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# Review Article

## Past and Present of Reverse Genetics in Animal Virology with Special Reference to Non–Segmented Negative Stranded RNA Viruses: a Review

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ABSTRACT

Reverse genetics is a cutting–edge tool that has revolutionized molecular virology through which viruses possessing artificial genomes can be rescued from cloned cDNA. This gave the researchers the choice and flexibility to get the modifications in the progeny virions that would be done at the genome level while constructing the cDNA. The idea led to two significant discoveries, with the first that gave an impetus in the area of live attenuated "Differentiation of infected and vaccinated animals" (DIVA) vaccines, and the second that led to a better understanding into the host–virus relationship. The DIVA vaccines developed through the reverse genetics tool have advantages of stable expression of the foreign protein coupled with the fundamental characteristics of the background virus that equates with the wild type. Rescue of DNA viruses and positive sense RNA viruses have been made easy, thanks to the less complicated replication strategies followed by them, but the non–segmented negative sense RNA viruses needs Ribonucleoprotein (RNP) complex to be provided *in vitro* to aid in anti–genome complex necessary for their replication. This technology has also played an effective role in identifying the intricacies in viral biology, evolution and replication. This in turn has made a phenomenal progress in identifying the nuances in host–pathogen interactions, thereby establishing new insights in molecular pathogenesis. The complex interplay of viral moieties and the cellular mechanisms that respond to these variations has been simplified to a greater extent with the advent of reverse genetics, and that has changed the way the virulence mechanisms of virus have been addressed so far.



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## INTRODUCTION

The paradigm of "genetics" is usually explained as *phenotype to genotype*, and this has led to the idea of *genotype to phenotype* being called as "reverse genetics". This neo–paradigm has culminated to a major breakthrough of genetically manipulating the genomes of viruses for decoding their structure–function relationship with respect to their virulence and pathogenicity (Conzelmann and Meyers, 1996) and generation of safe attenuated vaccine strains of virus. Earlier works on generation of recombinant virus were concentrated in DNA virus, in which the naked DNA was transfected into permissive cell lines to rescue the progeny virus. This was followed by homologous recombination of certain type of genes between the wild–type virus and mutated virus leading to rescue of defined mutated viruses. Later, the rescue of progeny virus from cDNA was established for positive sense RNA (PS–RNA) viruses like polio virus (Racaniello and Baltimore, 1981; Kaplan *et al*., 1985), bacteriophages (Taniguchi *et al*., 1978) etc. However the biggest challenge was for negative sense RNA (NS RNA) viruses, because the genomic RNA was not mRNA sense, and that it could not initiate replication by itself. The NS–RNA viruses additionally require certain elements like ribonucleoprotein (RNP) complex for their encapsidation and functional replication as in the case of Pestes des petit ruminants virus (PPRV), Rabies virus etc. By taking into consideration, the biggest task of constructing a genomic cDNA in a cloning plasmid along with helper plasmids (RNP cloned into expression plasmids) and then transfection into a cell line that could generate the progeny virus (Schnell *et al*., 1994). Most pathogenic viruses of veterinary importance fall under the category of RNA viruses with negative sense genome, and hence the rescue of Rabies virus from cDNA marks the



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beginning of a new era in the history of virology and reverse genetics. Advances in molecular techniques has reduced the burden on the researcher and provided more insights in the construction of infectious clone. One of the most important discoveries is the use of RNA polymerase promoter in the cDNA genomic construct followed by T7 RNA polymerase expressing Vaccinia virus infection in the permissive cell lines for rescuing viruses with NS RNA genome. The rescue was made further more easy with the development of constitutive expression of T7 RNA polymerase by cell lines itself, with the very instance of BSRT 7/5 cell line derived from a BHK–21 base (Buchholz *et al*., 1999). This was soon followed by the RNA pol I promoter being used for the rescue of segmented viruses like Influenza A, wherein the viral transcription plasmids carrying the eight segments of the virus were individually cloned into vector under the control of RNA pol I promoter (Fodor *et al*., 1999; Neumann *et al*., 1999). More advances led to a bidirectional strategy using RNA pol I promoter wherein the 12 plasmid system was reduced to 8 (Hoffmann *et al.* 2000). Further refinement in the application of promoters has led to rescue of virus from non–specialized simple cell lines, like in the case of successful rescue of mesogenic NDV strain Mukteswar using RNA pol II as promoter (Li *et al*., 2011).

***Rescue of Different Groups of Viruses by Reverse Genetics***

***Rescue Strategies for DNA Viruses***

The viruses that possessed DNA as their genome were the first to be reportedly amenable for manipulations by reverse genetics. This breakthrough was achieved for the first time with SV40 virus in which the cloned genomic DNA was transfected to permissive cells resulting in the rescue of recombinant SV40 virus, and in another version of the technique, mutated genomes after being transfected into permissive cells had generated defined mutated viruses (Goff and Berg, 1976). Later, this technique allowed rescue of large DNA viruses like Herpes virus, by homologous recombination (Post and Roizmann, 1981). This study reported the generation of chimeric Herpes viruses carrying a viable selectable marker like thymidine kinase (TK) gene, when wild type genome and marker DNA flanked by viral sequences were co– transfected in appropriate cell lines lacking the thymidine kinase, allowed the rescue of unique recombinant viruses by homologous recombination carrying the TK gene (Post and Roizmann, 1981). Other DNA viruses including pox viruses (Panicalli and Paoletti, 1982; Mackett *et al*., 1982), Adeno viruses (Jones and Shenk, 1978; Samulski *et al*., 1989) and parvo viruses were successfully rescued by a similar technique. Cosmids with overlapping portions of viral genomes have been used extensively to generate recombinant viruses through recombination between the cosmids, and this system was reportedly used to rescue herpes simplex 1 viruses (Cunningham and Davison, 1993), cytomegaloviruses (Kemble *et al*., 1996) and Epstein –Barr viruses (Cohen *et al*., 1989).

***RNA Viruses and their Rescue***

***Positive Sense (PS) RNA Viruses***

Among the positive sense RNA viruses, application of reverse genetics was relatively easy because the genomic RNA is mRNA and they directly act as template for viral RNA replication. This makes the deproteinated genomes of these viruses the ability to utilize the host cell machinery directly to initiate their replication. Hence, plasmid encoded or *in vitro* synthesized genomic RNA of these viruses is infectious, when transfected to permissive cells (Conzelmann and Meyers, 1996). Soon, the reverse genetics system has been established in RNA viruses like Poliovirus (Racaniello and Baltimore, 1981; Kaplan *et al*., 1985) and Bacteriophages (Taniguchi *et al*., 1978). Recovery of poliovirus was performed by transfecting the plasmids containing the cDNA or RNA transcribed by plasmids containing the polio virus genome into suitable cell lines. There were reports on other plus–stranded RNA viruses like Sindbis virus (Rice *et al*., 1987) and Semiliki forest virus (Liljeström *et al*., 1991), in which the cloned cDNA derived RNAs of these viruses in the form of vector with promoter elements were transfected to cell lines, resulting in the recovery of infectious viruses. The studies on these viruses have paved the way for an extensive analysis of the promoter elements of viral RNA and the structure–function studies of viral proteins. Another virus that belongs to family *Coronaviridae*, Avian infectious bronchitis virus (AIBV) belonging to PS RNA viruses were extensively subjected to reverse genetics applications for studying the virus biology and replication (Stirrups *et al*., 1998), virulence (Hodgson *et al*., 2004) and generation of live attenuated recombinant vaccines (Zhou *et al*., 2013). Recombinant AIBV strain H120 was successfully rescued with the reverse genetics technique, wherein contiguous subgenomic fragments spanning the entire virus genome was cloned using the modified "no– seem" ligation strategy into pMD19–T and it was co–transfected along with the nucleocapsid gene into BHK–21 cells, and this virus gave 85 % immune protection to chickens challenged with IBV Mass–41 strain (Zhou *et al*., 2013).

***Negative Sense (NS) RNA Viruses***

The negative stranded RNA viruses are divided into those with non–segmented genomes (under order Mononegavirales) including the four families namely *Paramyxoviridae*, *Rhabdoviridae*, *Filoviridae*, *Bornaviridae*; and three families carrying segmented genomes including *Orthomyxoviridae* (6–8 segments), *Bunyaviridae* (3 segments), and *Arenaviridae* (2 segments) (van Regenmortel *et al*., 2000). These groups of viruses include many pathogens that cause fatal diseases in humans and the animals such as Measles, Mumps, Rabies, Human respiratory syncytial virus (HRSV), Human parainfluenza I viruses, Rinderpest, PPR, Newcastle disease, Bovine respiratory syncytial virus, Avian influenza etc., and also many emerging and re–emerging viruses like Hendra, Nipah, Ebola, Marburg, Rift valley fever, Crimean congo hemorrhagic fever (CCHF).

The NS RNA viruses follow two different ways to bind to a host cell receptor to initiate an infection viz., a pH dependent pathway, where the viral surface interacts with the endosomal membranes in an acidic environment of late endosomes and a pH independent pathway in which the viral glycoprotein binds to the plasma membrane (Knipe and Howley, 2001).

***Segmented Negative Sense (SNS) RNA Viruses***

The SNS RNA viruses differ from the other non–segmented negative stranded (NSNS) RNA viruses in their replication and mRNA transcription. Each segment of these viruses


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represents a separate transcription and replication unit, and each 3' and 5' termini of every segment showing conservations and partial inverted complementarity, resulting in base paired terminal ends which together constitute a core functional promoter. Initiation of replication was observed from the 3' end of the genome (vRNA) and antigenome (cRNA). In contrast to NSNS viruses, the transcription initiates only on the vRNA template, except in some viruses belonging to bunyaviridae like phlebovirus and tospovirus, which uses ambisense coding strategy in one or more of their genome segments (Knipe and Howley, 2001; Neumann *et al*., 2002) .

Among the SNS RNA viruses, influenza viruses were the one that was extensively studied and the first of the kind of NS RNA virus (Enami *et al*., 1990) to be rescued using reverse genetics. The influenza virus RNPs that comprises of the proteins PA, PB1 and PB2, upon their release into cytoplasm of host cell, enters the nucleus and begins to transcribe the genomic RNA into mRNA and a positive–sense antigenomic RNA that serves as template for the production of genomic RNA. Genetic manipulation of the segmented genomes was through the isolation of the reassortant viruses of the arena, bunya and influenza viruses. Infectious clone of Influenza A can be rescued by transfecting MDBK cells with RNAs derived from specific recombinant DNAs corresponding to NA (Neuraminidase) gene of Influenza A (WSN/33) followed by addition of purified RNA polymerase complex and a superinfection with helper virus (Influenza A virus lacking the WSN –NA gene) [Enami *et al*. (1990]. Neumann *et al*. (1999) has reported a unique technique in which the subgenomic segments were individually cloned as eight plasmids under the control of RNA polymerase I (Pol I) promoter and transcription terminator, which were co–transfected with four plasmids encoding the polymerase complex proteins and nucleoproteins cDNAs under the control of RNA polymerase II (pol II) promoter. The use of pol I transcripts to produce recombinant influenza virus RNA segments became more popular, in which the primary RNA transcripts produced by the pol I are ribosomal RNAs that reportedly do not possess either a 5′ cap structure or a 3′ poly (A) tail, thereby making precise 3′ and 5′ ends that aids in effective encapsidation (Neumann and Hobom, 1995; Neumann *et al*., 1999). Another group of coworkers produced artificial influenza RNA segments with precise 3′ and 5′ ends to ease encapsidation and packaging of replication complex in progeny influenza virions (Zobel *et al*., 1993). The pol I system has several advantages over the other systems like T7 promoter, because pol I enzyme is present in the nucleolus of all eukaryotic cells and therefore it does not require the genetic elements in *trans*, and produce a precise 3′ and 5′ termini due to lack of the 3′ poly(A) tail and a 5′ cap, and more importantly it requires simple permissive cells compared to other systems that requires a stably T7 polymerase expressing specialized cell lines or eliminates the use of a helper virus. A more efficient ambisense expression for rescue of influenza A virus was developed, in which the RNA pol I cassette was cloned between an RNA pol II promoter sequence and polyadenylation signal, where the viral RNA and mRNA were generated simultaneously from these constructs, which has considerably reduced the total number of plasmids to eight compared to previous experiments which used a total of 15 plasmids to rescue influenza A virus (Hoffmann *et al*., 2000). The first ever generated report on rescue of a SNS RNA virus entirely from cDNA using a helper free system was Bunyavirus (Brigden and Elliot, 1996). Through this chimeric Bunya virus was generated with specific genetic tags through three segments of the RNA genome cDNA copies with a precise leader and trailer regions flanked by bacteriophage T7 promoter and HDVRz, respectively. In 2001, another SNS RNA virus namely Thogoto virus was rescued using a combination of T7 promoter and pol I systems for protein expression and genomic RNA transcription, respectively (Wagner *et al*., 2001)

***Non–Segmented Negative Sense (NSNS) RNA Viruses***

In contrast to the replication of PS RNA virus counterpart, the negative sense RNA viruses cannot initiate a life cycle, since it requires a *de novo* protein synthesis catalyzed by the viral RNA polymerase, because the input genomic or antigenomic RNA has to be encapsidated with the nucleoprotein before it can serve as functional template to initiate replication and transcription (Knipe and Howley, 2001; Sedlmeier and Neubert, 1998). The possible constraints that hay–wired the rescue of NS RNA viruses from cDNA (Pekosz *et al*., 1999) is as listed below:

i) Precise leader and trailer extracistronic elements are required for replication and packaging of the genomic RNA
ii) The polarity of the genome of negative sense RNA viruses are opposite to the mRNA sense
iii) The RNA genome of these viruses are encapsidated co–transcriptionally with the nucleoprotein to form NP–RNA complex of helical symmetry and associate with phosphoprotein and Large polymerase protein to form a minimal replication initiation complex
iv) Both genomic and anti–genomic RNA exist as viral RNP complex

To overcome the problem of anti–sense RNA, initial work on Rabies virus rescue concentrated on filling the gap between the above–listed constraints and a successful rescue. The solution was identified and the RNP expression plasmids were co–transfected along with the cDNA (whole genome cloned plasmid construct) into cell lines that allowed selection and successful rescue of recombinant progeny rabies virion. Successful rescue of rabies virus from cloned cDNA resulted as a trendsetter in the rescue of first NSNS RNA virus (Schnell *et al*., 1994). This study has provided the much needed impetus for further work on other NSNS viruses like Vesicular stomatitis virus (Lawson *et al*., 1995 and Whelan *et al*., 1995), Measles virus (Radecke *et al*., 1995), Human respiratory syncytial virus (Collins *et al*., 1995) and Sendai virus (Garcin *et al*., 1995 and Kato *et al*., 1996).

***Reverse Genetics of NSNS RNA Viruses***

***Different Strategies Adopted to Rescue NSNS RNA Virus from cDNA***

The technique of manipulation at the genome level with the aid of helper plasmids has increased the scope for rescuing other viruses with non–segmented negative sense single stranded RNA genome like Newcastle disease virus. Under this section, most discussions had been related to NDV,


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since numerous contributions in this field has come from researchers who have been studying with this virus. The initial work on NDV rescue from cloned cDNA was reported by Romer–Oberdörfer *et al.* (1999) followed by Peeters *et al.* (1999). Romer–Oberdörfer *et al.*, (1999) applied a vaccinia virus free system to rescue a lentogenic NDV strain Clone 30 using a pBluescript II based modified vector. In this study, the complete antigenome was under the control of T7 promoter, and helper plasmids with NP, P and L individually cloned in pCite2a based expression vector transfected to a cell line called BSR T7/5 clone that constitutively expresses T7 RNA polymerase. Whereas, the technique used by Peeters *et al.* (1999) involved recombinant helper virus of Fowl Pox origin that expresses T7 RNA polymerase to rescue NDV strain LaSota from a cloned cDNA in a transcription vector (pOLTV5) which has the genome length cDNA between T7 promoter and autocatalytic Hepatitis delta virus ribozyme (HDVRz) co–transfected into chicken embryo fibroblast (CEF) and QM5 cells, along with helper plasmids (NP, P and L) individually cloned in pCIneo based expression vector. In another contemporary study, an almost similar system was employed for the rescue of mesogenic strain NDV Beaudette C using recombinant Vaccina virus based T7 RNA polymerase expression system along with a modified pBR322 vector backbone containing the infectious clone and the helper plasmids in a Hep–2 cell line (Krishnamurthy *et al.*, 2000). While there was also recent reports that stated that, apart from helper viruses like recombinant Vaccinia virus (Ankara strain) or fowl pox virus (FPVT7) or a cell line like BSR T7/5 that helps in the rescue of the NDV, a different promoter based reverse genetics system that can exploit the rescue of Newcastle disease virus strain like Mukteswar, used RNA polymerase II promoter, in which, the full length viral antigenome was assembled between a cytomegalovirus promoter and hammer–head ribozyme and, along with co–transfection of helper plasmids NP, P and L coding sequences cloned in correct reading frame into pCIneo expression vector (Li *et al.*, 2011).

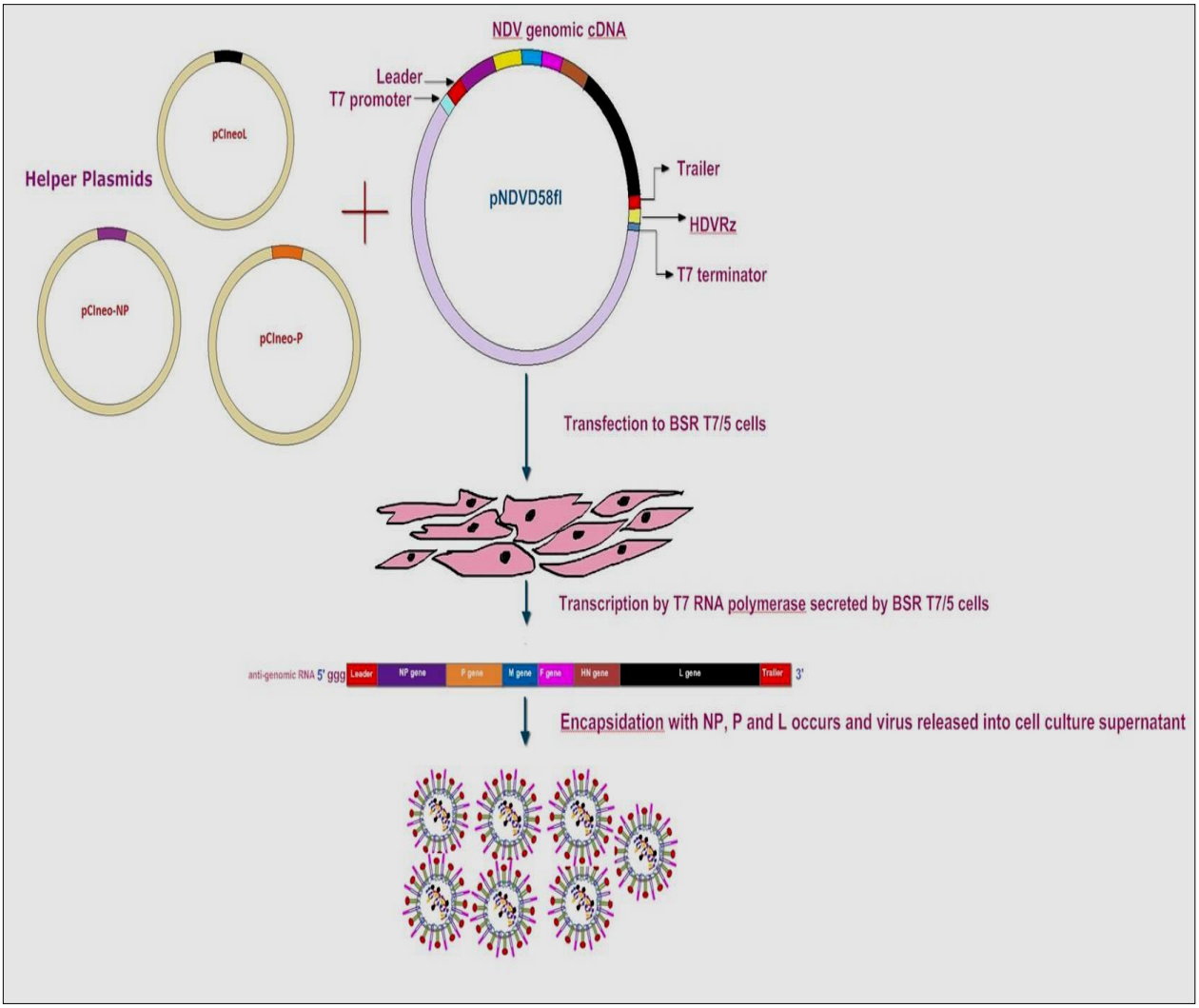


Figure 1: Diagram depicting the strategy for co–transfection of viral genomic cDNA and helper plasmids into a cell line for rescue of recombinant progeny virus in case of NSNS–RNA viruses


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***Marker Vaccine Development against NDV with Reverse Genetics***

Marker vaccines are defined as those that can differentiate the infected animals from the vaccinated animals by means of companion diagnostic tests (Van Oirschot, 1999). A DIVA vaccine against NDV was generated by reverse genetics system (Peeters *et al*., 2001) in which the gene encoding the hemagglutinin–neuraminidase (HN) has been replaced by a hybrid HN gene that consisted cytoplasmic domain, transmembrane region and stalk region of HN of NDV LaSota strain, while the immunogenic globular domain of HN was replaced with that of the APMV–4, resulting in a chimeric virus carrying immunogenic HN from APMV–4. The chimeric virus had an ICPI value of 0.00 and induced protection against chicken challenged with a lethal dose of virulent virus. Moreover, it differentiated the conventional vaccinated and infected chickens serologically by using a diagnostic test that used a *Pichia pastoris* secreted HN protein (Peeters *et al*., 2001). This was soon followed by a marker vaccine against NDV, with an epitope completely from an unrelated virus, which was developed by deleting an immunodominant B –cell epitope present in the NP gene coding region and by inserting a B cell epitope of the S2 glycoprotein of Murine hepatitis virus (MHV) in the deleted region by reverse genetics. The recombinant virus generated by this technique carried MHV epitope, and that was evidenced by the fact that the chickens that were immunized with this virus expressed S2–MHV antibodies but completely lacked antibodies directed against B–cell NP immunodominant epitope (Mebatsion *et al*., 2002). NDV is also used as a vaccine vector for conferring immunity to other viral diseases as in the case, NDV–Hitchner B1 used to protect against avian influenza by introducing hemagglutinin of influenza A/WSN/33 virus between the intergenic regions of P and M genes of NDV genome. The rescued virus induced a strong humoral antibody response against influenza and provided complete protection to mice challenged with a lethal dose of influenza virus (Nakaya *et al*., 2001).

In another instance, an NDV based live attenuated vaccine carrying HA epitope of H5 subtype avian influenza virus was generated by reverse genetics technique (Ge *et al*., 2007). The experiment was carried out with both mutated and wild–type HA open reading frame from Highly pathogenic avian influenza virus (HPAIV) wild bird isolate (A/Bar–headed goose/Qinghai/3/2005 [H5N1]), that was inserted into the intergenic region between the P and M genes of the NDV LaSota vaccine strain, and upon immunization of the chimeric viruses, induced antibodies against both NDV and HA epitope, and protected the immunized population against both homologous and heterologous challenge.

Another group of workers (Cho *et al*., 2008) developed a recombinant vaccine virus (KBNP–C4152R2L) carrying the HN and F gene of genotype VIId virus (KBNP–4152) against a LaSota background. The F cleavage motif was changed from $^{112}$RRQKR$^{116}$↓ to $^{112}$GRQAR$^{116}$↓ to attenuate the virulence of recombinant strain. To reduce the pathogenic instability due to random mutations, a single point mutation was inserted at codon 115, which blocked any changes to basic amino acids and a six nucleotide insertion was created in the intergenic region between the M and F genes complying with the 'rule of six' for viral attenuation. The length of the HN protein was mutated from 571 to 577 amino acid residues to function as a marker. The recombinant strain KBNP–C4152R2L had a similar antigenicity as of KBNP–4152, but differed from wild type LaSota and it was completely avirulent with an ICPI of 0.00, making it a promising candidate for marker vaccine. Further, to strengthen the role of FPCS, a virulent genotype VII strain belonging to class II from Indonesia namely Banjarmasin/010/10 (Ban/010) was used as a candidate (Xiao *et al*., 2012) for development of live attenuated vaccine to protect the flocks against the circulating virulent genotype VII viruses. The experiment was carried out with the generation of an attenuated Ban/010 virus that was mutated in the FPCS region from virulent "RRQKR↓F" to avirulent "GRQGR↓L "by three amino acid substitutions, and the resultant recombinant virus has not reportedly produced any syncytia or plaques even after supplementing with an external protease, and was found to be completely avirulent. The rBan/010 virus effectively neutralized the circulating virulent viruses as evidenced by protective HI titers and its protection conferred against a clinical infection and mortality after challenge with virulent Ban/010 or Texas GB, and also reduced shedding of the virulent virus compared to B1 vaccine.

***Reverse Genetics – Role in Molecular Pathogenesis***

***NDV Proteins and its Virulence***

***Fusion Protein as a Determinant of Virulence***

Many yardsticks have been followed to measure the virulence for NDV, but the amino acid composition at the fusion protein cleavage site (FPCS) of F protein has been shown to be a major factor that determines NDV virulence (Nagai *et al*., 1976; Ogasawara *et al*., 1992; Collins *et al*., 1993). It has been reported in many instances (Nagai *et al*., 1976; Garten *et al*., 1980a; Rott and Klenk, 1988) that the cleavage of F precursor protein ($F_0$) into F1 and F2 by the host cell proteases was an essential factor for the progeny viruses to become infective. The presence of monobasic amino–acid motif at the C–terminus of F2 fraction and a leucine at the N–terminus of the F1 protein are seen in lentogenic viruses and are cleaved extracellularly by trypsin–like proteases present in the respiratory and alimentary tract of the host. Whereas the amino acid motif present in the C–terminus of F2 is polybasic and at the N–terminus of F1 is phenylalanine in the case of velogenic and mesogenic NDV strains and are cleaved by intracellular ubiquitous Furin–like proteases, resulting in a fatal systemic infection (Nagai *et al*., 1976; Ogasawara *et al*., 1992). This data has been corroborated later by a series of reverse genetics studies (Peeters *et al*., 1999; Romer–Oberdörfer *et al*., 2006) that swapped the lentogenic viral FPCS with that of a velogenic, and it was found that the pathogenicity of the chimeric virus containing artificial velogenic FPCS was augmented. The vice versa was also reported to be true to the fact that the ICPI was decreased from 1.89 to 0.13 with a three nucleotide change at the FPCS of ZJ1 recombinant NDV generated by reverse genetics, whose wild type was highly virulent (Hu *et al*., 2009). There were reports on contrary to these findings that FPCS as sole determinant of virulence may not be universal, because the ICPI increase in lentogenic chimeric viruses with artificial velogenic cleavage sites were higher


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but not phenomenal, from 0.0 to 1.13, compared to wild–type velogenic viruses whose ICPI range from 1.6–1.9 (Panda *et al*., 2004a; Peeters *et al*., 1999; Romer–Oberdörfer *et al*., 2006). There had been reports to throw more light on this aspect, suggesting that there would be proteins other than F that contributes to pathogenicity of NDV (Kommers *et al*., 2003; De Leeuw *et al*., 2003; Wakamatsu *et al*., 2006a; Subbiah *et al*., 2011).

***HN Protein and its Role in Virulence***

The HN protein is involved in the tropism of the virus and it has a major role to play in the attachment and release of the virus, thereby making an important candidate for virulence determinant studies. Further the HN also triggers the fusion activity of F protein during the entry of the virus into the host cell (Lamb and Parks, 2007). The HN ORF reportedly exists as three genotypes resulting in proteins of 571, 577 or 616 amino acids, with the lentogenic strains of viruses having 616 amino acids that further processes into an active HN by proteolytic removal of a small glycosylated C–terminal moiety (Nagai *et al*., 1976; Nagai and Klenk, 1977; Garten *et al*., 1980b; Sato *et al*., 1987; Gorman *et al*., 1988).

In an experiment reported by Huang *et al*. (2004) where a recombinant LaSota virus backbone containing HN protein of mesogenic Beaudette C strain showed a significant virulence increase from lentogenic to mesogenic. There were also sufficient reports (Huang *et al*., 2004; Romer–Oberdörfer *et al*., 2006; Wakamatsu *et al*., 2006b) to strengthen the possible role of the HN in virulence, by generating an infectious clone, with changes introduced in the amino acids either in the glycosylation sites or by mutating specific residues in HN gene have shown a moderate effect on the pathogenicity of the chimeric NDV. But there had been reports (De Leeuw *et al*., 2005) contrasting these facts wherein, the HN protein of lentogenic LaSota virus was swapped with that of a velogenic Hertz strain or a HN chimera consisting of stem region of Hertz HN and the globular head of LaSota or vice versa, that the recombinants did not differ in virulence from that of the parent strain as witnessed by the ICPI value. But the IVPI value has shown a significant increase, not undermining the fact that both the stem region and the globular head region of the HN protein playing a major role in the virus tropism and its virulence. In a study conducted by Estevez *et al*., (2007), where NDV chimeras were created by exchanging the HN gene of mesogenic strain with that of neurotropic as well as viscerotropic velogenic strain and interestingly found that there had been no significant difference in the virulence between the chimeric viruses and the parent strain. In an another study similar findings were reported in which an infectious clone with an anhinga backbone containing artificial velogenic HN, did not exhibit any virulence, as witnessed by the intraocular route of inoculation of the recombinant virus in both day old and four week old chickens (Susta *et al*., 2010). These findings suggest that the virulence of NDV is not controlled by single determinant, but is influenced by 'group effect' of multiple genes.

Post translational modification like glycosylation has a paramount role in the expression and proper functioning of the HN protein in the NDV life cycle (Vigerust and Shepherd, 2007) and mutations at these N–linked glycosylation sites has been found to undermine the virulence of the virus (Panda *et al*., 2004b).

Studies have also been targeted at correlating the length of the HN ORF on virulence; however its role in virulence could not be established (Romer–Oberdörfer *et al*., 2003). But by mutating some of the key amino acid residues in HN protein has been shown to influence the virus activity. By creating a mutation of key amino acid, like the Tyrosine residue at position 526, near the receptor binding site in the globular head region to glutamine resulted in a decrease in viral hemadsorption, neuraminidase and fusion activity, causing virus attenuation and influencing the growth kinetics in cell culture, MDT and ICPI value (Khattar *et al*., 2009). But substitution of a methionine residue instead of Isoleucine at the position 192 influenced the functional properties of HN protein, without affecting the virulence of the virus (Estevez *et al*., 2011).

***Role of V Protein in Virulence***

The V protein, which is generated by RNA editing from the polycistronic P gene, is rich in cysteine residues in the carboxyterminal end and is more conserved cutting across the paramyxoviruses, with the exception of human parainfluenza virus type I (HPIV–1), which lacks an intact V ORF (Matsuoka *et al*., 1991). It has been reported that the chimeric NDV viruses that completely or partially lack the V protein or mutations in the V protein showed severe growth impairment in vitro and their replication in embryonated eggs was found to be age–dependent (Huang *et al*., 2003; Mebatsion *et al*., 2001; Park *et al*., 2003). This has been explained due to the fact that the chimeric viruses with mutated V protein in contrast to the wild type viruses are unable to degrade the STAT1 protein (Huang *et al*., 2003), which is an important part of the interferon signaling pathway (Haller *et al*., 2006). Reports have been generated to corroborate the role of V protein in virulence and studies on measles (Patterson *et al*., 2000) and SeV (Huang *et al*., 2000) has shown that deletion of V protein attenuated their virulence. Apart from these findings, further reports that conducted *in vitro* experiments on the V protein mutated viruses have shown that they have an increased rate of apoptosis (Park *et al*., 2003), and *in vivo* studies have found that the apoptotic rates corresponded to the severity of the disease caused by various strains (Harrison *et al*., 2011). In another study (Alamares *et al*., 2010), it has been reported that the V protein of a mesogenic strain, beaudette C showed a hiked anti–interferon response compared to a less virulent strain LaSota, *in vitro*.

***Viral Replication Complex and Virulence***

Efficiency of viral replication has been correlated to the virulence in many viruses, because higher levels of replication leads to an increased virus titer and in turn has a cascading effect in jeopardizing the immune response by the host, enhancing the pathogenesis. Reduced levels of RNA synthesis due to mutation in the components of replication complex has been reportedly associated with the reduced virulence of NDV (Madansky and Bratt, 1981) and several other paramyxoviruses viz., measles virus (Bankamp *et al*., 2002; Takeda *et al*., 1998), respiratory syncytial virus and parainfluenza virus (Murphy and Collins, 2002; Skiadopoulos *et al*., 1998). The role of NP, P and L proteins in the virulence of NDV has been studied (Rout and Samal,


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2008) using a reverse genetics system, wherein chimeric viruses were generated by exchanging these genes between the lentogenic strain LaSota and the mesogenic strain Beaudette C, both belonging to genotype II of the class II ND viruses (Aldous *et al*., 2003). Interestingly, LaSota L gene in a beaudette C background replicated at a higher level, and was more virulent than its wild–type virus, and no significant effects could be observed with the chimeric viruses with NP and P proteins generated under different pathotype backbone. In a similar study (Dortmans *et al*., 2010) conducted with a different lineage and genotype of viruses, where it was found that all the proteins that make the viral replication complex (NP, P and L) played a significant role in determining the virulence of NDV. In this experiment, all the three genes of viral replication complex have been simultaneously exchanged between a low virulent virus of PPMV origin AV324 and a virulent NDV strain Hertz (genotype IV). The low virulent RNP complex genes in a Hertz background, attenuated the chimeric virus, whereas the virulent genes in AV324 (avirulent PPMV–1 strain) avirulent background became more virulent. This further reported that the individual genes has contributed their part of virulence, but the virulence was either conferred or attenuated because of the synergistic effect of these genes together, rather than alone. This could be explained with another experiment in the same study in which the matrix protein of AV324 virus in a Hertz chimeric virus generated by reverse genetics showed a decrease in virulence, but didn't show an increase in virulence when the experiment was conducted vice versa, because the M protein of Hertz lacked the ability to increase the virulence of AV324, alone.

***Non–Coding Regions as Determinant of Virulence***

Apart from these findings, it has been fascinating to observe that the transcription and translational control signals also modulate the virulence, by controlling the protein expression, with the studies on NDV (Yan and Samal, 2008; Yan *et al*, 2009; Kim and Samal, 2010), Vesicular stomatitis virus (Barr *et al*., 1997; Stillman and Whitt, 1998), measles virus (Parks *et al*., 2001) and canine distemper virus (Anderson and von Messling, 2008). Especially in measles virus, the 3' UTR of M (Takeda *et al*., 2005) and in canine distemper virus (Anderson and von Messling, 2008), the 5' UTR of F gene has an important role in replication and virulence of the virus. The deletion of the entire 5' UTR of HN gene could affect the transcription and translation of HN mRNA and has subsequently reduced the virulence (Yan *et al*., 2009). The position of the UTR in its associated gene and in the genome is very specific (Kim and Samal, 2010).

## CONCLUSION

An overall insight into the biology of viruses and their structure –function relationship has made the genetic manipulation of the viruses plausible. Thanks to the recent advances in the molecular techniques like reverse genetics through which the difficult targets are drawn close to success. A comprehensive strategy using these cutting–edge tools could decipher more understanding towards the mechanism of virulence. The viral determinants of pathogenicity would be more important in interpreting the disease mechanism to enable an effective prophylactic measure to curb the disease.

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## CONFLICT OF INTEREST

There is no conflict of interest among authors..

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