CLONING AND PROKARYOTIC EXPRESSION OF HEMAGGLUTININ GENE OF RINDERPEST VIRUS*

Mehmet Ziya DOYMAZ[1] Ali Osman KILIÇ[2] Hakan BULUT[3] Aykut ÖZDARENDELİ[3] Yusuf BOLAT[3]

[1]Faculty of Medicine, Fırat University, Elazıg-TURKEY
[2]Faculty of Medicine, Karadeniz Technical University, Trabzon-TURKEY
[3]Faculty of Veterinary Medicine, Fırat University, Elazıg-TURKEY

Geliş Tarihi: 08.03.2000

Rinderpest Virüsü (RPV) Hemaglütinin (H) Geninin Klonlanması ve Bakteriyel Açıklanması*

SUMMARY: In this study, cloning and prokaryotic expression of rinderpest virus (RPV) hemagglutinin gene was reported. For this purpose, Vero cells were infected with Bovine O Kabete (RBOK) strain of RPV and total RNA was isolated at the height of the infection. Reverse transcription was carried out with random primers and viral cDNA was obtained. Hemagglutinin specific primers were used in polymerase chain reaction (PCR). Later, PCR amplified H gene segment was electrophoresed on 1,5% agarose gel and was found to be approximately 1830 base pair long. Hemagglutinin gene segment was cloned into the prokaryotic expression vector pEZZ18 and the resulting plasmid pRPVH4 was used in transforming *Escherichia coli*. Cloned H gene segment was subjected to restriction endonuclease analysis in order to confirm the identity of H gene and it was noticed that the product contained the unique restriction endonuclease sites present in the published H sequences of RPV. Recombinant H protein synthesized and secreted by *E.coli* was purified over affinity chromatography and electrophoresed on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Recombinant H protein was observed as a 65 kDa protein in Coomassie Brillant blue staining. Furthermore, in Western immunoblot experiments, recombinant H protein was identified with RPV hyperimmune rabbit serum.

Key words: Rinderpest virus, hemagglutinin, cloning, bacterial expression.

ÖZET

Bu çalışmada, sığır vebası virüsünün (RPV) hemaglütinin (H) proteinini şifreleyen gen segmenti klonlanmış ve klonlanan gen segmentinden H proteinin *Escherichia coli*'de açıklanması gerçekleştirilmiştir. Bu amaçla, Rinderpest Bovine O Kabete (RBOK) suşu ile infekte edilen Vero hücrelerinden toplam RNA izole edilmiş ve tersine transkripsiyon yöntemiyle viral RNA, cDNA haline getirilmiştir. Daha sonra, RPV gen segmentine özgül primerler kullanılarak gerçekleştirilen polimeraz zincir tepkimesi (PCR) ile H genine karşılık gelen segment çoğaltılmıştır. Çoğaltılan gen segmentinin %1.5'luk agaroz jelde büyüklüğü tespit edilmiş ve yaklaşık 1830 baz ikilisi uzunluğunda olduğu gözlenmiştir. Çoğaltılan gen segmenti prokaryotik açıklama vektörü olan pEZZ18 içine yerleştirilmiştir. Ortaya çıkan rekombinant plazmid (pRPVH4) içinde RPV H geni nükleotid dizgisinin bulunduğu PCR amplifikasyon ve restriksiyon endonükleaz analizi ile doğrulanmıştır. Rekombinant plazmid, *E. coli* transformasyonunda kullanılmıştır. Transforme edilen *E.coli*'nin salgıladığı rekombinant H proteini affinitte kromatografisi ile saflaştırılmış ve sodyum dodesil sülfat-poliakrilamid jel elektroforezi ile belirlenmiştir. Bu testlerde yaklaşık 65 kDa büyüklüğünde ve tek protein bandı halinde H proteini Coomassie Brillant mavisi boyama metoduyla tespit edilmiştir. Daha sonra gerçekleştirilen immünoblot yöntemiyle de rekombinant H proteininin immünolojik olarak kimliklendirilmesi yapılmış ve rekombinant H proteinin RPV'ye karşı hiperimmünize edilen tavşan serumuyla tepkime verdiği gösterilmiştir.

Anahtar Kelimeler: Rinderpest virüsü, sığır vebası virüsü, hemaglütinin, klonlama, bakteriyel açıklama.

* This work was supported by a grant from the Turkish State Planning Organisation (Devlet Planlama Teşkilatı, Proje No: 95K120370)

INTRODUCTION

Rinderpest virus is a member of the genus *Morbillivirus* (5). This genus contains several important human and animal pathogens such as measles virus of human and distemper virus of dogs and other canines. Rinderpest virus, the causative agent of devastating bovine pestis is completely eliminated from the developed Western countries. The disease occasionally appears in the Far and Middle Eastern Countries and Africa (5, 15). Turkey is located on the gateway of the virus to the Balkans and to Europe and occasional epidemics are also reported from different regions of Turkey (2).

Viruses in the genus *Morbillivirus* have single stranded RNA genome with negative polarity. The nucleotide sequence of several members of this genus are determined. Six open reading frame is determined on the *Morbillivirus* genom. These genes encode 5 structural and 1 non-structural proteins (3, 7).

Hemagglutinin proteins (H) of morbilliviruses are the attachment proteins for the virus. Most of the neutralizing antibodies are direceted towards H proteins. Utilization of H as an immunogen against paramyxoviruses has been proposed and various strategies in using H as immunogen have been explored (15, 18, 19). Utilization of H as an immunogen against RPV may not be justified in the face of the fact that an effective attenuated vaccine against RPV is available. Current vaccine for RPV is a cell culture derived vaccine and its effectiveness has been demonstrated by the fact that almost all Western world is freed from the disease as a result of the combined vaccination and control efforts (15). Rinderpest virus H protein has already been cloned and expressed by other researchers (13, 20, 21, 22, 24). The expressed protein is found to be immunogenic in animals (13, 23, 24). In this study, cloning and expression of H protein of RPV vaccine strain is aimed. The present study poses to be one of the early investigations on viral proteins and genes carried out in this country.

MATERIALS AND METHODS

Virus and Cells; In the study, cell culture adapted RPV vaccine strain was used. Vero cells (Foot and Mouth Disease Institute, Ankara, Turkey) were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Sigma Chemical Co. St. Louis, MO, USA) containing 10% fetal calf serum (Sigma), penicillin (100 IU/ml) and streptomycin (100 µg/ml). *Escherichia coli* DH5α cells were grown on Lurie-Bertani (LB) medium. To grow tranformed *E. coli* DH5α and JM109, LB medium containing ampicillin (Sigma, 60µg/ml) was used.

RNA Isolation, Reverse Transcription and Polymerase Chain Reaction; Monolayer Vero cells were infected with RPV at high multiplicity of infection and allowed to develop about 80% cytopathic effect (4 days). Afterwards total RNA from infected cells were extracted with TRI reagent (Sigma) as described by the manufacturer (16). One fifth of the total RNA extracted from a 75 cm^2 infected Vero cells was used in reverse transcription (RT) assay. Reverse transcription assay was carried out as described by the manufacturer using random hexanucleotide primers supplied in the commercial RT kit (12). cDNA obtained in RT reaction was used in PCR assay using forward (5'-CAU CAU CAU CAU CCG AAT TCA CCA TGT CTC CCC CAA GA-3') and reverse (5'-CUA CUA CUA CUA CCA AGC TTC TAT TTC CCA TTG CA-3') primers at 50pmol/reaction concentrations. In a 32 cycle PCR assay in which each cycle consisted of the incubations at 95°C for 1 min, at 52°C for 1 min and at 72°C for 2 min, H gene segment was amplified (1). Amplified PCR product was visualized on a 1.5% agarose gel (Figure 1).

Cloning of H Gene into pEZZ18 Vector; Standart molecular biological procedures were used in all cloning experiments (14). pEZZ18 vector is a prokaryotic expression vector and allows the cloned protein to be secreted into the bacterial culture medium (10). In the vector, IgG binding domain from Staphylococcal protein A was placed in tandem in front of the cloning sites. Thus, the expressed recombinant proteins from pEZZ18 is fused to IgG binding domain of Protein A. This feature allows the recombinant proteins to be purfied easily over IgG-Sepharose affinity column in one step procedure (10). The expression of recombinant fusion protein is driven by the protein A promoter. For directional cloning, *Eco*RI and *Hind*III restriction sites were included in the primers. In the cloning of H protein gene, PCR product specific for H gene were first digested with *Eco*RI and *Hind*III and then cloned into similarly digested cloning vector pAMPI (8). Later, H gene was excised from the pAMPI and placed into pEZZ18 which was also doubly digested with *Eco*RI and *Hind*III. The schema for the cloning is depicted at Figure 2. Ligation of *Eco*RI-*Hind* III cut H gene insert and *Eco*RI-*Hind* III cut pEZZ18 vector was carried out at 16°C for 12 hr in the presence of 50U T4 ligase (Promega) at suggested conditions by the manufacturer (11). Transformation of *E.coli* JM 109 with ligated vector was performed and colonies

growing on ampicillin containing agar plates were selected. Plasmids containing H gene inserts were identified with *Eco*RI and *Hind* III digestion (Figure 3-A). Further confirmation of the presence of inserts was carried out with additional restiction enzyme digestions (Figure 3-B).

Affinity Purification of Recombinant H Protein. *Escherichia coli* strain JM109 carrying the recombinant plasmid pRPVH4 was grown overnight in LB containing ampicillin at 37^{0}C shaker. The media was centrifuged at 3500 rpm for 10 min and the supernatant was collected (14). Recombinant H protein was produced as a fusion protein which had two IgG binding motif of Staphylococcal protein A (PrA) fused to N terminus of RPV H protein. Thus, binding of recombinant H present in the culture medium through its PrA domain to IgG Sepharose beads (IgG Sepharose 6-Fast Flow, Pharmacia Biotech, Uppsale, Sweden) was allowed to take place for 12 hr at 37 ^{0}C. Later, IgG-sepharose beads were separated from bacterial supernatants and washed 5 times in 5mM ammonium acetate pH 4.8. Bound recombinant H fusion protein was eluted from the beads in 0.2 M acetic acid pH 3.2 (10). Concentration of recombinant H protein in the eluates was determined in Bradford Assay (Sigma) as suggested by the manufacturer (17). Affinity purified H protein was stored at –20 ^{0}C until used .

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Immunoblotting: Affinity purified H protein was electrophoresed on a 10% SDS-PAGE and gel was stained with Coomassie Brillant Blue as described elsewhere (4, 6). After electrophoresis, proteins were transferred onto nitrocellulose papers and Western immunoblotting assay was performed (4, 9). In immunoblotting assays, hyperimmune rabbit anti-RPV serum was used as the primary antibodies.

RESULTS

Amplification of the gene segment corresponding to RPV H coding sequences was readily accomplished in PCR assay. In PCR amplification, primers deduced from the published sequences of RBOK strain of RPV was used. Accordingly, an amplification product of 1830 base pair long was predicted and the amplified product was at the expected size (Figure 1). To confirm the cloned fragment was indeed the H encoding gene segment, restriction endonuclease analysis on the vector pRPVH4 was carried out. Since, *Hind*III plus *Eco*RI enzymes were used the cloning of H gene into pEZZ18, we wanted to confirm that the inserted H gene would be excised with *Eco*RI plus *Hind*III digestion. The result of this experment showed that the piece excised with *Hind*III plus *Eco*RI was about 1830 bp long (Figure 3A). In the published sequence, unique restriction sites of *Kpn* I and *Eco*RV were placed at 1345 and 403 bases, respectively. When the amplified and purified PCR product was digested with *Kpn*I, expected digestion products of 1344 and 486 bp long pieces were obtained. Similarly, *Eco*RV digestion of PCR product gave 1428 and 402 bp long fragments (Figure 3B).

Since pEZZ18 was an expression vector, we directly proceeded to produce recombinant H protein in *E. coli*. Recombinant protein produced by *E. coli* transformed by pRPVH4 is an H protein fused to IgG binding domain of protein A and secreted into the liquid media. Thus, after the seperation of cellular debris from the liquid media, recombinant H protein was purified over IgG Sepharose affinity column. The eluted recombinant H protein was electrophoresed on 10% SDS-PAGE and stained with Coomassie Brillant blue. In Coomassie blue staining a single band of approximately 65 kDa was demonstrated (Figure 4A). In Western blot experiments, reactivity of this protein band with RPV hyperimmune rabbit serum was confirmed (Figure 4B).

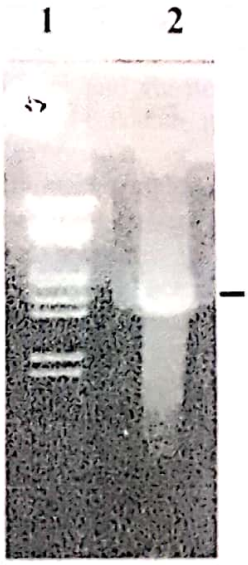

Figure 1. Agarose gel electrophoresis of RT-PCR product. Lane 1; Lambda DNA *Hind*III cut marker. Lane 2; About 1830 bp long PCR product obtained after a 32 cycle PCR.

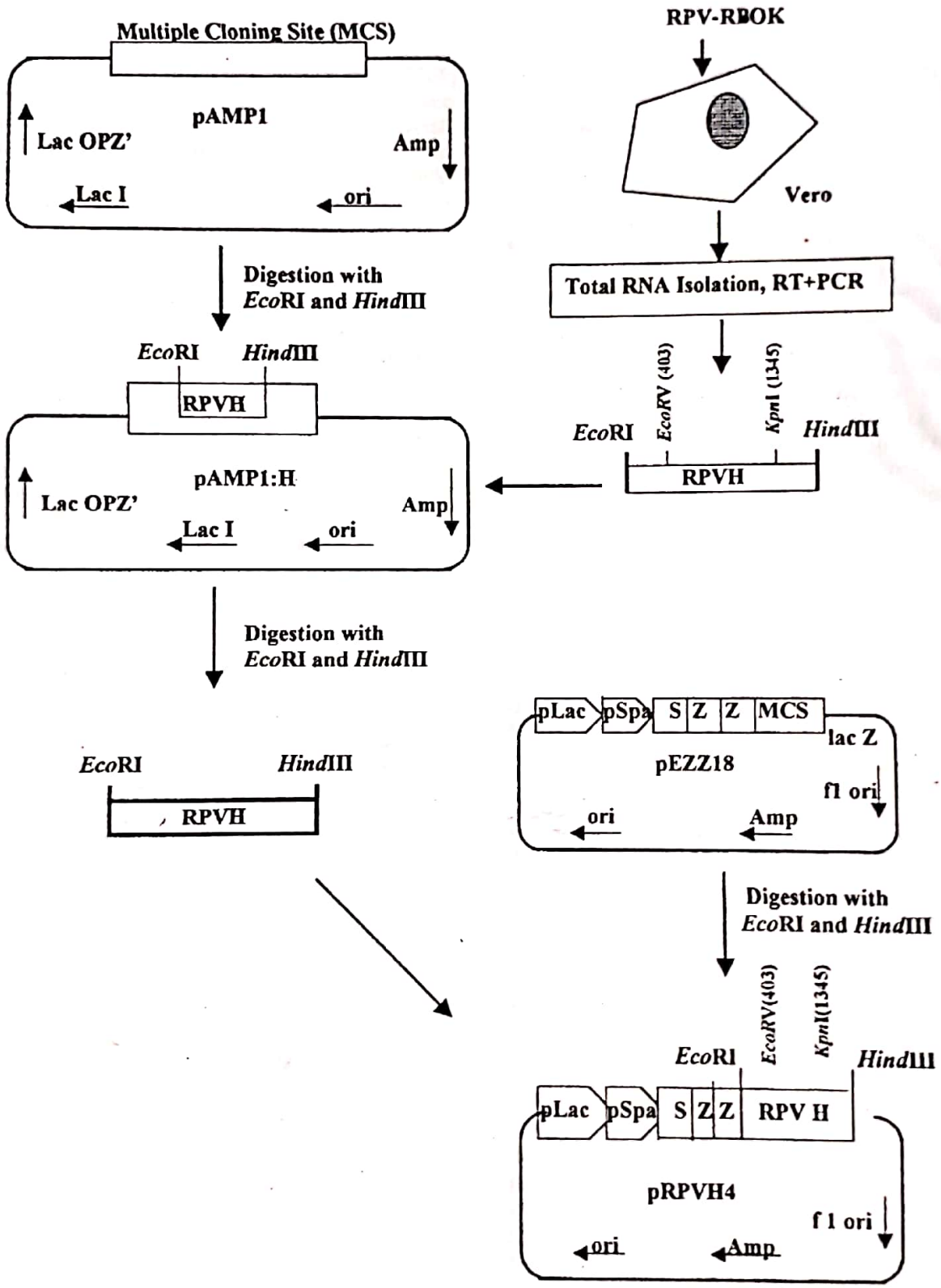

Figure 2. Cloning strategy for H protein of RPV. pEZZ18 bacterial expression vector has lactose and staphylococcal protein A promotors driving the transcription. Two tandem IgG binding domains of protein A (Z) were placed following protein A signal sequence (S) and the vector allows the protein whose coding sequence is placed in the multiple cloning site (MCS) to be expressed as fusion protein. pRPVH4 was generated by the ligation of *Eco*RI-*Hind*III cut H gene excised from pAMP1 with pEZZ18.

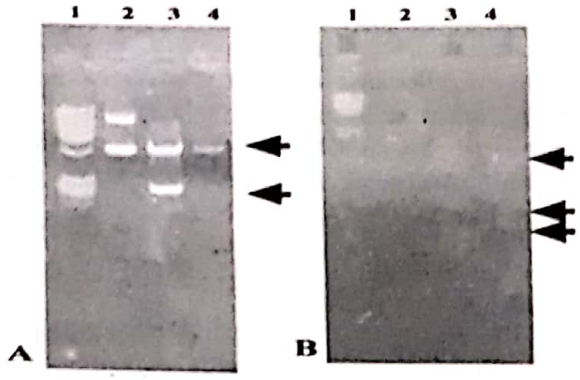

Figure 3. **A.** Demonstration of H gene segment in pRPVH4. H gene segment in pRPVH4 was excised with *Eco*RI and *Hind*III restriction enzymes and the resulting fragments were electrophoresed on 1.5 % agarose gel. Lane 1; Lambda DNA *Hind*III marker. Lane 2; Uncut pRPVH4 plasmid DNA. Lane 3; *Eco*RI and *Hind*III cut pRPVH4 plasmid DNA. Lane 4; pEZZ18 plasmid DNA. **B.** Confirmation of H gene segment with PCR and restriction analysis. Lane 1; Lambda DNA *Hind*III cut marker. Lane 2; 1830 bp PCR product. pRPVH4 plasmid DNA was used as template in PCR assay where RPV H gene segment specific primers were used. Lane 3; *Eco*RV cut PCR product. Lane 4; *Kpn*I cut PCR product.

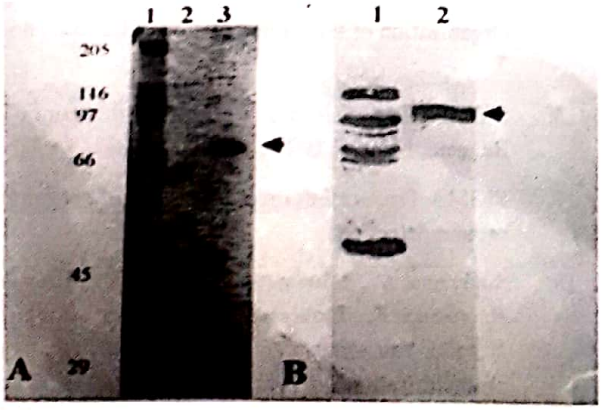

Figure 4. **A.** Demonstration of recombinant H protein on 10 % SDS-PAGE with Coomassie blue staining. Lane 1; Molecular weight markers. Lane 3; Affinity purified recombinant H protein. **B.** Western immunoblot staining of recombinant H protein. After the transfer of RPV-infected vero cell lysates (Lane 1) and recombinant H protein (Lane 2) onto nitrocellulose filters, detection was carried out with hyper-immune rabbit serum as described in reference 4.

DISCUSSION

In this study, cloning and expression of H protein of RPV vaccine strain RBOK were reported. Hemagglutinin protein of paramyxoviruses has paramount importance in the life cycle of the virus (18, 19). The virus initially attaches to the host cell via this protein and H protein is the primary target for host humoral and cellular responses (3, 7, 19). Cloning and expression of H protein from various RPV strains were reported earlier (13, 20, 21, 22, 24). Immunization studies carried out with recombinant H protein has produced promising results (13, 20, 23). However, these studies were not extended to the field. The primary aim the current study was to initiate molecular cloning experiments on one of the most important viral pathogens of veterinary importance in this country. Rinderpest virus poses particular threat to animal husbandry because of the aforementioned reasons. However, studies on RPV or any other virus seem to be concentrated on seroepidemiology. Investigations at molecular level appear to be the most rewarding approaches in developing efficient antiviral strategies. Furthermore, information gained from molecular studies will probably be utilized at more frequency in the future.

Recombinat H protein which was fused to IgG binding domain of Protein A was efficiently expressed in *E.coli*. The recombinant protein was at the expected size and reacted with hyperimmune rabbit serum. From this data, it could be inferred that prokaryotic expression of H protein did not completely eliminate antigenic epitopes. It is usually considered that viral surface proteins expressed in bacteria loose antigenic properties (14). Since proteins synthesized by bacteria are not glycosylated, the loss of some conformational epitopes are likely. However, there must have been some unchanged epitopes (possibly lineer epitopes) which were enough to be detected in Western blot experiments.

The size of the RPV H was reported to be 75 kDa (3, 7). Molecular weight (MW) of the recombinant H protein produced in this study was about 65 kDa. The lack of glycosylation should reduce the MW of the protein somewhat. However, proteins expressed from pEZZ18 vector is fused to 115 amino acid fragment of Protein A and their size is increased correspondingly (about 15 kDa). Thus, the reduction caused by the lack of glycosylation was probably compensated somewhat by Protein A fragment and in electrophoresis roughly 65 kDa H protein was obtained (10).

In this study, recombinant H protein was detected as a single protein species. In eukaryotic cells, H protein is normally cleaved into two pieces, H1 and H2 respectively. The cleavage of H protein is considered to be the function of cellular trypsin-like proteases (5, 15, 3). In the present study, demonstration of a single H band show that bacterial cells do not contain appropriate proteases.

Recombinant proteins expressed in prokaryotic or in eukaryotic systems are usually used in immunization studies. We plan to proceed with similar approaches. In recent years, immunizations with nucleic acids encoding viral proteins are also attempted (14, 23, 24). The present study will also provide with important reagents carry out similar experiments.

REFERENCES

1. Aşçı, Z., Akbulut, A., Doymaz, M. Z., Felek, S., and Kılıç, S. Evalution of DNA of hepatitis B virus in serum by PCR and comparison with serological parameters. Viral Hepatit Derg. 1996; 2: 6-9.
2. Buharalılar, N., and Okay, G. The control of rinderpest in Turkey. Cento Seminar on Viral Diseases. 1972; 35-38.
3. Chanock, R. M., and McIntosh, K. M. Parainfluenzaviruses. *In* "Fields Virology" (B. N. Fields, D. M. Knipe, R. M. Chanock, M. S. Hirsch, J. L. Melnick, T. P. Monath, and B. Roizman, eds.). 2nd Ed., Raven, New York. 1990; 963-975.
4. Doymaz, M. Z., Bulut, H., Özdarendeli, A., and Bolat, Y. Production and characterization of monoclonal anti-ovalbumin antibodies. Tr. J. Med. Sci. 2000; 30: 411-415.
5. Fenner, F. J., Gibbs, E. P. J., Murphy, F. A., Rott, R., Studdert, M. J., and White, D. O. *Paramyxoviridae. In* Veterinary Virology. Academic Press, San Diego. 1993; 471-487.
6. Harlow, E., and Lane, D. *In* Antibodies; A Laboratory Manual. Cold Spring Harbor Laboratory Press, New York. 1985.
7. Kingsbury, D. W. *Paramyxoviridae* and their replication. *In* "Fields Virology" (B. N. Fields, D. M. Knipe, R. M. Chanock, M. S. Hirsch, J. L. Melnick, T. P. Monath, and B. Roizman, eds.). 2nd Ed., Raven, New York. 1990; 945-955.
8. Life Technologies. pAMPI System for Rapid Cloning of Amplification Products. 1993; 2-5.
9. Mahy, B. W. J., and Kangro, H. O. Western (immuno) blotting and Radioimmunoprecipitation. *In* Virology Methods Manual. Academic Press. London. 1996; 261-265.
10. Nilsson, B., Forsberg, G., and Hartmanis, M. Expression and purification of recombinant insulin-like growth factors from *Escherichia coli*. Methods in Enzymology. 1991; 198: 3-15.
11. Promega Protocols and Application Guide. Second Ed. 1991; 52-53.
12. Promega Technical Bulletin. AMP Reverse Transcriptase. No 502. 1993.
13. Romero, C. H., Barrett, T., Chamberlain, R. W., Kitching, R. P., Fleming, M., and Black, D. N. Recombinant capripoxvirus expressing the hemagglutinin protein gene of rinderpest virus: Protection of cattle against rinderpest and lumpy skin disease viruses. Virology. 1994; 204: 425-429.
14. Sambrook, J., Fritsch, E. F., and Maniatis, T. *In* Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, New York. 1989.
15. Scott, G. R., Taylor, W. P., and Rossiter, P. B. Epizootiological background. *In* Manual on the diagnosis of rinderpest. Food and Agriculture Organization of the United Nations, Rome. 1986; 3-7.
16. SIGMA Bio Sciences Technical Bulletin. TRI Reagent. No 205. 1995.
17. SIGMA Product Information. Bradford reagent for protein detection.
18. Sugiyama, M., Minamoto, N., Kinjo, T., Hirayama, N., Asano, K., Tsukiyama-Kohara, K., Yoshikawa, Y., and Yamanouchi, K. Antigenic and functional characterisation of rinderpest virus envelope proteins using monoclonal antibodies. J. Gen. Virol. 1991; 72: 1863-1869.
19. Sugiyama, M., Minamoto, N., Kinjo, T., Hirayama, N., Sasaki, H., Yoshikawa, Y., and Yamanouchi, K. Characterisation of monoclonal antibodies against four structural proteins of rinderpest virus. J. Gen. Virol. 1989; 70: 2605-2613.
20. Sugiyama, M., Minamoto, N., Kinjo, T., Ito, H., and Yamanouchi, K. The nucleotide sequence of the

hemagglutinin gene of the LA strain of rinderpest virus, a seed virus strain used for vaccine production in Japan. J. Vet. Med. Sci. 1996; 58: 769-771.

21. Tsukiyama, K., Sugiyama, M., Yoshikawa, Y., and Yamanouchi, K. Molecular cloning and sequence analysis of the rinderpest virus mRNA encoding the hemagglutinin protein. Virology. 1987; 160: 48-54.

22. Yamanaka, M., Hsu, D., Crisp, T., Dale, B., Grubman, M., and Yilma, T. Cloning and sequence analysis of the hemagglutinin gene of the virulent strain of rinderpest virus. Virology. 1988; 166: 251-253.

23. Yamanouchi, K., Unui, K., Sugimoto, M., Asano, K., Nishimaki, F., Kitching, R. P., Takamatsu, H., and Barrett, T. Immunisation of cattle with a recombinant vaccinia vector expressing the hemagglutinin gene of rinderpest virus. Vet. Record. 1993; 13: 152-155.

24. Yilma, T., Hsu, D., Jones, L., Owens, S., Grubman, M., Mebus, C., Yamanaka, M., and Dale, B. Protection of cattle against rinderpest with vaccinia virus recombinants expressing the H or F gene. Science. 1988; 242:1058-1061.