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## Original Article

# Cloning & Expression of F Protein Gene (HR1 region) of Newcastle Disease Virus NR43 Isolate from Iran in *E.coli*

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## Abstract

**Background and Aims:** NDV (Newcastle Disease Virus) is one of the viruses that cause disease in avian with severe economic losses in the poultry industry in many countries. Fusion protein (F) which plays a major role in the virus pathogenicity contains several regions that have a role in the fusion process. Mutation in the sequence of HR1 & HR2 regions of this protein prevents fusion of the virus to host cell. In addition, the proteins of HR1 and HR2 regions have antitumor properties that are related to their pathogenicity.

**Methods:** In this investigation we used Newcastle disease virus NR43 isolate, from poultry diseases diagnostic department of Razi vaccine and serum research institute. RNA was extracted using SDS and proteinase K procedure. In the next step, RT-PCR was carried out and then cDNA cloned in pTZ57RT vector. After sequencing and alignmenting of the cDNA, a pair of proper primers for cloning HR1 in expression vector Pet32a(+) was designed. The HR1 expression was carried out by SDS–PAGE Western- Blotting in which the peptides were blotted onto nitrocellulose membrane using Ni-NTA anti His tag (1:1500 dilutions) coupled to HRP enzyme.

**Results:** A peptide with 23.76 kD/a molecular weight s peptide was obtained.

**Conclusion:** By cloning and expression of HR1 region of protein F, it will be possible to express the whole gene that could be introduced as a novel vaccine against NDV.

**Keywords:** Cloning; Newcastle Disease Virus (NDV); F protein gene; Heptad repeat

## Introduction

Members of the family Paramyxoviridae are enveloped, negative – stranded RNA viruses that infect a great variety of mammalian and avian species (1, 2). According to the current taxonomy and nomenclature, the family of paramixoviridae consists of two subfamilies. The subfamily paramyxoviridae contains three genera, Rubulavirus, Respirovirus (Formally known as the genus paramyxovirus) and Morbilivirus. There are nine serotypes of avian paramyxovirus (APM). APMV type 1 (APMV-1), also called Newcastle disease virus (NDV), is assigned into the genus of Rubulavirus (3). Outbreaks of NDV have been causing severe economic loses in many countries (4-6). The genome of NDV is nearly 15186 nucleotides in length and encodes at least seven proteins, nucleocapsid protein (NP), phosphoprotein (P),

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matrix protein (M), haemagglutinin-neurammidase (HN) protein, fusion protein (F) and large protein (L) (7, 8). Pathogenicity of the virus is related to F protein. The function of the protein is fusing cellular & viral membranes to each other that is accompanying with penetrating virus particle into the host cell. F protein contains at least three heptads repeat regions including HR1, HR2 & HR3 (2, 4-6, 9-12).

HR1 is located at the carboxyl terminus of the fusion peptide, while HR2 is located adjacent to the amino terminus of transmembrane domain. Both HR1 & HR2 are important for fusion function. HR3 function seems to be a requirement of HN for fusion. The HR1 helical wheel contains 62 amino acids. As peptides from HR1 & HR2 regions inhibit F protein activity, they have antiviral property. NDV contains anti tumoral effects especially on human tumor xenografts including neuroblastoma, fibrosarcoma xenografts, which is linked to the F protein properties (11-13).

Considering the importance of nucleotide and amino acid sequence of the F protein and particularly its HR regions with focusing on mentioned regions motive related to naïve isolated from Iran can help more investigations in designing recombinant vaccines & NDV molecular diagnosis.

It is possible to design a recombinant virus which can be used for NDV vaccine.

## Methods

### Virus

Avian NDV NR43 isolate was obtained from Iranian poultry industry in Ghom city. The virus was cultured on specific pathogen free eggs. Mean dead time of NDV NR43 infected chick embryos was less than 40 hours.

### Extraction of viral RNA

NDV RNA was extracted using RNX procedure (CinnaGen co). The concentration of extracted RNA was determined by absorption spectroscopy at 260nm using a UV spectrophotometer.

### RT-PCR of NDV Fusion Protein Gene

The specific primers used for the synthesis and amplification of cDNA encoding the F protein were forward primer, 5′-CAT CTT CTA CCA GGA TCC A-3′ and reverse primer, 5′-CCA AGA GTT GAG TCT GTG AGT C-3′ for segment A, and forward primer 5′ GAC TCA CAG ACT CAA CTC TTG G-3′ and reverse primer 5′-TTT GTA GTG GCT CTC ATC TG-3′ for segment B. The cDNA was synthesized at 50˚C for 30 min in the presence of AMV Reverse Transcriptase and High Expand enzymes (Roche, Germany), containing Taq DNA polymerase and PWO, dNTP mix (final concentration each at 2mM). After preparation of cDNA, the PCR amplification was carried out under the following condition: initial denaturation at 94˚C for 2 min, followed by 35 cycles amplification (94˚C for 10 sec, 51˚C for 30 sec, and 68˚C for 30 sec) , with final extension for 5 min at 68°C. The cDNA was analyzed by 1% agarose gel electrophoresis (size marker No 9, Roch co). The products were identified by endonuclease digestion using Ps1, HindI and HindIII restriction enzymes. For cloning of cDNA into TA-vector pTZ57/RT; both segments A and B were run on LMT gel and purified. Then ligated using T4 DNA ligase at 22°C for 1 hr.

### Cell transformation

Competent cells (DH5α) were prepared under 50mM $CaCl_2$ conditions. The cloned pTZ57/RT vectors were transformed into the competent cell under heat –shock condition. The transformed cells were grown on LB-ampicillin plate containing IPTG & Xgal for screening the cells containing cloned vectors. The transformed cells appeared as white colonies on the LB-ampicillin plate after 24 hr of incubation.

### Designing specific primers and amplification of HR1

The specific primers for HR1 region were designed by Generunner package. The cloned pTZ57/RT vectors (containing sequence A) were purified from E coli (DH5α) by alkaline lysis procedure similar to that of Russell D *et al* (12). The primers used for amplification of HR1 (AY678224) region cDNA were as follow:

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5′ –ACA GGG ATC CGC AGC TGC GGC TCT AAT AC-3′ (Forward) and
5′-TAATT AAGCTTCAGTCCAA TTC TCG TGC TGT ATT A-3′ (Reverse)
The specific primers (above) were used for amplification of HR1 region cDNA by Hot-start PCR technique under following conditions: initial denaturation at 94˚C for 10 min, a 35 cycle amplification (94˚C for 20 sec, 62˚C for 20 sec, and 72˚C for 20 sec), and a final extension at 72˚C for 5 min.

**Cloning NDV (NR43) HR1 region cDNA**

The PCR product of NDV (NR43) HR1 region was loaded into 0.6% LMT agarose gel and extract. HR1 region cDNA and pET32a(+) expression vector were digested by BamHI/HindIII restriction enzymes (40 Unit/µl, respectively) in buffer-B (Roche). The digested pET32a(+) expression vector was purified and ligated to the extracted PCR product according to the manufacturer's instructions.

**PCR and DNA sequencing**

To verify the presence of NDV(NR43) HR1 region cDNA inserted to pET32a(+) expression vector, Hot-start PCR was carried out under former conditions (above mentioned).The purified PCR product (amplified cDNA) was sequenced using an automatic sequencer (MRD-MDR. ab 1,2004,Korea).

**Expression**

For expression the procedures of Novagen (pET system manual) was followed. The promoter of T7 of pET32a(+) was induced using IPTG and growing the E coli cells in presence of 1mM IPTG at 30°C in shaking incubator for 6 hr. Cells were pelleted by centrifugation (3000rpm). The pellet was suspended in lysis buffer (NaCl: 500mM, Imidazole 5mM, Metheamen: 0.3mM, Tris (pH:7.9): 20mM, PMSF: 0.12mg/ml, MgCl2 : 200 mg/30ml). Cells were sonicated, for 7 cycles, 15 sec each in ice. The sonicated cells were mixed with lysis mix (Tris-HCl, pH:6.8; 10% 2-ME, 4% SDS and boiled for 10 min and electrophoresed on 12% SDS polyacrylamide gel.

**Peptide Estimation**

Peptide estimation was done by Bradford method. One ml of Bradford solution poured as blank, control and test tube; separately, 100µl of D.W for blank tube, 5µl crude extraction of uninduced cells for control tube, 95µl D.W for test and control tubes; were added separately. The concentration of the interested expressed peptide was determined by absorption spectroscopy at 595nm. The estimated concentration of the peptide was 2.4 mg/ml.

**Western-blotting analysis**

Peptide was transferred from the gel on to Nitrocellulose paper using wet blotting electrophoresis (14). The bands of molecular Weight marker on the nitrocellulose sheet were stained by Ponceaus dye. The membrane (containing transferred peptides) was coated with 3% BSA/PBS blocking solution. After washing with PBS three times, Ni-NTA anti His tag (1: 1500 dilutions) coupled to HRP enzyme were added and incubated at room temperature for followed by subs. Finally DAB (Diaminobenzidine) substrate was added and further incubated at room temperature for 20 min.

## Results

**Construction of cDNA, and cloning**

The cDNAs of segments A & B were constructed from NDV (NR43) RNA and then amplified by PCR technique (Fig. 1). The amplified segment A & B cDNA were cloned

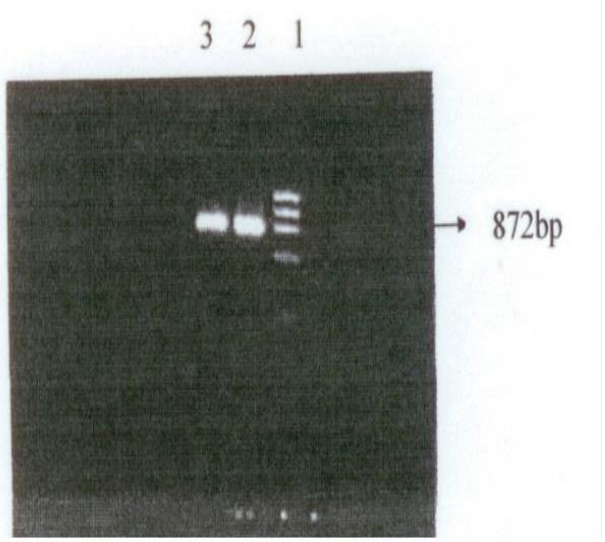


**Fig. 1.** The cDNA of segment A (791bp) and segment B(701bp) amplified in 1% Agaros gel wells 3, 2 respectively.



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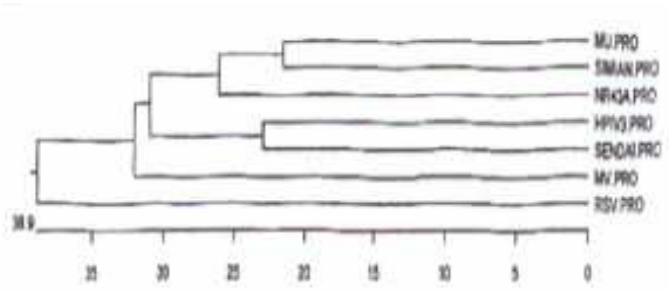


**Fig. 2.** Phylogenetic tree of Q.MEG, using Clustal method with PAM250 residue weight table.

into pTZ57/RT and transferred into *E.coli* DH5α.

**Sequencing and computational analysis Bioinformatic**

The cDNAs of the inserted F protein gene were sequenced using an automatic sequencer (VII 234-FA2-r5 and IV 232-F88-t7.). The sequences of A & B were deposited at GenBank (Ac. No. AF 548623 and AY342002, respectively). Alignmenting sequence A to other sequences showed high similarity with Russian strain Vol 95 (Identity: 98%) and Chinese viruses (similarity: 92%). Amino acid sequences of segments A & B were compared with other NDVs and with other paramixoviruses. Phylogenic analysis showed the position of NDV (NR43) in other paramixoviruses (Fig. 2). Amino acid sequences of NDV (NR43) F protein gene were made pairwise in comparisons with known paramixoviruses (Table 1). HR1 region of cDNA (NR43) F protein gene was discerned on the basis of heptad repeat amino acid sequence (abcdefg)n that the position a and d must be hydrophobic. HR1 amino acid and nucleotide sequence was aligned and deposited at GenBank (AY678224). Peptide localization sites (PLS) in *E.coli* Bl21 ($DE_3$) were predicted using PSORT software. Final results showed that PLS was present in bacterial cytoplasm. Restriction enzymes digestion of HR1 region and its amino acid sequences were recognized

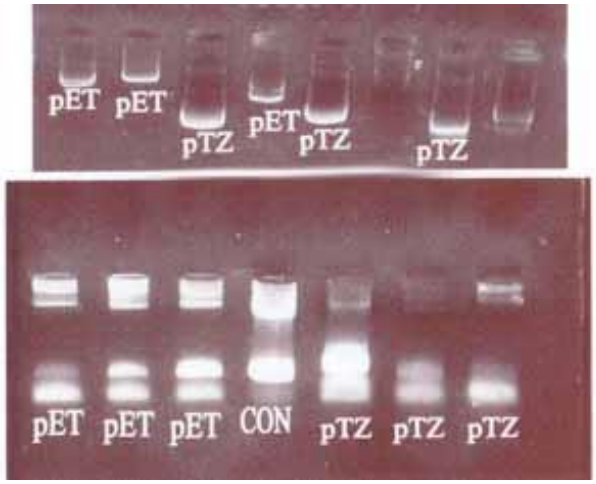


**Fig. 3.** Enzymatic digestion pattern of the vectors containing the interest in pET32a(+) & pTZ57/RT using PCR.

using Generunner package (Table 2) following the specific primers designed using Gene runner package. Finally practical plan for amplifying HR1 region of cDNA was was amplified using specific primers and master circler ® gradient (Eppendorf) by Hot-Start PCR technique. The interest amplified cDNA was digested with BamHI/HindIII enzymes and the results of cloned pTZ57/RT and pET32a(+) vectors are shown in Figure 3. The cloned pET32a(+) expression vectors were purified from *E.coli* and $Bl_{21}$ ($DE_3$) and then digested with BamHI/HindIII enzymes for detecting the presence of insert in vector MCS (Fig. 4). The insert PCR product was sequenced using an automatic sequencer.

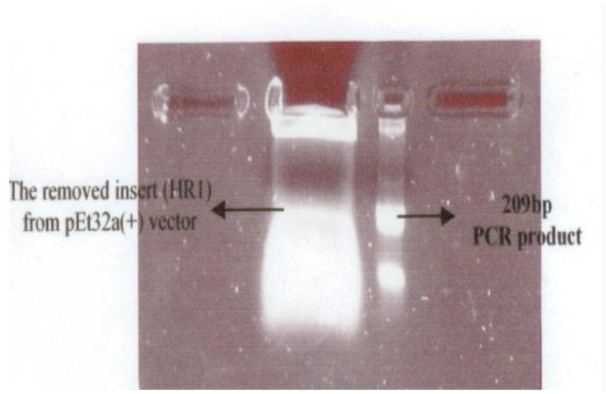


**Fig. 4.** Removing 209bp fragments (HR1 cDNA) from pET32a (+) vector using enzyme digestion.

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**Table 1**. Sequences pair distances of Q.MEG. Using Clustal method with PAM250 residue weight table.

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | | |
|---|---|---|---|---|---|---|---|---|---|
| 1 | | 43.5 | 48.9 | 46.7 | 38 | 37 | 14.1 | 1 | NR43A.PRO |
| 2 | 56.5 | | 35.7 | 42.4 | 54 | 32.6 | 12.8 | 2 | HPIV3.PRO |
| 3 | 51.1 | 64.3 | | 56.8 | 29.4 | 40.5 | 13.5 | 3 | MU.PRO |
| 4 | 53.3 | 57.6 | 43.2 | | 32.8 | 41.6 | 16.8 | 4 | SIMIAN.PRO |
| 5 | 62 | 46 | 69.8 | 66.4 | | 32 | 12.2 | 5 | SENDAI.PRO |
| 6 | 63 | 67.4 | 59.5 | 58.4 | 67.4 | | 14.1 | 6 | MV.PRO |
| 7 | 76.5 | 79.4 | 80 | 76.1 | 79.7 | 77.2 | | | RSV.PRO |
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | | |

$T_7$ promoter of cloned pET32a(+) expression vector in *E.coli* $Bl_{21}$ ($DE_3$) was induced by IPTG. The expressed peptides were analysed using SDS-PAGE technique (Fig. 5). The peptides were blotted onto nitrocellulose membrane for Western-Blotting analysis by the help of Ni-NTA anti His tag (1: 1500 dilutions) linked to HRP enzyme to obtain a peptide with 23.76 kDa molecular weight as an expressed peptide (Fig. 6).

## Discussion

Segment A contains sequences encoding cleavage site, fusion peptide, and HR1 domain in fusion protein. Segment B contains the sequence encoding HR2 domain (containing 55 amino acid residues). The sequence is highly conserved since it was compared with other NDV strains. There are three mutations in the sequence at sites 33, 46 and 48 including alanin, arginine and leucine replacing glutamic acid, lysine and tyrosine, respectively. There is a substitution of alanine for arginine in Chinese viruses including AFF2240, ch2000,ch98-c,ch98-3,JS9,L2 NDV H, TW 2000, Wawer, ypn,ZgI, but there is isoleucin in JS2 virus. As a result, it is likely that the origin of NDV (NR43) is from China. It is likely that the virus was transported from China to Iran and Russia.

We have cloned HR1 region cDNA into pET32a(+) expression vector. The cloning was done into BamHI/HindIII sites. The

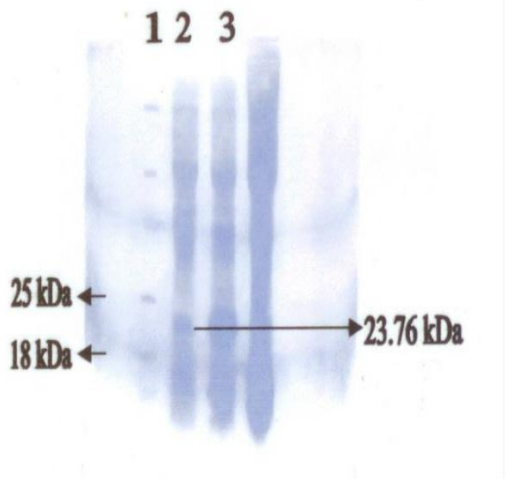


**Fig. 5.** PAG analysis of peptide showing the 2.76kD peptide.

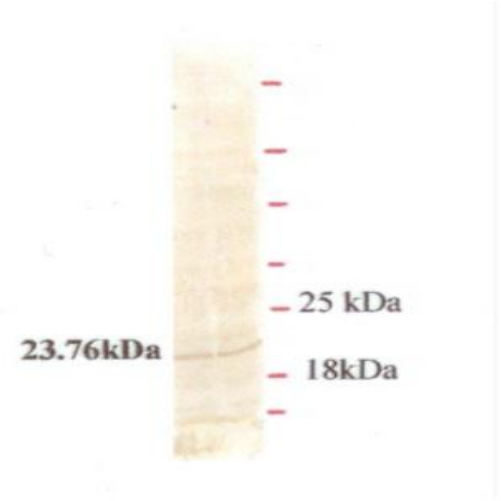


**Fig. 6.** Considering 23.76 kDa peptide using Western-Blotting technique.

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**Table 2.** The restriction enzymes that digest HR1 nucleotide sequence.

| | | | |
|---|---|---|---|
| Aci I | Eco65 I | Msp I | Sfc I |
| AspA I | EcoR I | MspA I | Tsp509 I |
| Bfa I | Fok I | Mwo I | Mnl I |
| BsaM I | Hap II | Nla III | Alu I |
| Bsm I | Hpa II | NspB II | Bbv |
| BsbW | Hph I | Pvu II | Bst71 I |
| BsrG I | MaeI | Rma I | Mse I |
| BstE II | Mae III | Rsa I | Tru9 I |
| Csp6 I | Mbo II | SfaN I | Fnu4H I |

combinations of BamHI/HindIII enzymes are compatible when used together in B buffer (Roche). Since BamHI and HindIII enzymes contain identical restriction sites which are more than 10 bp apart in pET32a(+) & in cDNA, they were used in the same reaction (Novagen). For optimizing expression, the following notes must be considered.

Growth at 37˚C causes some proteins to accumulate as inclusion bodies, while incubation at 30˚C leads to soluble, active protein. So we cultivated and induced the cloned E.coli $Bl_{21}$ ($DE_3$) cells at 30˚C for expressing soluble peptides and preventing the formation of inclusion bodies. The Trx. Tag ™ fusion tag is highly soluble polypeptide. pET32a(+) vector has been designed for cytoplasmic expression, and folding can be improved into the host cell that is permissive for the formation of disulfide bonds in N-terminal thioredoxin (Trx. Tag) sequence to become soluble. As HR1 peptide does not have any cystein amino acid residue and does not contributes in any disulfide bond formation, so we didn't use TrxB mutants.

Excessive rare codon usage in the target gene has been implicated as a cause for low level expression (10, 13, 14) as well as truncation products. The effect seems to be most severe when multiple rare codons occur near the amino terminus (12, 15). A number of studies have indicated that high usage of the arginine codons AGA and AGG can have severe effects on protein yield. As HR1 peptide does not have any rare codon near the N-terminus, so we didn't use certain host cells including Rosetta™, Rosseta Blue™ and Rosetta-gami™strains. Finally, the inhibition mechanism of two HR-derived peptides from the fusion protein of the paramyxovirus NDV has been investigated. Peptide $N_{24}$ derived from HR1 has been found to be 145-fold more inhibitory in a syncytium assay than peptide C24, derived from HR2.When both peptides had mixed at equimolar concentrations, their inhibitory effect had been abolished.

In addition, both peptides can induce the aggregation of negatively charged and Zwitterionic phospholipid membranes. The ability of the peptides to interact with each other in solution suggests, that these peptides may bind to the opposite HR region on the protein where as their failure to interact with membranes as well as their failure to block lipid transfer suggest a second binding site (16). So we induced to express HR1 peptide not only is a blocker, but also is an antigen for detecting NDV NR43 isolate.

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