Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
DOI: 10.29261/pakvetj/2022.059

RESEARCH ARTICLE

An Efficient Approach for the Recovery of LaSota Strain of Newcastle Disease Virus from Cloned cDNA by the Simultaneous use of Seamless PCR Cloning Technique and RNA-POL II Promoter

Faisal Masoud[1], Muhammad Shahid Mahmood*[1], Sajjad-ur-Rahman[1] and Rao Zahid Abbas[2]

[1]Institute of Microbiology, University of Agriculture, Faisalabad
[2]Department of Parasitology, University of Agriculture, Faisalabad
*Corresponding author: shahiduaf@gmail.com

ARTICLE HISTORY (22-027)

Received: January 14, 2022
Revised: April 11, 2022
Accepted: April 13, 2022
Published online: August 25, 2022

Key words:
Newcastle disease virus
LaSota strain rescue
cDNA
RNA- Polymerase – II Promoter
Reverse genetic system
Seamless PCR cloning

A B S T R A C T

The reverse genetic system for the LaSota strain of Newcastle disease virus (NDV) based on RNA POL II promoter is currently unavailable. This study was designed to produce the LaSota virus from the cDNA in the laboratory. The cDNA was cloned in mammalian expression vector pcDNA 3.1 and it was flanked by hammerhead and hepatitis delta virus ribozyme. The virus was rescued from the Vero cell line by simultaneous in-vitro transcription of full-length LaSota strain clone along with support plasmids i.e. NP, P, and L genes. The RNA polymerase was produced by cell lines themselves; there was no need to provide it by outside means. The progeny of the recombinant NDV shows the presence of an artificially introduced genetic marker (A set of three nucleotides) in the intergenic space of the F and HN genes. The haemagglutinin titer (HA) of rescued and parenteral viruses indicates values of 2^8 and 2^7; while the mean death time assay (MDT) score of the viruses was 106 and 104 hrs, respectively. The embryo infective dose (EID_{50}/mL) was lower in recombinant virus ($10^{9.10}$) as compared to the wild virus ($10^{9.24}$). The growth curve of both viruses was indistinguishable from each other, which shows that recombinant LaSota is a faithful copy of the parenteral virus. The results predict that; this system can facilitate the easy, efficient and robust rescue of viruses, for their use as a vaccine vector.

To Cite This Article: Masoud F, Mahmood MS, Sajjad-ur-Rahman, Abbas RZ 2022. An efficient approach for the recovery of LaSota strain of Newcastle disease virus from cloned cDNA by the simultaneous use of seamless PCR cloning technique and RNA-POL II promoter. Pak Vet J, 42(3): 346-351. http://dx.doi.org/10.29261/pakvetj/2022.059

INTRODUCTION

Newcastle disease (ND) is an important avian disease that produces huge losses in terms of mortality and morbidity (Mehmood *et al*., 2019; Mahmood and Sabir, 2021). This disease is caused by Newcastle disease virus (NDV), which belongs to genus *Avulavirus* of family *paramyxoviridae*. The NDV is classified into three groups depending upon the severity of the disease i.e. lentogenic, mesogenic and velogenic. The velogenic strains are further divided into viscerotropic and neurotropic classes. This virus has a non-segmented, single-stranded, negative-sense RNA genome (Ganar *et al*., 2014). The genome of NDV encodes for six structural proteins which are; fusion protein (F), haemagglutinin-neuraminidase protein (HN), matrix protein (M), nucleoprotein (NP), phosphoprotein (P) and Large (L) RNA dependent RNA polymerase in the order 3' NP-P-M-F-HN-L 5' (Miller *et al*., 2010). While two other proteins; V and W can be produced by RNA editing of the P gene during transcription (Jadhav *et al*., 2020).

The reverse genetic system has provided us an opportunity to genetically engineer the viruses to produce vaccines and has increased our knowledge about their molecular biology (Stobart and Moore, 2014). As we look back in history, the NDV was the first pathogen for which a reverse genetic system was reported. After the early success different strains of NDV has been rescued from cloned cDNA like clone-30 (Ramp *et al*., 2012), Beaudette C, Hitchner B1 (Molouki and Peeters, 2017b), Mukteswar (Li *et al*., 2011), LaSota (Hu *et al*., 2011) and NDV4-C (Zhang *et al*., 2013).

The genomic RNA of NDV (a negative sense RNA virus) cannot be translated into viral proteins, until it is encapsidated with NP, P and L proteins, which altogether makes the ribonucleoprotein (RNP) complex. For this purpose, the genes of viral RNP complex and full-length

viral cDNA are provided in the form of cloned plasmids and those are transcribed by the polymerase (Song *et al*., 2019). The previous research reveals that T7 RNA polymerase was used for this purpose and it was provided by either cell lines expressing the T7 RNA polymerase or by the vaccinia virus. But both systems have some drastic effects on the recovery of viruses (Molouki and Peeters, 2017a; Hasni *et al*., 2021). So, the scientists shifted to the use of cellular polymerases like the Pol-II system and recovered the different strains of NDV (Li *et al*., 2011; Zhang *et al*., 2013).

Similarly, cloning a full length NDV genome in the plasmid is a very tricky job that's why only a few labs in the world have adopted this system. The previous studies have used the traditional methods based on restriction-ligation cloning procedures that are very hectic and the data generated from one strain cannot be used for others as such due to different patterns of restriction enzymes (Hu *et al*., 2011; Li *et al.,* 2011; Zhang *et al*., 2013). While on the other hand LaSota strain is an important vaccine candidate for the control of Newcastle disease and is being used all over the world (Dimitrov *et al*., 2017). But the reverse genetic system with sophisticated modifications has not yet been developed for this strain of NDV. So, it was desperately needed to introduce a new rescue system that can facilitate the whole process. Here, we describe the rescue system for the LaSota strain of NDV by using RNA-POL II promoter and the seamless PCR cloning technique. This system is devoid of traditional cloning procedures and does not require T7 RNA polymerase. The results suggest that this new system will improve the recovery of the LaSota strain. It will also pave the way to use this strain as an attenuated vaccine or vaccine vector.

MATERIALS AND METHODS

Virus, Cell line and expression vector: This study was executed on the lentogenic LaSota strain of NDV. The Vero cells (African monkey kidney cells) were grown in a 25 cm^2 tissue culture flask (orange, UK) by providing Dulbecco's modified Eagle's medium (DMEM) (Cat # 11965092) (gibco) supplemented with 10% fetal bovine serum (FBS) at 37°C with 5% CO_2 in the incubator (Sanyo) (Yurchenko *et al*., 2019). For the plasmid construction, mammalian expression vector pcDNA $3.1^{(TM)}$ (+) (cat no. V790-20) (Invitrogen) was used (Inoue *et al*., 2003). While the cloning procedures were carried out by using the GeneArt seamless PLUS cloning and assembly kit (cat no. A14603) (Thermofisher Scientific, USA).

RNA Extraction cDNA synthesis and Construction of plasmids: The LaSota strain virus was subjected to RNA extraction by using TRIzol reagent® (Life Technology, Carlsbad, CA, USA) with some modifications (Yang *et al*., 2021) and cDNA synthesis by using Revert $Aid^{(TM)}$ First-strand cDNA synthesis kit (Thermo scientific) (cat # K 1622).

As the length of the NDV genome is long, it was divided into four fragments; A, B, C and D; cloned sequentially in the expression vector. To produce the exact 3´and 5´end of the viral genome, the cDNA was placed in between the Hammerhead ribozyme (HamRz) (3´) and hepatitis delta virus ribozyme (HdvRz) (5´) (Zhang *et al*., 2013).

The PCR reactions (for amplification of inserts and linearization of pcDNA 3.1 vector) were carried out by using AccuPrime™ *Pfx* SuperMix (Cat no. 12344-040) (Invitrogen) in the Bio-Rad C1000 Thermal cycler (Ali *et al*., 2021). After that PCR products were analyzed on gel electrophoresis and then subjected to a purification process by using the GeneJET PCR purification kit (Thermo Scientific, USA) (Cat no. K0701). In the next step, a ligation reaction was performed by using GeneArt™ seamless PLUS cloning and assembly kit (cat no. A14603) (Thermofisher scientific, USA) as described by the manufacturer. Then transformation was done in the Top-10 strain (*E. coli*), and the correct clone was screened by using blue-white screening procedure. While for the support plasmids, the open reading frame (ORF) of these NDV genes i.e. NP, P and L were cloned in the pcDNA 3.1 vector (Ayllon *et al*., 2013).

Transfection and recovery of virus: In this study, the Lipofectamine® 3000 (Invitrogen) (Cat # L3000-008) was used as a transfection reagent and Opti-MEM ® (gibco) (Cat # 31985062) as a reduced serum medium (Ayllon *et al*., 2013). The Vero cells were grown to 80% confluency in a 6-well cell culture plate and on the next day these were fed with 1mL of DMEM (with 10% FBS) and 200 µL of DNA mix (which contains Lipofectamine, Opti-MEM, Full length genome plasmid and support plasmids) in each well. After the incubation, the supernatant was collected from the wells of cell culture plate, clarified in centrifuge tube and inoculated in the 9-days old embryonated chicken eggs (ECEs). The allantoic fluid was harvested from ECEs after 72 hrs post-inoculation and was subjected to haemagglutination test (HA).

Characterization of rescued virus: The rescued LaSota and parenteral virus were tested for growth kinetics by infecting Vero cells (80% confluency) at a multiplicity of infection 0.01 (MOI). The cell culture plates were kept at 37°C in a CO_2 incubator. The supernatant was harvested after 08 hrs post-infection and replaced with an equal quantity of cell culture medium up to 72 hrs. It was preserved and then subjected to estimation of viral titer by plaque assay. In the next step EID_{50} and mean death time assay (MDT) were performed to estimate the biological properties of recombinant and parenteral LaSota viruses (Reed and Muench, 1938).

RESULTS

Cloning of full-length NDV genome and support plasmids: To add the ribozymes at 3´ and 5´ of NDV anti-genome, the pre-cloning was done in the pcDNA 3.1 vector. In the pre-cloning, the whole NDV genome was divided into A1, B1, C1 and D1 fragments. The sequences of ribozymes (HamRz and HdvRz) were added by the use of extremer primers (Eurofins). The forward primer of pre-clone A1 was made extremer by adding the sequence of HamRz on its 5'end. Similarly, the sequence of HdvRz was added in the reverse primer of pre-clone D1 on its 5'end. These pre-cloned plasmids (A1, B1, C1 and D1)

were used as a template for the generation of A, B, C and D fragments of the NDV genome. In this way, the NDV leader sequence was preceded by the HamRz while the trailer region was followed by HdvRz. The inserts and vector were linearized by a set of primers and cloned in the MCS of the pcDNA vector with help of GeneArt enzyme mix as shown schematically in Fig 1-A.

The genes encoding the viral polymerase complex of NDV (NP, P and L gene) were cloned in the pcDNA 3.1 vector. The open reading frame (ORF) of each gene was preceded by the "Kozak sequence", so that efficient transcription can be carried out by eukaryotic polymerase. The full-length construct was designated as 'Pc-LaSota, while the support plasmids i.e. NP, P and L genes were named as Pc-NP. Pc-P and Pc-L. These expression vectors having full-length LaSota cDNA and helper genes were used for *in-vitro* transfection.

The transcription was carried out by RNA POL II; provided by eukaryotic cell lines, it binds to the CMV promoter in the pcDNA 3.1 vector and starts transcribing the cDNA sequence in plasmids. The HamRz sequence was transcribed first and it detaches automatically when the RNA POL II reads the 3′ of the ribozyme sequence and hence produces the exact 5′ of the NDV leader region. While the HdvRz cleaves on its 5′ end and leads to the generation of the exact 3′ end of antigenomic viral RNA.

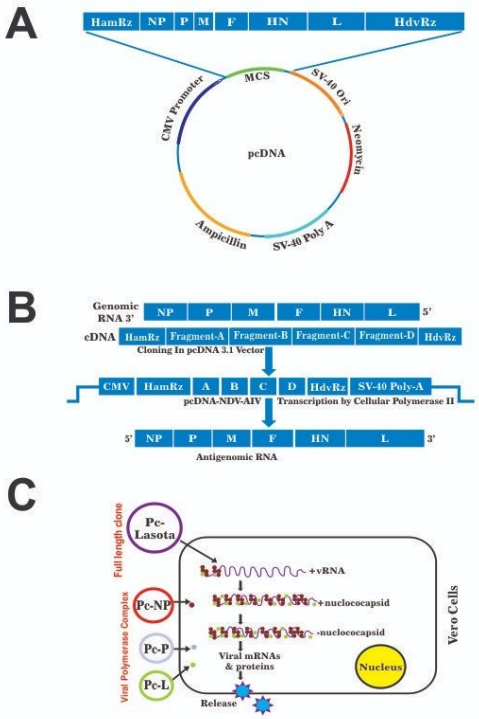

Fig. 1: This figure is to describe the cloning and transfection process of recombinant LaSota strain **A:** construction of full length plasmid in the MCS of pcDNA vector, which shows the cDNA of LaSota virus is cloned along with ribozyme. **B:** The detailed procedure was as follows; the genomic RNA was taken; cDNA was prepared and ribozymes were attached at its terminal ends. This was cloned in pcDNA vector under the control of CMV promoter and as result antigenomic RNA was obtained. **C:** The transfection process was carried out by simultaneous application of full length and support plasmids to Vero cells and it produces the complete virus.

Recovery of virus: The Vero cells were grown in Dulbecco's modified eagles' medium (DMEM) medium up to 80% confluency as shown in Fig. 4. On the next day, these were washed with 1mL of DMEM (Cat # 11965092) (gibco) supplemented with 10% FBS. In the next step, the cells were covered with 1mL of DMEM (with 10% FBS) and 200 µL of DNA mix (which contains Lipofectamine, Opti-MEM, Pc-LaSota 1.5 µg, Pc-NP 1 µg, Pc-P 0.5 µg, Pc-L 0.5 µg) in each well. The Pc-NP, Pc-P and Pc-L encode for ribonucleoprotein complex (RNP) of Newcastle disease virus as shown schematically in Fig. 1C. After 72 hrs of transfection, the supernatant was collected and then injected into 9-days old embryonated chicken eggs via allantoic cavity. In the next step after 48 hrs of post-inoculation, the allantoic fluid was harvested. The 50 µL of allantoic fluid was used for haemagglutination test (HA) and an equal quantity of parenteral virus was subjected for this test. The results demonstrated 2^7 and 2^8 for the parenteral and recombinant LaSota viruses respectively as shown in Table 1.

Identification of genetic markers: To identify whether the virus is rescued from the cloned cDNA of plasmids or from a wild type virus. The genetic marker was added for the identification of recombinant LaSota virus; the nucleotide bases on positions 6296, 6297 and 6298 in the intergenic space of F and HN gene; GTT were replaced by CGG to create the restriction site for the ksp1 enzyme. The parenteral LaSota and recombinant LaSota RNA were extracted and then subjected to cDNA synthesis, in the next step a pair of primer was used to amplify the specific part (which have a genetic marker) and later on digested by the restriction enzyme. The restriction enzyme (ksp1) cuts at the specific point and confirms the genetic markers. When the parenteral LaSota was digested with the same restriction, it does not produce any cleavage, which also confirms that recombinant LaSota was generated from cloned plasmid and not from a wild virus. To rule out the possibility of the contamination of plasmids with viral RNA, which can give false-positive results. The PCR reaction was performed by using the RNA directly as a template and omitting the reverse transcriptase process (RT). The RNA of both viruses (Parenteral and recombinant LaSota) did not produce any amplification, which means the RNA was not contaminated with any type of plasmids and the recombinant virus is produced from cloned plasmids. The diagrammatic description is shown in Fig. 2.

Table 1: Biological properties of parenteral and recombinant LaSota strains

Comparison of biological properties of LaSota and recombinant LaSota viruses			
Virus	HA	MDT (hrs)	EID_{50} /mL
rLaSota	2^8	106	$10^{9.10}$
Parenteral LaSota	2^7	104	$10^{9.24}$

HA = heamagglutination test MDT = Mean death time EID = Embryo infective dose, the statistical analysis was performed for each character of rLaSota and parenteral virus by using least significant difference test (*LSD*). The non-significant difference was found in the results of HA titer, MDT (hrs) and EID_{50} /mL at $P \leq 0.05$.

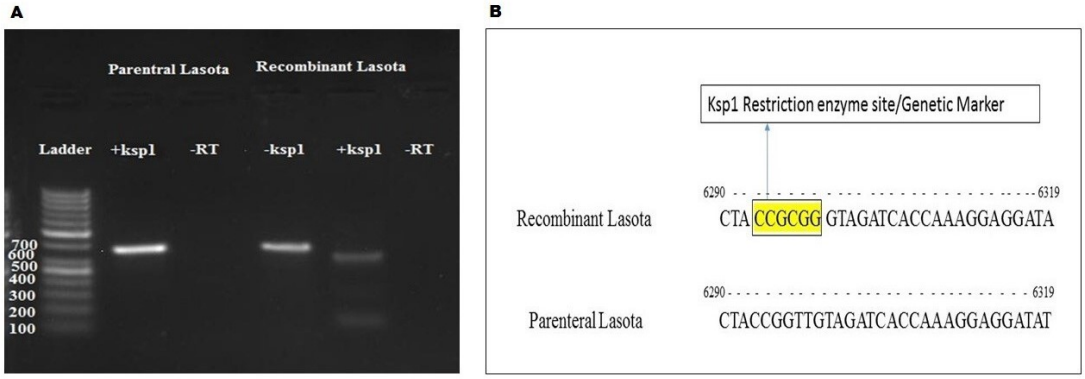

Fig. 2: A: The gel image for identification of genetic marker is shown. (Parenteral LaSota) +ksp1 means the PCR product was treated with restriction enzyme and no fragment was produced because no genetic marker at that position and -RT means RNA was used as template and it produces no results. In the (recombinant LaSota) -ksp1 means the PCR product was not treated with restriction enzyme and no fragment was produced and +ksp1 means the product was treated with enzyme and it produces the fragment, -RT means RNA was used as template and it produces no results in PCR. **B:** Addition of genetic marker by replacing the GTT (nucleotides) with CGG to produce restriction site for ksp1 (CCGCGG) in recombinant LaSota. The aforementioned three nucleotides have position on LaSota genome at 6296, 6297 and 6298; in the intergenic space in between F and HN gene.

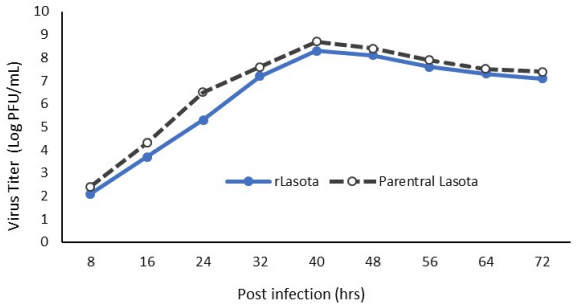

Fig. 3: Multistep growth analysis of rLaSota and Parenteral LaSota viruses. The Vero cells were grown in cell culture plates and for each virus 0.01 MOI of infection was given in duplicate. The supernantant was harvested at interval of 08 hrs post infectin and titer was deterimiend by the plaque assay. The non-significant growth rate difference was found between rLaSota and Parenteral virus at $p \leq 0.05$ according to least significant difference test (LSD).

Characterization of recovered virus: The recombinant LaSota and parenteral virus were tested in terms of growth properties and virulence with the parenteral virus. The Vero cells were infected with the recovered and parenteral virus in duplicate at multiple of infection 0.01. The supernatant was collected at the interval of 08 hrs post-infection up to 72 hrs and samples were quantified by plaque assay. It was observed that there is no significant difference in the titer and growth kinetics of both viruses as shown in Fig. 3.

The virulence of recombinant virus (rLaSota) was tested by the mean death time assay (MDT). The 9-days old embryonated chicken eggs were infected with the recovered and parenteral virus. The death time for embryos was monitored for the specific. The resulting value indicates that the MDT of recombinant (rLaSota) and parenteral virus was 106 and 104, respectively. These values indicate that the recovered virus has same potential as that of the parenteral virus. In the same way, the EID_{50} of the parenteral and recombinant LaSota virus was $10^{9.24}$ /mL and $10^{9.10}$ /mL, respectively. The biological properties of both viruses reveal that there is no significant difference between the rLaSota and parenteral virus as shown in Table 1.

DISCUSSION

The Newcastle disease virus remains the interest of poultry virologists for its vaccine development. In this study, we have successfully recovered the LaSota virus from the cloned cDNA, and to the best of our knowledge, this strain of NDV has never been rescued from cDNA by using eukaryotic polymerases. The LaSota strain has been used for vaccination purposes as a first candidate for controlling ND throughout the world. Still, this LaSota strain; in combination with others strains of NDV has been used for vaccination in Pakistan and around the world (Dey *et al*., 2014; Rauf *et al*., 2019; Manar *et al.*, 2020). Due to the proven track record of safety and immunogenicity, this strain was selected for the current study, so that in the future it can be used for further research related to vaccine development.

The rescue system of the negative-sense RNA viruses depends on the intra-cytoplasmic reconstruction of the ribonucleoprotein complex (RNP). It encodes the viral polymerase, which is essential for the infectious cycle of the virus (Stobart and Moore 2014). The different types of polymerases (Bacterial and eukaryotic origin) are provided during the *in-vitro* transcription by cell lines themselves or by viruses. So that plasmids encoding the genes of viral polymerase or template can be transcribed. The T7 polymerase is commonly used but it has some drastic effects on the recovery of viruses, so scientist gives weightage to the eukaryotic polymerase (Molouki and Peeters, 2017a). So, the current study was planned by using RNA POL II of eukaryotic cells. The pcDNA 3.1 vector which has cytomegalovirus (CMV) promoter and simian virus-40 (SV-40) poly-A tail; was used for the construction of plasmids. This vector has already been used in different strategies of virus rescue (Inoue *et al*., 2003). The RNA POL II can bind to the CMV promoter and carry on the transcription process. This polymerase was used to produce the genomic RNA from the full-length plasmid and mRNAs of proteins NP, P and L protein from the helper plasmids. The RNA II polymerase has been used to rescue different viruses i.e. Newcastle disease virus genotype II (Wang *et al*., 2015), measles virus (Martin *et al*., 2006), Mukteswar strain of NDV (Li

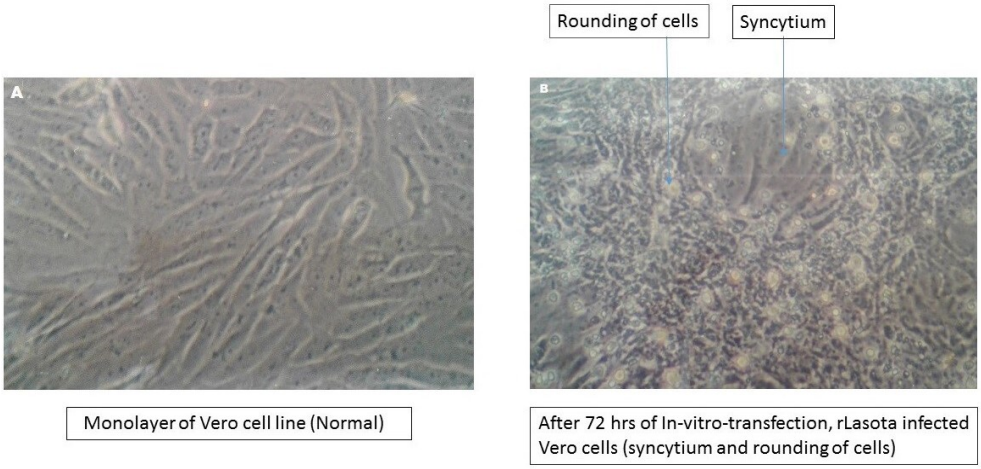

Fig. 4: Vero cell line images before and after invitro-transcription. **A:** Image of cell before transfection **B:** Cytopathic effects (syncytium formation and rounding of cells were seen) after invitro-transfection.

et al., 2011) Borna disease virus (Yanai *et al.,* 2006) and rabies virus (Inoue *et al*., 2003). The plasmid-derived LaSota virus particle can replicate in the Vero cells up to limited numbers due to the unavailability of trypsin-like proteases which have prime importance in the pathogenicity of lentogenic strains of NDV (Römer *et al*., 1999). So, the supernatant from the Vero cells was taken, clarified and then inoculated in the allantoic cavity of the embryonated chicken eggs for their amplification. In this way, a recovery system for the LaSota strain from the cloned cDNA by employing the cellular polymerase involves the recovery of a few virion particles and then amplification in embryonated chicken eggs.

The members of the paramyxoviridae family obey the "rule of six" for efficient replication, which narrates that the genome length of the virus should be multiple of six (Kolakofsky *et al*., 2005). All the isolates of NDV, which have been discovered till now, have genome length according to the above-mentioned rule (Ganar *et al*., 2014). This point was taken into consideration while constructing the full-length plasmid encoding the viral cRNA (Peeters *et al*., 1999; Peeter *et al*., 2001; Wang *et al*., 2015; Zhang *et al*., 2013). Similarly, to produce the exact 3′ and 5′ end of the viral RNA, the ribozymes were added at the respective sites in the plasmid encoding the full-length viral genome. This strategy has also been used for the recovery of different strains of NDV (Molouki and Peeters, 2017b) and other viruses i.e. rabies (Inoue *et al*., 2003), Borna disease virus (Yanai *et al*., 2006).

Due to the large size of the NDV genome, it is not an easy task to clone the error-free complete cDNA of the virus in the expression vector. This was a major problem in the last two decades and that's why only a few laboratories in the world have got expertise in reverse genetics of this virus. If we review history, the scientist used the restriction-ligation based cloning method for this purpose but that method has a problem, as the different strains of NDV (i.e. LaSota, Mukteswar, Clone-30, Beaudette C, Hitchner B1, NDV4-C, NDV-genotype II and Genotype VII.1.1. etc.) have a different pattern of restriction enzymes (REs) in their genome, so the data generated from the cloning of one strain cannot be used for the cloning of others (Krishnamurthy *et al*., 2000; Li *et al*., 2011; Nakaya *et al*., 2001; Peeters *et al*., 1999; Römer-Oberdörfer *et al*., 1999; Wang *et al*., 2015; Zhang *et al*., 2013; Lebdah *et al*., 2022; Sultan *et al*., 2022).

So, to avoid all the above-mentioned problems related to cloning procedures; in the current study, restriction independent cloning system was adopted by using GeneArt® Seamless PLUS Cloning and Assembly Kit (Cat # A14603**)** (Thermofisher, USA). This kit does not require restriction-ligation procedures. It can clone and assemble the inserts. The primers for the vectors and inserts can be designed with homologues overhangs by using web-based software (https://www.thermofisher.com/order/oligoDesigner/seamlessplus). One can adjust the size of the insert according to his strategy and clone them. We think this cloning technique will make the target easier. So, we have maintained the full-length cDNA as a multiple of six and this was flanked by HamRz and HdvRz. The full-length LaSota strain of NDV with exact 3′and 5′ends was rescued by this system.

The *in-vitro* characterization of the recombinant LaSota and the parenteral virus was tested in terms of pathogenicity, growth kinetic and titer development and no significant differences were found between two isolates. These results depict that artificially induced mutation has no drastic effects on the virus and secondly a faithful copy of the desired virus can be produced by using this recovery system.

Conclusions: In summary, we have rescued the recombinant LaSota strain of NDV by simultaneous co-transfection of four transcription plasmids; encoding the complete genome of NDV and the viral polymerase in the cell line. The identification of recombinant LaSota strain was confirmed by the presence of a genetic marker. The biological characteristics and growth kinetics of rescued virus were indistinguishable from the parenteral virus. In contrast to earlier virus recovery methods; this system is based on the seamless PCR cloning technique and cellular RNA POL II enzyme for the construction and expression of plasmids respectively. That's why it is free from the limitations of T7 RNA polymerase-provision either by

cell lines or helper viruses and drawbacks of traditional cloning methods. This system will provide an efficient method for the analysis of NDV biological properties, molecular mechanisms related to its pathogenesis, and for the development of genotype-matched vaccines.

Authors contribution: FM: Conceptualization, Methodology, writing – original draft. MSM: Funding acquisition; Project administration; Writing - review & editing. SUR: software, Resources, interpreted the results RZA: Formal analysis and critically reviewed the draft.

Declaration of competing interest: The authors declare that they have no known competing financial interests.

Acknowledgments: This research was funded by Higher education commission (HEC) of Pakistan under the scheme of National research program for universities (NRPU) with project ID 6348/Punjab/NRPU/R&D/HEC/2016.

REFERENCES

Ali M, Khan MUR, Aslam A, et al., 2021. Comparative molecular characterization and pathogenicity of H9N2 avian influenza virus in commercial poultry flocks in Pakistan. Pak Vet J 41:451-5.

Ayllon J, García-Sastre A and Martínez-Sobrido L, 2013. Rescue of recombinant Newcastle disease virus from cDNA. J Vis Exp 1–9.

Dey S, Chellappa MM, Gaikwad S, *et al*., 2014. Genotype Characterization of Commonly Used Newcastle Disease Virus Vaccine Strains of India. PloS One 9:e98869.

Dimitrov KM, Afonso CL, Yu Q, *et al.,* 2017. Newcastle disease vaccines—A solved problem or a continuous challenge? Vet Microbiol 206:126-36.

Ganar K, Das M, Sinha S, *et al*., 2014. Newcastle disease virus: Current status and our understanding. Virus Res 184:71-81.

Hasni MS, Chaudhry M, Mushtaq MH, *et al*., 2021. Prevalence and associated risk factors of avian influenza H9 in backyard poultry populations of two agroecological zones of Pakistan. Pak Vet J 41:132-6.

Hu H, Roth JP, Estevez CN, *et al*., 2011. Generation and evaluation of a recombinant Newcastle disease virus expressing the glycoprotein (G) of avian metapneumovirus subgroup C as a bivalent vaccine in turkeys. Vaccine 29:8624-33.

Inoue KI, Shoji Y, Kurane I, *et al*., 2003. An improved method for recovering rabies virus from cloned cDNA. J Virol Methods 107:229-36.

Jadhav A, Zhao L, Ledda A, *et al*., 2020. Patterns of RNA Editing in Newcastle Disease Virus Infections. Viruses 12:1249.

Kolakofsky D, Roux L, Garci D, *et al*., 2005. Paramyxovirus mRNA editing, the "rule of six" and error catastrophe: a hypothesis. J Gen Virol 86:1869-77.

Krishnamurthy S, Huang Z and Samal SK, 2000. Recovery of a virulent strain of Newcastle disease virus from cloned cDNA: Expression of a foreign gene results in growth retardation and attenuation. Virology 278:168-82.

Lebdah M, Tantawy L, Elgamal AM, *et al*., 2022. Molecular detection and characterization of virulent Newcastle disease viruses from different avian species in Egypt. Int J Vet Sci 11:189-95.

Li B, Li Y, Rui X, Lan X, *et al*., 2011. Rescue of Newcastle disease virus from cloned cDNA using an RNA polymerase II promoter. Arch Virol 156:979-86.

Mahmood MS and Sabir R, 2021. Preparation and evaluation of avian influenza (H9) and Newcastle disease (thermostable i-2 strain) bivalent vaccine for commercial poultry. Agrobiol Rec 3:17-23.

Manar AAK, El-Kady MF and Shaheed IB, 2020. Pathogenesis of Newcastle disease virus genotype vii in chickens vaccinated with LaSota and inactivated Newcastle disease vaccines. Int J Vet Sci 9:196-202.

Martin A, Staeheli P and Schneider U, 2006. RNA Polymerase II-Controlled Expression of Antigenomic RNA Enhances the Rescue Efficacies of Two Different Members of the Mononegavirales Independently of the Site of Viral Genome Replication. J Virol 80:5708-15.

Mehmood A, Rasool MH, Anjum AA, *et al*., 2019. Pathotyping and genetic characterization of a highly virulent newcastle disease virus strain isolated from recent outbreak in District Okara, Pakistan. Pak Vet J 39:353-8.

Miller PJ, Decanini EL and Afonso CL, 2010. Newcastle disease: Evolution of genotypes and the related diagnostic challenges. Infect Genet Evol 10:26-35.

Molouki A and Peeters B, 2017a. Rescue of recombinant Newcastle disease virus: current cloning strategies and RNA polymerase provision systems. Arch Virol 162:1-12.

Molouki A and Peeters B, 2017b. Rescue of recombinant Newcastle disease virus: a short history of how it all started. Arch Virol 162:1845-54.

Nakaya T, Cros J, Park MS, *et al*., 2001. Recombinant Newcastle Disease Virus as a Vaccine Vector. J Virol 75:11868-73.

Peeters BPH, de Leeuw OS, Koch G, *et al*., 1999. Rescue of Newcastle Disease Virus from Cloned cDNA: Evidence that Cleavability of the Fusion Protein Is a Major Determinant for Virulence. J Virol 73:5001-9.

Peeters BPH, De Leeuw OS, Verstegen I, *et al*., 2001. Generation of a recombinant chimeric Newcastle disease virus vaccine that allows serological differentiation between vaccinated and infected animals. Vaccine 19:1616-27.

Ramp K, Topfstedt E, Wackerllin R, *et al.,* 2012. Pathogenicity and Immunogenicity of Different Recombinant Newcastle Disease Virus Clone 30 Variants After *In Ovo* Vaccination. Avian Dis 56:208-17.

Rauf I, Wajid A, Hussain I, *et al*., 2019. Immunoprotective role of LaSota vaccine under immunosuppressive conditions in chicken challenged with velogenic avian avulavirus-1. Trop Anim Health Prod 51:1357-65.

Reed LJ and Muench H, 1938. A simple method of estimating fifty per cent endpoints. Am J Epidemiol 27:493-7.

Römer-Oberdörfer A, Mundt E, Mebatsion T, *et al*., 1999. Generation of recombinant lentogenic Newcastle disease virus from cDNA. J Gen Virol 80:2987-95.

Song X, Shan H, Zhu Y, *et al*., 2019. Self-capping of nucleoprotein filaments protects the Newcastle disease virus genome. eLife 8:e45057.

Stobart CC and Moore ML, 2014. RNA virus reverse genetics and vaccine design. Viruses 6:2531-50.

Sultan HA, Elfiel WK, Nour AA, *et al*., 2022. Efficacy of the Newcastle Disease Virus Genotype VII.1.1-Matched Vaccines in Commercial Broilers. Vaccines 10:29.

Wang J, Wang C, Feng N, *et al*., 2015. Development of a reverse genetics system based on RNA polymerase II for Newcastle disease virus genotype VII. Virus Genes 50:152-5.

Yanai H, Hayashi Y, Watanabe Y, *et al*., 2006. Development of a novel Borna disease virus reverse genetics system using RNA polymerase II promoter and SV40 nuclear import signal. Microbes Infect 8:1522-9.

Yang F, Xiao Y, Liu F, *et al*., 2021. Molecular Characterization and Antigenic analysis of reassortant H9N2 subtype avian influenza viruses in Eastern China in 2016. Virus Res 306:198577.

Yurchenko KS, Jing Y and Shestopalov AM, 2019. Adaptation of the newcastle disease virus to cell cultures for enhancing its oncolytic properties. Acta Naturae 11:66-73.

Zhang X, Liu H, Liu P, *et al*., 2013. Recovery of avirulent, thermostable Newcastle disease virus strain NDV4-C from cloned cDNA and stable expression of an inserted foreign gene. Arch Virol 158:2115-20.