloning vector using CloneJET PCR Cloning Kit (Thermo, USA). Several independent clones were sequenced from both directions to verify the plasmid insertion.

Sequence analysis

All alignment and phylogenetic analyses were performed with MEGA5 software (Molecular Evolutionary Genetics

Analysis, version 5.0) by neighbor-joining method at 1,000 bootstrap values.

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Conflict of interests The authors declare that they have no competing interests.

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