Turk J Vet Anim Sci
29 (2005) 297-301
© TÜBİTAK

Research Article

Cloning of Nucleoprotein Gene (NP) of Rinderpest Virus (RPV) RBOK Vaccine Strain*

Şükrü TONBAK
Department of Virology, Faculty of Veterinary, Fırat University, Elazığ - TURKEY
Aykut ÖZDARENDELİ
Department of Microbiology, Faculty of Veterinary, Fırat University, Elazığ - TURKEY
Yusuf BOLAT
Department of Virology, Faculty of Veterinary, Fırat University, Elazığ - TURKEY

Received: 07.04.2003

Abstract: In the present study cloning of the NP gene in a cloning vector is reported. For this purpose, Vero cells were infected with rinderpest RBOK vaccine strain. Total RNA was obtained from the infected cells and reverse-transcription (RT) was performed to get cDNAs of NP gene. Afterwards, the NP gene was amplified by polymerase chain reaction (PCR) with the primers specific to the NP gene. The NP gene, 1575 bp in length, was cloned into pGEM-3Zf (-) cloning vector. PCR screening and enzyme digestion assays carried out to verify the presence of the NP gene.

Key Words: Rinderpest virus, nucleoprotein gene, cloning

Sığır Vebası Virüsü RBOK Aşı Suşu Nükleoprotein (NP) Geninin Klonlanması

Özet: Bu çalışmada nükleoprotein (NP) geninin bir klonlama vektörüne klonlanması amaçlanmıştır. Bu amaçla Vero hücreleri sığır vebası RBOK aşı suşu ile infekte edildi. İnfekte hücrelerden toplam RNA'lar elde edildi ve NP geni kopya DNA'larını elde etmek için tersine transkripsiyon (TT) yapıldı. Daha sonra NP genine özgül primerler ile NP geni polimeraz zincir reaksiyonu (PZR) ile çoğaltıldı. 1575 baz uzunluğuna sahip NP geni pGEM-3Zf (-) klonlama vektöründe klonlandı. NP geninin klonlanması PZR tarama ve enzim kesim deneyleri ile teyit edildi.

Anahtar Sözcükler: Sığır vebası virüsü, nükleoprotein geni, klonlama

Introduction

Rinderpest virus (RPV) infection is a viral disease with an acute and peracute course and having infections showing high mortality and morbidity (1). The disease causes necrosis and erosions on the mucosa of respiratory and digestion systems (2-4).

RPV belongs to the morbilli virus genus of paramyxoviridae family, and has single stranded, negative polarity and nonsegmented RNA genome (5-7). RPV, which has serologically single serotype, has antigenic affinities with the measles virus, canine distemper virus and the peste-des-petits ruminantes virus, which causes plague in small ruminants, included within the morbilli virus genus (8-11). It has six structural proteins, namely nucleoprotein (N), phosphoprotein (P), matrix (M), fusion (F), hemagglutinin (H), and large (L), and two nonstructural V and C proteins. Using transcription mapping method, the sequence of the genes on the viral genome was found to be N–P–M–F–H–L from 3' direction to 5' direction (7,12,13).

The nucleoprotein of the RPV plays a role not only in the formation of nucleocapsid, but also in transcription and replication (6,14-18). Since NP, which is an internal protein, stimulates cellular immunity, it is important for the development of vaccination against the RPV (19). Moreover, the determination of NP protein is also used in

* This study is a part of PhD thesis of Şükrü TONBAK and was funded by the Scientific Research Projects Unit (Project No: 645) of the University of Fırat.

the diagnosis of viral diseases (19,20). NP gene has a 1668 nucleotide length and expresses the NP protein, which has a molecular weight of 65 kDa (21). The NP gene has an open reading frame (ORF) encoding 525 amino acids with a 1575 nucleotide length. 3' nontranslating region has 53 nucleotides, and 5' nontranslating region has 40 nucleotides (11,13,19).

Cloning activities provide great convenience for gene manipulations. Cloning of viral genes and expression of them in different systems enable studies on the biological activities of these genes (22).

In this study, total RNAs obtained from Vero cells infected with the RPV vaccine strain are used for multiplication of nucleoprotein gene region through reverse transcription and polymerase chain reaction (PCR), for the placement of the amplified NP gene to the cloning vector, for the transformation of recombinant plasmid to *Escherichia coli* cells and for the cloning of NP gene in the *E. coli* cells.

Materials and Methods

Cells and virus: African green monkey kidney (Vero) cells (Sigma, St. Louis, MO, USA) was produced in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum and 100 IU/ml penicillin and 100 µg/ml streptomycin. In the study, RPV RBOK vaccine strain adapted to cell culture as produced in the Etlik Research Institute was used.

Total RNA isolation: After approximately 65-75% of the 25 cm^2 cell culture flask monolayer with Vero cells, it was infected with the RPV RBOK vaccine strain. On the 5^{th} day of the infection when cytopathological effect (CPE) was observed in approximately 80% of the cells, total RNA isolation was performed with TRI-Reagent (Sigma, St. Louis, MO, USA) in accordance with the protocol of the manufacturer (23).

Reverse transcription (RT) and PCR: In the establishment of the primers specified to the NP gene, EMBL gene bank X68311 RPV RBOK vaccine strain gene sequence was utilized. According to the gene sequence, the forward primer (NP1: 5' GG AAG CTT CCG CTA GCC CAC CAT GGC TTC TCT C 3') and the reverse primer (NP2: 5' GGG TCG ACT CAG TTG AGA AT 3') were designed. *Hind*III and *Nhe*I enzyme cut sites and Kozak DNA sequence were added to the forward primer; and *Sal*I enzyme cut sites was added to the reverse primer. The reverse primer was used to make cDNAs of the nucleoprotein gene by using reverse transcription assay. Using these cDNAs as templates, 10 pmol reverse and forward primers, 1.25 mM dNTP (Promega), 25 mM $MgCl_2$ (Promega), 10X PCR buffer (Promega), 2U Taq DNA Polymerase (Promega), and 50 µl PCR mixture were prepared. After preheating at 95 °C for 2 minutes, the PCR was established containing 32 cycles with denaturation at 94 °C for 1 minute, annealing at 44 °C for 1 minute, extension at 72 °C for 5 minutes and ending with final extension at 72 °C for 15 minutes. The amplified NP gene PCR products were visualized on 1.5% agarose gel stained with ethidium bromide (24).

Purification of the nucleoprotein gene from the agarose gel: The NP gene was purified from the low melting agarose gel using the Wizard PCR Preps DNA Purification System (Promega) in accordance with the protocol of the manufacturer (25).

Formation of recombinant plasmid and transformation to *E. coli*: pGEM-3Zf (-) (Promega) plasmid was used as the cloning vector. After the purified nucleoprotein gene, both the cloning vector and the amplified NP gene PCR products were cut with *Hind*III and *Sal*I enzymes. The ligation reaction using T4 DNA ligase enzyme with a gene/vector ratio of 3:1 was performed at 16 °C for 16 hours. The ligation product was transformed to JM109 cells of *E. coli* prepared in advance with $RbCl_2$ method. The cells were kept at 4 °C for 30 minutes. Subsequently, they were kept at 42 °C for 1 minute to ensure the transformation of recombinant plasmid to the cells. 400 µl Luria Bertani (LB) solution was added to the cells and they were stirred at 37 °C for 1.5 hours. The transformed cells were inoculated to LB plate containing 60 µg/ml ampicillin and kept at 37 °C for 16 hours (24).

PCR screening and verification with enzyme digestion assays: In order to determine the recombinant plasmids, PCR screening (PCR-S) test was performed (26). Briefly, a sterile toothpick tip was touched on the enumerated colonies and transferred to another plate containing ampicillin. Touching with the toothpick tip again to the colony, the mixture containing 2 µl 25 mM $MgCl_2$ (Promega), 2 µl 10X PCR buffer (Promega), 3 µl 1.25 mM dNTP, 4 µl 10 pmol/µl nucleoprotein gene specific primer and 4 µl dH_2O was contacted. It was preheated at 95 °C for 10 minutes. Following a quick spin, 1 U Taq DNA polymerase (Promega) was added and

mineral oil was dropped. The PCR-S program was adjusted to 25 cycles containing preheating at 95 °C for 5 minutes, separation at 94 °C for 1 minute, binding at 55 °C for 1 minute and extension at 72 °C for 1 minute. PCR products were visualized on 1.5% agarose gel stained with ethidium bromide.

Showing the NP gene in recombinant plasmid: Using alkali lysis method, the recombinant plasmid DNA was obtained from the transformed *E. coli* cells (24). In brief *E. coli* JM109 which recombinant DNAs were transformed was growth in 100 ml LB with ampicillin at 37°C for 16 hours. To harvest, growth cells in suspension was centrifuged, then supernatant was discarded and pellet was resuspended with 1 ml STE (0.1 M NaCl, 10 mM Tris.HCl [pH 8.0], 1 mM EDTA [pH 8.0]) and centrifuged at 12,000 rpm for 10 minutes. Duration of this lysis mixing was centrifuged at 12,000 rpm for 10 minutes. Following this procedure to degrade RNA, pancreatic RNAse (10 µg/ml) was mixed at 37 °C for an hour. For the deproteinization of DNA, an equal volume of sample phenol-chloroform was performed in same speed centrifuge. For DNA precipitation upper phase was replaced new tube and added 2 volumes of ethanol and 1:10 volume 3 M Na-acetate. Mixing left at –80 °C for an hour precipitation and centrifuged at 12,000 rpm for 15 minutes. To remove salt from DNA, DNA was washed twice with 70% ethanol. Supernatant was discarded and pellet dried. Resuspended DNA with sterile was kept at –70 °C until used. The presence of the NP gene in the recombinant plasmid was determined using *Nhe*I, *Hind*III and *Sal*I restriction enzymes. Moreover, the PCR was established to the recombinant plasmid DNA using the primers specific to the NP gene.

Results

On the 5th day of the infection at the Vero cells infected with RPV vaccine strain, it was observed the cytopathological changes specific to paramyxoviruses were obvious in 70-80% of the cells. The cDNAs were obtained through reverse transcription (RT) using the total RNAs obtained from these infected cells and were amplified with the PCR method. Following the PCR, the NP gene with a length of approximately 1575 nucleotides was shown in 1.5% agarose gel (Figure 1, lane 1).

In order to determine the recombinant plasmids which were obtained after the ligation of the NP gene to the pGEM-3Zf (-) cloning vector and the transformation of these ligation products to the *E. coli* cells, PCR-S was performed using the primers specified to the NP gene and the presence of the NP gene with a length of 1575 nucleotides was shown in these recombinant plasmids (Figure 2).

With a view to determining the presence of the NP gene in the recombinant plasmids, enzyme digestion assay and PCR were performed using the plasmid DNA obtained through alkali lysis method. By cutting the recombinant plasmid with *Hind*III and *Sal*I enzymes, a fragment of 1575 nucleotides was obtained (Figure 3 lane 3). Moreover, following the cutting with the *Nhe*I enzyme, which does not recognize any site on the cloning vector, but which is placed at 5' end of the NP1 primer, it was observed that the plasmid was opened in a linear structure as expected (Figure 3 lane 2).

The primers specific to the NP gene were used in order to show the presence of the NP gene in the recombinant plasmid. The NP gene with length of 1575 nucleotides as amplified by the PCR was shown in the agarose gel stained with 1.5% ethidium bromide (Figure

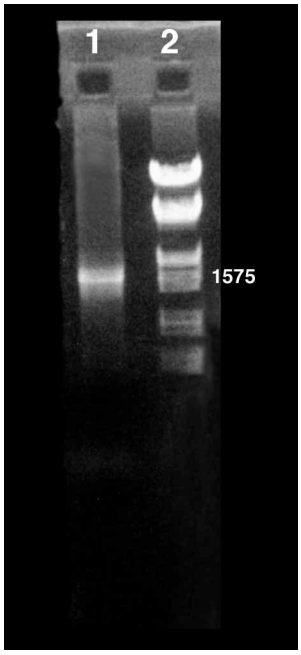

Figure 1. Agarose gel electrophoresis of RT-PCR products of NP gene of RPV RBOK vaccine strain.
Lane 1: PCR product (1575 bp) obtained from Vero cells infected with RPV RBOK vaccine strain. Lane 2: DNA ladder (Lambda DNA *EcoR*I/*Hind*III marker)

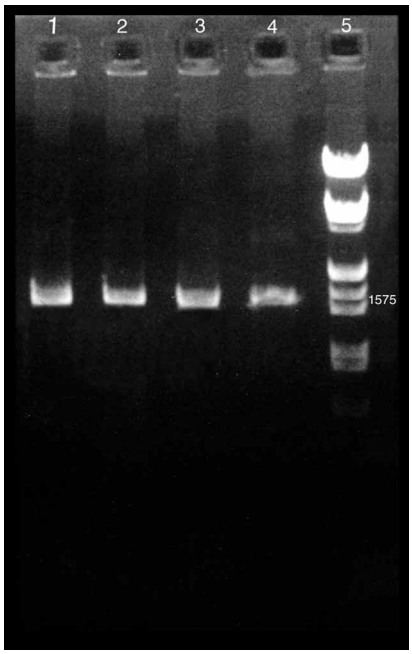

Figure 2. Agarose gel electrophoresis of PCR-S products.
Lane 1, 2, 3 positive colonies with PCR-S. Lane 4: RPV-RBOK NP gene purified from agarose gel. Lane 5: DNA ladder (Lambda DNA *Eco*RI/*Hind*III marker)

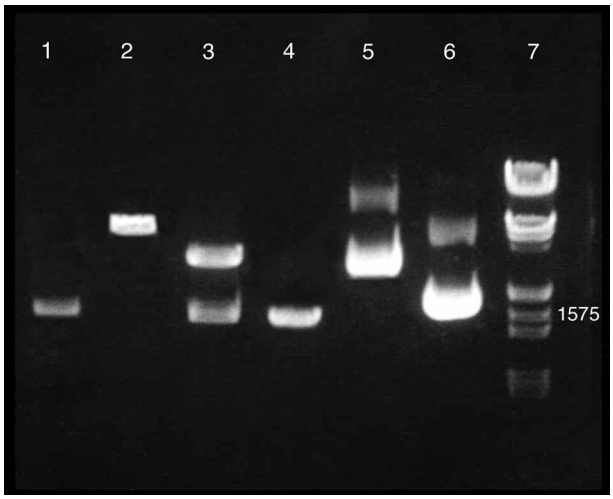

Figure 3. Demonstration of recombinant plasmid pGEM-SV-NP.
Lane 1: RPV-RBOK NP gene purified from agarose gel. Lane 2: *Nhe*I cut pGEM-SV-NP plasmid DNA. Lane 3: *Hind*III/*Sal*I cut pGEM-SV-NP plasmid DNA. Lane 4: pGEM-SV-NP plasmid DNA was used as template in PCR assay where RPV-NP gene specific primers were used. Lane 5: Uncut pGEM-SV-NP plasmid DNA. Lane 6: Uncut pGEM-3Zf (-) plasmid DNA. Lane 7: DNA ladder (Lambda DNA *Eco*RI/*Hind*III marker).

3 lane 4). Moreover, as a result of the ligation of the NP gene to the pGEM-3Zf (-) cloning vector having a length of 3200 nucleotides, it was shown to have acquired a length of 4775 nucleotides (Figure 3 lane 5). The recombinant plasmid obtained was called pGEM-SV-NP.

Discussion

In this study, gene of NP of rinderpest virus RBOK strain was cloned successfully. To confirm cloning of NP gene, restriction enzyme assay was used and almost 200 base pair of region NP gene was sequenced as a manual. As a result it was confirmed by enzyme digestion and sequencing assay. RPV RBOK is a vaccine strain. The whole sequence of NP was known and so NP gene, 1575 nucleotide base pair, was partially sequenced.

Nucleoprotein is an internal protein of the viruses within the *paramyxoviridae* family (19). Nucleoprotein takes part in the formation of ribonucleoprotein (RNP) structure (12).

Nucleoprotein is important for the development of vaccines against the viral infections, especially for the formation of cellular immunity (19,20,27). Moreover, the determination of the NP protein is used in the diagnosis of viral disease (19,20).

For the effective control and elimination of RPV disease, it is an important step to distinguish serologically the infected and vaccinated animals (28). Recently, the utilization of recombinant vaccines for effectively fighting with RPV has become dominant (1). These vaccines are very successful in forming a protective immune response to the disease. However, it is impossible to distinguish the vaccinated and infected animals concerning the vaccinations with this vaccine. One of the most reliable methods in the diagnosis of RPV disease is the serum-neutralization test (2,19). Yet, this test is time consuming and expensive as well as requires experienced laboratory staff. On the other hand, using the diagnostic kits prepared from nucleoprotein, it is definitely possible to distinguish the animals vaccinated with recombinant vaccine from the infected animals and it takes much less time (20).

For these reasons, we attempted to clone the nucleoprotein gene of RPV. Works for placing the cloned NP gene to the expression vector and for preparing the diagnostic kits are underway.

References

1. Ohishi, K., Inui, K., Barrett, T., Yamanouchi, K.: Long-term protective immunity to rinderpest in cattle following a single vaccination with a recombinant vaccinia virus expressing the virus hemagglutinin protein. J. Gen. Virol., 2000; 81: 1439-1446.
2. Diallo, A., Libeau, G., Couacy-Hymann, E., Barbron, M.: Recent developments in the diagnosis of rinderpest and peste des petits ruminants. Vet. Microbiol., 1995; 44: 307-317.
3. Bolat, Y., Doymaz, M.Z.: Paramyxoviridae Ailesi. Fırat Üniversitesi Veteriner Fakültesi Viroloji Ders Notları. Fırat Üniversitesi Yayınları, Elazığ. 1998; 302-304.
4. Tatar, N.: Değişik ısı derecelerinde farklı sürelerde tutulan sığır vebası aşısının Vero hücrelerinde aktivite kontrolü. Etlik Vet. Mikrobiyol. Derg., 1994; 7: 5-25.
5. Blixenkrone-Möller, M., Sharma, B., Varsanyi, T.M., Hu, A., Norrby, E., Kövamees, J.: Sequence analysis of the genes encoding the nucleocapsid protein and phosphoprotein (P) of phocid distemper virus, and editing of the P gene transcript. J. Gen. Virol., 1992; 73: 885-893.
6. Shaji, D., Shaila, M.S.: Domains of rinderpest virus phosphoprotein involved in interaction with itself and the nucleocapsid protein. Virology., 1999; 258: 415-424.
7. Kumar, S.S., Renji, R., Saini, M., Goel, A.C., Sharma, B.: Use of prokaryotically expressed nucleocapsid protein as a positive antigen in ELISA. Biochem. Mol. Biol. Int., 1998; 46: 1093-1100.
8. Baron, M.D., Kamata, Y., Barras, V., Goatley, L., Barrett, T.: The genome sequence of the virulent Kabete 'O' strain of rinderpest virus: Comparison with derived vaccine. J. Gen. Virol., 1996; 77: 3041-3046.
9. Diallo, A., Barrett, T., Barbron, M., Subarao, S.M., Taylor, W.P.: Differentiation of rinderpest and peste des petits ruminants viruses using specific cDNA clones. J. Virol. Meth., 1989; 23: 127-136.
10. Baron, M.D., Barrett, T.: Rinderpest viruses lacking the C and V proteins show specific defects in growth and transcription of viral RNAs. J. Virol., 2000; 74: 2603-2611.
11. Kamata, H., Tsukiyama, K., Sugiyama, M., Kamata, Y., Yoshikawa, Y., Yamanouchi, K.: Nucleotide sequence of cDNA to the rinderpest virus mRNA encoding the nucleocapsid protein. Virus Genes, 1991; 5: 5-15.
12. Ghosh, A., Joshi, V.D., Shaila, M.S.: Characterization of an in vitro transcription system from rinderpest virus. Vet. Microbiol., 1995; 44: 165-173.
13. Baron, M.D., Barrett, T.: The sequence of the N and L genes of rinderpest virus, and the 5' and 3' extra-genic sequences: The completion of the genome sequence of the virus. Vet. Microbiol., 1995; 44: 175-186.
14. Fooks, A.R., Schadeck, E., Liebert, U.W., Dowsett, A.B., Rima, B.K., Steward, M., Stephenson, J.R., Wilkinson, G.W.G.: High-level of the measles nucleocapsid protein by using a replication-deficient adenovirus vector: Induction of an MHC-1 restricted CTL response and protection in a murine model. Virology., 1995; 210: 456-465.
15. Conzelmann, K.K.: Nonsegmented negative-strand RNA viruses: Genetics and manipulation of viral genomes. Annu. Rev. Genet., 1998; 32: 123-162.
16. Buchholz, C.J., Retzler, C., Homann, H.E., Neubert, W.J.: The carboxy-terminal domain of sendai virus nucleocapsid protein is involved in complex formation between phosphoprotein and nucleocapsid-like particles. Virology., 1994; 204: 770-776.
17. Curran, J., Homann, H., Buchholz, C., Rochat, S., Neubert, W., Kolakofsky, D.: The hypervariable C-terminal tail of the sendai paramyxovirus nucleocapsid protein is required for template function but not for RNA encapsidation. J. Virol., 1993; 67: 4358-4364.
18. Ryan, K.W., Portner, A., Murti, K.G.: Antibodies to paramyxovirus nucleoproteins define regions important for immunogenicity and nucleocapsid assembly. Virology., 1993; 193: 376-384.
19. Ismail, T., Ahmad, S., D'souza-Ault, M., Bassiri, M., Saliki, J., Mebus, C., Yilma, T.: Cloning and expression of the nucleocapsid gene of virulent Kabete O strain of rinderpest virus in baculovirus: Use in differential diagnosis between vaccinated and infected animals. Virology., 1994; 198: 138-147.
20. Kamata, H., Ohkubo, S., Sugiyama, M., Matsuura, Y., Kamata, Y., Tsukiyama-Kohara, K., Imaoka, K., Kai, C., Yoshikawa, Y., Yamanouchi, K.: Expression in baculovirus vector system of the nucleocapsid protein gene of rinderpest virus. J. Virol. Meth., 1993; 43: 159-166.
21. Grubman, M.J., Mebus, C., Dale, B., Yamanaka, M., Yilma, T.: Analysis of the polypeptides synthesized in rinderpest virus-infected cells. Virology., 1988; 163: 261-267.
22. Buckland, B.R., Giraudon, P., Wild, F.: Expression of measles virus nucleoprotein in *Escherichia coli*: Use of deletion mutants to locate the antigenic sites. J. Gen. Virol., 1989; 70: 435-441.
23. SIGMA Bio Sciences Technical Bulletin. TRI Reagant. No 205. 1995.
24. Sambrook, J., Fritsch, E.F., Maniatis, T.: Molecular cloning: A Laboratory Manual. Second Edition. Cold Spring Harbor Laboratory Press, New York. 1989.
25. Promega Technical Bulletin. Wizard PCR preps DNA purification system. No 118. 2001.
26. Özdarendeli, A., Toroman, A.Z., Kuk, S., Tonbak, Ş., Bulut, Y., Kalkan, A.: Polimeraz zincir reaksiyonu ile transforme bakteriyel kolonilerin belirlenmesi (PZR-tarama). Fırat Tıp Derg., 2003; 8: 79-82.
27. Nakamura, K., Ohishi, K., Ohkubo, S., Kamata, H., Yamanouchi, K., Fujiwara, K., Kai, C.: Immunizing effect of vaccinia virus expressing the nucleoprotein of rinderpest virus on systemic rinderpest virus infection in rabbits. Comp. Immunol. Microbiol. Infect. Dis., 1998; 21: 91-99.
28. Walsh, E.P., Baron, M.D., Anderson, J., Barrett, T.: Development of a genetically marked recombinant rinderpest vaccine expressing green fluorescent protein. J. Gen. Virol., 2000; 81: 709-718.