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Rapid Generation of a Recombinant Genotype VIII Newcastle Disease Virus (NDV) Using Full-Length Synthetic cDNA

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Abstract

Rescue of (−)ssRNA viruses involves the sequential assembly and cloning of the full-length cDNA, which is often a challenging and time-consuming process. The objective of this study was to develop a novel method to rapidly clone the full-length cDNA of a very virulent NDV by only one assembly step. A completely synthetic 15 kb cDNA of a Malaysian genotype VIII NDV known as strain AF2240-I with additional flanking *Bsm*BI sites was synthesised. However, to completely follow the rule-of-six, the additional G residues that are traditionally added after the T7 promoter transcription initiation site were not synthesised. The synthetic fragment was then cloned into low-copy number transcription vector pOLTV5-phiX between the T7 promoter and HDV Rz sequences through digestion with *Bbs*I. The construct was co-transfected with helper plasmids into BSRT7/5 cells. A recombinant NDV called rAF was successfully rescued using transfection supernatant harvested as early as 16 h post-transfection. Virus from each passage showed an intracerebral pathogenicity index (ICPI) and a mean death time (MDT) similar to the parent strain AF2240-I. Moreover, rAF possessed an introduced mutation which was maintained for several passages. The entire rescue using the one-step assembly procedure was completed within a few weeks, which is extremely fast compared to previously used methods.

Abbreviations

rAF	Recombinant AF2240-I
RNP	Ribonucleoprotein complex
h p.t.	Hours post-transfection
h p.i.	Hours post-infection
ICPI	Intracerebral pathogenicity index
NDV	Newcastle disease virus
HDV Rz	HDV virus ribozyme
(−)ssRNA	Negative-sense single-stranded RNA

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Introduction

Newcastle Disease (ND) is a potential threat to commercial poultry production. This disease spreads rapidly in chickens and other birds and is caused by avian orthoavulavirus-1, commonly known as Newcastle disease virus (NDV) [1]. The genome of NDV, abiding by the rule-of-six [2], contains a non-segmented single-stranded negative-sense RNA with a length of 15,186, 15,192 or 15,198 nucleotides (nt) [3]. The structural genome contains six genes, which encode (in 3′–5′ order) the nucleoprotein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase (HN), and large (L) polymerase protein [4]. During replication, two non-structural proteins known as V and W are also produced through RNA editing of the P gene [5]. The initiation of an infectious cycle of a negative-sense RNA virus such as NDV requires the presence of a complete ribonucleoprotein (RNP) complex consisting of the genomic RNA and the NP, P and L proteins since the naked RNA genome is not infectious [6, 7].

The various strains of NDV can be classified into different pathotypes based on the degrees of disease severity in birds [8]; asymptomatic strains cause a subclinical enteric infection without any clinical manifestations. Lentogenic strains are of low-virulence and may produce mild respiratory signs

or enteric disease in young chickens. Mesogenic strains exhibit intermediate virulence causing respiratory disease in young birds and decreased egg production in laying flocks. Viscerotropic velogenic and neurotropic velogenic strains are highly virulent and cause different severe clinical signs such as diarrhoea and frequent haemorrhagic intestinal lesions, and neurotropic disease symptoms respectively [9]. However, NDV is also genetically classified based on the sequence of its F gene [10]. The Malaysian NDV strain AF2240-I is a viscerotropic velogenic genotype VIII strain [11] that was first isolated in Malaysia in the 1960s. The oncolytic potential of this strain has been studied both in vitro and in vivo. Overall, it has been demonstrated that AF2240-I possesses the ability to induce apoptosis in many cancer cell lines such as MCF7, HCT116, HT29 and HeLa [12–14].

Reverse genetics systems for several NDV strains have been developed in the past [6, 7, 15–20]. These systems rely on cloning of the full-length NDV cDNA in a suitable transcription vector followed by co-transfection with helper plasmids expressing the viral NP, P and L proteins in suitable cells [21]. Based on the preferred system, the cells are either pre-infected with helper recombinant poxviruses carrying the T7 RNA polymerase gene, or stably express this polymerase (e.g. BSRT7/5 cells). In addition, Pol II-based rescue systems have also been reported in recent years [22, 23].

Traditionally, there are two major methods used to sequentially assemble the NDV full-length cDNA into transcription vectors. The first and most frequently used strategy is based on using natural or artificially introduced RE sites [6, 7, 15, 17]. Several cDNA fragments spanning the entire genome of NDV are created and then they are joined at overlaps using shared RE sites. This strategy is based on a technique first published by Schnell et al. 1994 for rescue of rabies virus [24]. The other strategy for assembly of the full-length cDNA is based on a ligation-independent cloning (LIC) technique [18], in which Hu and colleagues used an In-Fusion® PCR system to amplify fragments using primers that contained a 15-nt overlapping region of homology on 5′ ends. These fragments were sequentially assembled in a modified pBluescript, which was linearized before each phase of In-Fusion® reaction. This strategy has been gaining more popularity in recent years for its ease of use compared to the RE-based strategy [19, 20, 25–27].

Both of the above procedures are time-consuming and challenging. In the current study, we report the generation of rNDV from a completely synthetic full-length cDNA, a new rapid strategy for cloning the whole ~ 15 kb genome, which requires only one cloning step. This work is patented [28] and was also recently used to generate an IL12-expressing rNDV which was then used in an anti-cancer study [29]. Here we describe the methodology by which the original rAF was generated.

Materials and Methods

Construction of pOLTV5-phiX

Transcription vector pOLTV5 [9] was modified to insert a *phi*X region (taken from the high-copy number vector pTVT7R(0,0)-*phi*X, kindly provided by Dr. Andrew Ball, Department of Microbiology, University of Alabama, Birmingham, AL, USA) by utilising the *Sap*I and *Bam*HI RE sites to generate low-copy number vector pOLTV5-*phi*X (3020 bp). The *phi*X region contains *Bbs*I RE sites at both ends (reverse at 5′ and forward at 3′ ends) to facilitate the insertion of full-length AF2240-I cDNA in pOLTV5-*phi*X between the T7 promoter and the Hepatitis Delta virus ribozyme (HDV Rz) by a single cloning step.

Construction of Full-Length cDNA

To assemble the full-length AF2240-I cDNA into the pOLTV5-*phi*X vector, the full-length genome cDNA was synthesised and cloned by GenScript, NJ, USA. The synthetic cDNA contained *Bsm*BI RE sites (forward at the 5′ end and reverse at 3′ end) and three overhanging nucleotides at both ends to facilitate cloning (Fig. 1). To follow the rule-of-six, the additional G residues that are traditionally added after the T7 promoter transcription initiation site were not synthesised. A single natural *Bsm*BI RE site, at position 6741 within the genome cDNA, was mutated by replacing G with A (CGTCTC to CATCTC) to serve as a genetic marker. The change did not alter the amino acid sequence of the HN protein. The synthesised cDNA was then digested with *Bsm*BI RE. Meanwhile, pOLTV5-*phi*X was digested with *Bbs*I RE resulting in overhangs complementary to full-length cDNA *Bsm*BI digestion. When digested, the vector would therefore produce cohesive 5′ ends bearing the sequence 5′-TATA-3′ and 5′-GGGT-3′ overhangs. Consequently, the synthesised rAF cDNA when digested with *Bsm*BI produced cohesive 5′ ends, 5′-TATA-3′ and 5′-ACCC-3′ overhangs. Subsequently, the rAF full-length cDNA genome was ligated into pOLTV5-*phi*X vector targeting the *phi*X region to generate the plasmid pOLTV5-AF (17,565 bp). The full-length cDNA was placed right after the T7 promoter (without any additional guanine residues behind the promoter) and before the HDV Rz motif on the plasmid (Fig. 1). To increase the plasmid copy number, CopyCutter™ Induction Solution (Epicentre, USA) was added to induce and artificially increase the plasmid copy number at its log phase followed by an overnight incubation at 37 °C and a constant shaking at 300 rpm for 18–20 h. The transformation of large plasmid was enhanced using DH10β™ competent *E. coli* cells

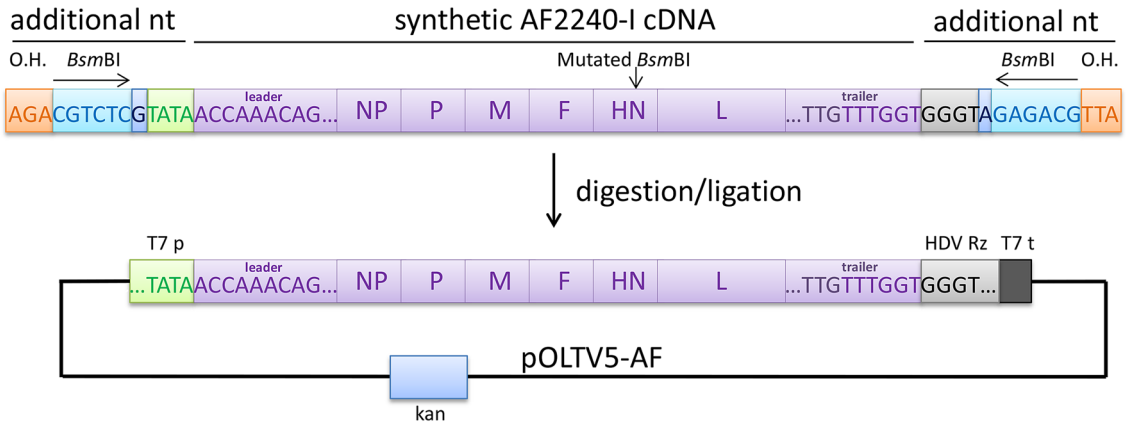

Fig. 1 Plasmid pOLTV5-AF was produced by utilising the *Bbs*I RE site in the *phi*X region of pOLTV5-*phi*X. AF2240-I genome was synthesised to contain *Bsm*BI RE at the 5′ and 3′ ends. Final size of the synthetic cDNA was 15,220 bp. A naturally present *Bsm*BI within the HN gene was mutated to not interfere with cloning. pOLTV5-*phi*X was digested with *Bbs*I to create matching sticky ends with the *Bsm*BI-digested synthetic genome. The AF2240-I genome was ligated to be located between the T7 promoter and HDV Rz region

(Thermo Scientific, USA) which are intended for higher transformation efficiency. Bacteria cultures were grown in 2.5 L of LB medium and a maximum concentration of 1 mg plasmid was obtained.

Construction of Helper Plasmids

To construct the three helper plasmids, the open reading frames (ORFs) of the NP, P, and L genes were amplified from the AF2240-I cDNA using specific primers, and then cloned into pJET cloning vector. The primers used to amplify the NP, P and L genes were NP-Fwrd (5′-CTTG**CTCGAGACC**ATGTCTTCCGTATTCGATGAA-3′), NP-Rvrse (5′-CTTG**GCGGCCGC**TCAATACCCCCAGTC GGT-3′), P-Fwrd (5′-AGAC**CTCGAGACC**ATGGCCACC TTTACAGAT-3′), P-Rvrse (5′-AGAC**GCGGCCGC**TTA GCCATTTAGTGCAAGGCG-3′), L-Fwrd (5′-CAGA**GCT AGC**TGGTCAGCATACATAATATTCGGCTGACGT -3′) and L-Rvrse (5′-CTTG**GTCGAC**GATAACTATGCG CACTCCTGTTTTAAGATAATTATTTGAATCCGAATA C–3′) (***XhoI***, ***NotI***, ***SalI*** and ***NheI*** are in bold and a Kozak's sequence in italic). The genes were then RE digested and cloned into pCIneo (Promega, USA) between *Xho*I and *Not*I to generate pCIneo-NP/AF and pCIneo-P/AF respectively and *Sal*I and *Nhe*I to generate pCIneo-L/AF.

Rescue of Virus

Baby hamster kidney cell line stably expressing the phage T7 RNA polymerase (BSRT7/5) (kindly provided by Prof. Karl-Klaus Conzelmann, Munich, Germany) were grown in Glasgow's modified essential media (GMEM) (Sigma Aldrich, USA), supplemented with 10% newborn calf serum (NCS), 1% tryptose-phosphate, 1% pen/strep and MEM amino acids. Selection for the T7 RNA polymerase gene was done by using 1 mg/mL of G418 (Sigma, USA) in every second passage. To rescue recombinant AF2240-I, BSRT7/5 cells were grown to 80% confluency in six-well plates and were co-transfected using pOLTV5-AF and different ratios of the helper plasmids [6, 7, 15, 16, 22] using Lipofectamine™ 2000 plus (Invitrogen, Thermo Fisher Scientific) according to the supplier's instructions. As a negative control, pOLTV5-AF was co-transfected with a set of helper plasmids in which pCIneo-L was replaced with pEGFP.N2 (Clontech, USA). At 16 h post-transfection (p.t.), the media was removed and stored in 4 °C. The cells were then cultured in fresh GMEM medium. After 4 days (96 h p.t.), the culture supernatant and cells from the transfected monolayers were harvested by scraping. The clarified and unclarified supernatants were inoculated separately into the allantoic cavities of 9- to 11-day-old specific pathogen-free (SPF) embryonated chicken eggs. After 48 h all embryos were checked for viability. Allantoic fluid of all eggs was harvested. The purified recombinant virus was checked for hemagglutinating activity (HA).

Determining Recombinant Virus Stability and Pathogenicity

To further check the stability of the genetic marker, the rAF virus was successively passaged 5 times in 9- to 11-day-old embryonated SPF eggs. The virulence of the recovered virus and the wild-type virus was determined by the mean death time (MDT) and the intracerebral pathogenicity index (ICPI) according to OIE guidelines [8]. Briefly, the MDT assay was performed by making ten-fold dilutions of allantoic fluid to obtain dilution series between 10^{-6} and 10^{-9}. For each dilution, 100 µL was inoculated into the allantoic cavity of

each of five 9-day-old SPF eggs. Another five eggs were inoculated 8 h later as recommended. The incubated eggs were examined twice daily for 7 days and the times of any embryo death were recorded. The MDT was the mean time in hours for the minimum lethal dose to kill all the inoculated embryos. Furthermore, the ICPI was performed by ten-fold diluting a fresh NDV-positive allantoic fluid in sterile isotonic saline and injecting 500 µL of it intracerebrally into each of ten 1-day-old SPF chicks. The chicks were examined every day for 8 days. At each observation the birds were scored 0 if normal, 1 if sick and 2 if dead. The ICPI was the mean score per bird per observation over the 8-day period. Generally, highly virulent strains will demonstrate scores between 1.5 and 2.

Ethical Clearance

Animal handling procedures were performed in line with the national animal welfare regulations. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC), Faculty of Veterinary Medicine, Universiti Putra Malaysia (UPM) (reference number UPM/IACUC/AUP-R051/2014) and conducted in a BSL-2 facility in UPM, Selangor, Malaysia.

Results

Full-Length cDNA Synthesis

The fully synthetic rAF cDNA was successfully inserted into the T7-transcription plasmid pOLTV5-*phi*X (a derivative of pOLTV5). In the resulting plasmid pOLTV5-AF, the full-length rAF cDNA is flanked by the T7 RNA promoter and the HDV Rz sequence. In the presence of T7 RNA polymerase, this allows the generation of full-length vRNA (full-length viral RNA transcribed from plasmid DNA) with the precise authentic 3′ and 5′ ends of the genomic (−)RNA of NDV.

Virus Recovery

Transfection of BSRT7/5 cells was performed using pOLTV5-AF and the, NP-, P- and L-helper plasmids. The cell culture medium was collected at 16 h and 96 h p.t. The 16 h p.t. samples were clarified to remove cell debris, whereas the 96 h p.t. samples were either clarified or not. Clarified and unclarified samples were subsequently inoculated into embryonated SPF eggs. Embryo death from all supernatant sources was observed after 48 h of incubation, indicating the presence of infectious virus. Embryo death was not observed in eggs inoculated with control supernatant from transfections in which the L-helper plasmid was omitted. Allantoic fluid was harvested and all showed positive HA.

To identify the recovered virus as recombinant AF2240-I the presence of the mutated *Bsm*BI RE site genetic marker was confirmed by RT-PCR and DNA sequencing (Fig. 2). These results convincingly indicate successful rescue of rAF from the pOLTV5-AF infectious cDNA clone.

Pathogenicity and Genetic Stability

rAF virus showed a MDT of 48 h in chicken embryos compared to 46.8 h for the wild-type strain AF2240-I. This indicated that the pathogenicity of rAF was similar to that of wild-type AF2240-I.

Since the ICPI allows for a closer characterisation of virus pathogenicity, rAF and wild-type AF2240-I were used to determine the ICPI in 1-day-old chicks. The ICPI for rAF was 1.83, whereas the ICPI for the wild-type strain was 1.81.

To verify that the recombinant strain is phenotypically and genetically stable, rAF was passaged up to five times in embryonated eggs. All passaged strains showed ICPI values which were close to that of the parent strain (Table 1). In virus from each of the five passages, the presence of the mutated *Bsm*BI RE site within the HN gene was confirmed by sequencing analysis (Fig. 2). This indicates that the synonymous mutation was stably maintained for five passages.

Furthermore, the effect of rAF on cancer cell lines such as HeLa was investigated (Fig. 3). Membrane blebbing and detachment of cells from surface were evident at 24 h p.i.

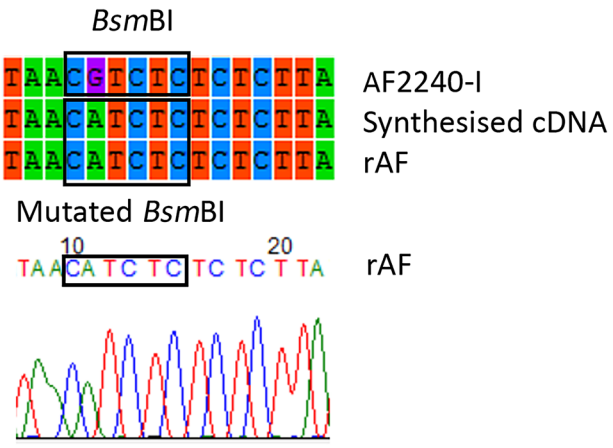

Fig. 2 Presence of genetic marker was confirmed. DNA sequencing showed that the naturally occurring *Bsm*BI RE site was mutated in rAF and the mutation maintained for five passages

Table 1 MDT and ICPI of rAF remained constant for five passages

Passage no.	MDT	ICPI
rAF-p1	48	1.83
rAF-p2	49	1.84
rAF-p3	47.8	1.85
rAF-p4	47	1.83
rAF-p5	48	1.84
Wild-type	46.8	1.81

The first rescued strain was tagged as rAF-p1, and thereafter passages labelled as rAF-p2, rAF-p3, rAF-p4, rAF-p5. All five passages killed all embryos within 49 h indicating that the strains are virulent. A more efficient determination is by ICPI which recorded at value between 1.81 and 1.85 which are within the range of a positive velogenic strain NDV

Discussion

In this study, the recombinant Malaysian strain AF2240-I was successfully rescued from a completely synthetic full-length cDNA clone. This is a new and rapid strategy for the generation of rNDV and, as far as we know, rAF is the first NDV to be rescued by this method. The design of the transfection vector pOLTV5-*phi*X greatly assisted in the ligation of the synthetic cDNA and may be a useful tool for future rescue work using other strains of NDV.

In pOLTV5-AF, the leader sequence was placed immediately after the T7 promoter without any additional G residue(s). Traditionally, at least two or three G residues are added after the T7 promoter to increase the efficiency of transcription. These residues are removed during replication and the recovered viruses do not carry them [24, 30, 31]. To fully follow the rule-of-six, the AF2240-I cDNA was synthesised without the additional G residues. The idea behind this was to include the original genome size (15,192 b), without any additional nucleotides, between the T7 promoter and the HDV Rz. The results showed that the first residues of leader sequence (the first residue is an A, which is also a purine) can efficiently rescue rAF from pOLTV5-AF. This suggests that, for a rule-of-six virus, the incorporation of G residues in such non-helper virus systems (in this case the BSRT7/5 cells) may not be needed. Furthermore, since the efficiency of virus recovery was high, no further comparison between rescue with or without additional G residues was performed.

The RE-based and LIC strategies are time-consuming and may take several weeks to complete, especially if a new strain has to be sequenced and cloned. These strategies require many cloning steps that are usually complicated and need a high level of molecular expertise. In contrast, pOLTV5-AF was created by only one ligation procedure. In this study, generation of rAF was completed within just a few weeks. Therefore, this approach certainly has major advantages over previously used strategies.

pOLTV5 originates from plasmid pOK12 which carries the replicon from P15A [32], resulting in a relatively low-copy number. A low-copy number plasmid was chosen as a transcription vector as it is particularly useful for large plasmids which may cause the metabolic burden to become too large for the host cell causing plasmid-bearing cells to grow slowly [33]. pOLTV5 vector is able to stably replicate large inserts such as the genome of NDV [7].

One of the most important requirements for successful virus rescue is the availability of the correct co-transfection components consisting of helper plasmids that express the functional NP, P and L proteins. The ratios of helper plasmids used in previous publications [6, 7, 15, 16, 22] varied accordingly to different strains hence in the current work we tested which ratio was best suited for the rescue of strain

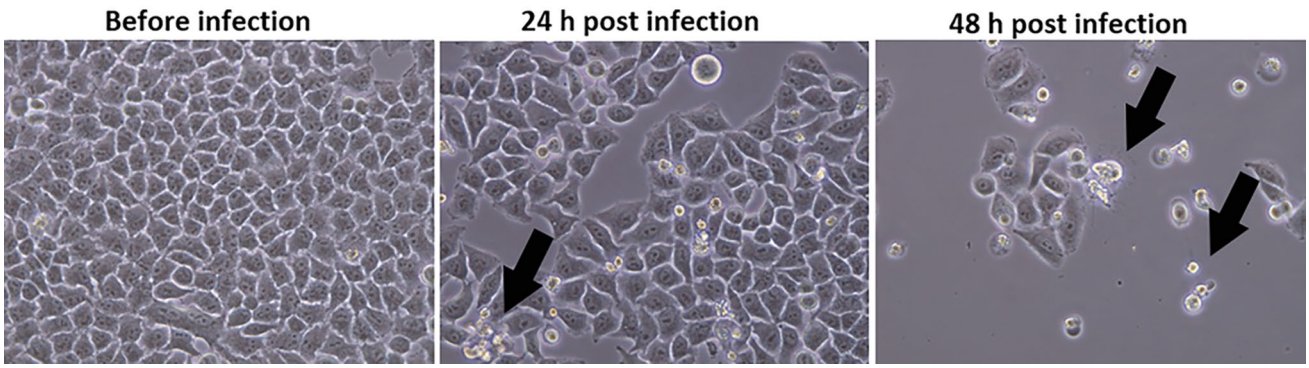

Fig. 3 Effect of rAF on HeLa cancer cell line (MOI = 10). The cells underwent membrane blebbing and detached from the surface. Black arrows indicate the apoptotic cells

AF2240-I. Conventionally, the ratios of the transfected NP, P and L plasmids were calculated considering the lengths of 6942 nt, 6660 nt and 12,087 nt of the expression plasmids, respectively. Particularly for RNA replication, a constant nucleocapsid synthesis is required [34] and a high level of NP expression may favour the initial nucleocapsid formation. Nevertheless, our results indicate that all ratios regardless of their correspondence to plasmid lengths were able to produce infectious viral particles (data not shown).

Typically, optimum transient gene expression from plasmid DNA can be noticed approximately 24–96 h post-transfection [35]. For NDV recovery, cell supernatant containing budded viral particles are harvested, usually optimally at 96 h p.t. In the current study, inoculation of 16 h p.t. supernatant resulted in infectious virus particle propagation in embryonated eggs. This suggests that, the viral titre in the supernatant 16 h p.t. was sufficient to initiate the production of infectious viral particles by embryonated eggs. Because of the frequent use, all possible means to reduce the time needed for the reverse genetics process will definitely help in speeding up the generation of recombinant virus. In addition, the cell supernatant is usually clarified (to remove cell debris) prior to inoculation into embryonated egg, although the use of cell lysate for inoculation has also been reported [18]. In this study we also showed that it was not necessary to remove cell debris from the cell supernatant and that the supernatant of cell media can be directly inoculated into embryonated chicken eggs without affecting virus recovery.

The mean times to kill all embryos are 48 h and 46.8 h respectively for rAF and AF2240-I. A highly virulent strain would supposedly take below 60 h to kill all embryonated eggs. Hence we can conclude that, based on the MDT, the rAF is a highly virulent strain similarly to its wild-type. Although the MDT of rAF showed a slight increase compared to wild-type AF2240-I by 2.8 h, both strains are still categorised as highly virulent strains. For the remaining passages of the strains, i.e. passage 2, passage 3, passage 4 and passage 5, the MDT were also determined and recorded at 49 h, 47.8 h, 47 h, and 48 h, respectively (Table 1). This shows that after five passages, the virulence of the recombinant virus remains high. The virulence of the passaged viruses was also tested in chickens by ICPI test. The index values indicated that recombinant virus from all passages was velogenic, equivalent to wild-type AF2240-I. The ICPI results coupled with MDT is an adequate indication of virulence stability.

Sequence analysis of the complete genome showed that the mutated *Bsm*BI RE site was maintained after five passages. This allowed us to ascertain that minor modifications to the genome that do not affect the rule-of-six or protein-encoding regions will be stable after at least five passages. In fact, over the last two decades recombinant NDVs have shown to be very stable viruses after several passages and may have very low mutation rates [36]. This also includes the stability of foreign genes inserted into the genome. Therefore, in this study mutation analysis after more passages was not conducted. However, a recent rescue of an IL12-expressing rAF proves the construct to be very reliable as its stability maintained for ten passages [29].

The successful rescue of the rabies virus [24], as well as the rescue of the two VSVs shortly thereafter [37, 38], were important milestones in virology as the technique provided the opportunity to genetically modify the genome of many other negative-sense RNA viruses such as Sendai virus [39, 40], measles virus [41], human para influenza [42, 43], Nipah virus [44] and NDV [7]. These developments have provided the opportunity to elucidate the function of NDV genes and proteins in determining virulence and tropism [45]. Reverse genetics has also allowed the development of NDV as a vaccine vector. Due to the ability of NDV to act as a vector to transiently but strongly express foreign genes, its application as a tool for gene therapy and vaccine vector has been studied. This applies for both animal and human use [46–48]. Another important application of the reverse genetics technology is the development of negative-strand viruses, particularly NDV, for cancer research [49–52].

New advances in whole genome sequencing, such as next-generation sequencing (NGS), as well as advances in chemical synthesis, such as the rapid synthesis of long cDNA fragments, may be very valuable if a recombinant virus or vaccine is urgently needed. By the rapid method introduced in this study the synthetic viral cDNA may be easily customised according to specific needs. Current research interests on recombinant NDV are mainly focussing on adding various additional properties [53]. Therefore it is of high interest to assure that the current rescue procedure is also applicable for all future work to produce rNDV for poultry vaccine development or human cancer research.

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Author Contributions KM and AM performed the experiments. KM wrote the manuscript. AM revised the manuscript. BP, AM and KM designed and planned the experiments. ARO and KY supervised the project and provided resources.

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Compliance with Ethical Standards

Conflict of interest The authors do not have any competing interests to declare.

Animal and Human Rights Statement All institutional and national guidelines for the care and use of laboratory animals were followed.

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