Molecular Cloning of VP1 gene of Foot-and-Mouth Disease Virus Type O1/Iran

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Received 8 Apr 2000; accepted 17 Jun 2000

Summary

Double stranded cDNA coding for the immunogenic capsid protein VP1 of foot-and-mouth disease virus type O1/Iran from the virion RNA was prepared .Two primer pairs for VP1 gene of virus were designed by comparison of published sequences and statistically analyzed using oligo and DNasis software. A PCR program was developed and optimized to amplify a segment of about 645 bp. Noninfected cells were used as negative control. The synthesized cDNA was then cloned into the NdeI and EcoR1 restriction enzymes site of pEt-23a+ plasmid using already attach linkers. The plasmid has been successfully propagated in and isolated from *Echerishia coli* bacterial culture and cloning of VP1 gene was confirmed by Dot Blot hybridization.

Key words: cloning, VP1 gene, FMD virus, *Echerishia coli*

Introduction

Foot-and-mouth disease (FMD) is an acute and highly contagious febrile and sometimes fatal disease, affecting primarily cloven-hoofed animals. The disease causes significant economic losses. The comprehensive vaccination of all susceptible animals to FMD virus (FMDV) is the basis of all sanitary programs for controlling and eradication of the disease (Brown 1992). Current methodology of vaccine production associated with some problems because culturing and manipulation of large amount of virulent virus is cost intensive and carries the risk of dissemination of

virulent virus (Beck & Strohmier 1987; King *et al* 1981). The causative agent, FMDV, comprises aphtovirus genus of the picornaviridae family. There are seven serotype of virus (A, O, C, Asia, and SAT 1,2,3). Like other picornaviruses its genome consist of a single stranded positive sense RNA (i.e. acts as mRNA *in vivo* and *in vitro*) molecule of about 8500 nucleotide (Fross *et al* 1984). It is polyadenylated at its 3 end, has a protein covalently attached to its 5′ end and a poly (C) tract of about 100-200 nucleotide in length close to the 5′ end. Only the region between the poly (C) tract and the 3′end of the virus genome carries the coding sequence. Viral genome is encapcidated in an icosahedral capsid consisting of 60 copies of each of four proteins VP1-VP4. FMDV proteins are synthesized as a precursor polyprotein that is subsequently processed by cellular and virus coded protease into four capsid (VP1-VP4) and noncapsid proteins (Rueckert 1996). Of these proteins VP1 is of particular interest because it carries epitops inducing neutralizing antibodies (Bittle *et al* 1982, Strohmaier *et al* 1982, Bachrach *et al* 1982). The cloning and expression of VP1 in different systems has been reported (Bachrach *et al* 1975, Kleid *et al* 1981, Bachrach *et al* 1982, Strohmaier *et al* 1981, Grubman *et al* 1993, Zamorano *et al* 1998, Wigdorovitz *et al* 1999). Results of these studies have demonstrated that VP1 is an effective antigen. In this regard, we interested in development of an alternative FMDV vaccine that would be safe and cost effective .The present paper describes the first stage of our try including the synthesis of double stranded cDNA of VP1 sequence of FMDV type O1/Iran and its cloning by means of Reverse Transcrioption–Polymerase Chain Reaction (RT-PCR).

Materials and Methods

Cell culture and virus propagation. One ampoule of frozen cell (approx.4×10) BHK-21/C13/P8 cells (Foot-and-Mouth disease Department) was cultured on Stocker™ medium in Roux bottle for 48h until complete confluence was seen. Five ml of seed virus of FMDV type O1/Iran in Earle's medium were added to each of four bottles containing monolayers of approximately 40×10 BHK-21 cells (OIE Manual). After incubation for 30min the medium was decanted and 80ml of fresh medium was added to each bottle and incubated until the onset of cytopatic effect.

RNA extraction. Virus infected cells were collected with trypsinization and sedimentation by centrifugation .In brief after the cells were trypsinized, they were

collected in a sterile 50ml polypropylene tube by centrifugation at 400*g* for 5min at 4°C. The cell pellets were washed 2-3 times with 25ml of ice cold 1×PBS pH7.2 and centrifuged at 400*g* for 5min at 4°C (Vanden Heuvel 1998). The supernatant were removed and the cells were resuspended in solution D [(100µl/10 cells) (4M guanidinium isothiocyanate, 25mM sodium citrate, pH7.0, 0.5% sarcosyl, 100mM 2-mercaptoethanol)]. The cells were homogenized by repeated pipetting followed by the addition of 0.2M sodium acetate pH4, 1 vol. phenol (DEPC-ddH2O saturated) and 0.2 vol. chloroform-isoamyl alcohol mixture (49:1). Then the sample was incubated for 10min on ice. Total RNA was extracted by phase separation. Isolated RNA was precipitated with isopropanol and washed with 70% cold ethanol. Purified RNA was resuspended in 25µl of RNase-free water. During the process, non-infected BHK-21 cells were used as negative control.

Denaturing gel electrophoresis of RNA. Assessment of integrity of RNA sample was determined by denaturing gel electrophoresis according to standard protocol (Farrell 1993). In brief 1% agarose gel was prepared by dissolving agarose in ddH_2O and then 10×MOPS buffer (0.2M MOPS, pH7.0; 50mM sodium acetate, 10mM EDTA, pH8.0), formaldehyde 37% and 0.5µg/ml EtBr were added to the melted agarose. 5µl of RNA sample was denatured in a mixture containing 10×MOPS, formaldehyde and formamide by heating to 55°C for 15min. Then 1µl of 6×loading buffer was added and mixed thoroughly. The samples were electrophoresed at 5v/cm of gel for 10min and at 30volt overnight in 1×MOPS buffer.

Synthetic oligoucleotides. Three primers, F, R1 and R2 were used. The sense primer F (5? -ACCACTTCTGCGGGCGAG-3′; nucleotides 2886-2903) and the antisense primer R1 (5′-CAAAGTCTGTTTCACCGGTGC-3'; nucleotides 3526-3502) are derived from conserved regions of the FMDV genome by comparison of published sequences of VP1 gene (Dopazo *et al* 1988) and further computerized analysis using oligo and DNasis software. The numbering used refers to the sequence of strain O1K reported by Fross *et al* (1984). The second antisense primer, R2 (5′-GCTTTGATTGCACCATAGTT-3'), has been described by Locher *et al* (1995). It is derived from the highly conserved 1A(VP1) region and corresponds to nucleotides 3485 to 3466 of sequence of strain O1K reported by Kurz *et al* (1981). With the

primer pairs F-R1 and F-R2, PCR products of complete sequence of VP1 gene, and 500bp were expected, respectively. NdeI restriction site, CATATG, was used at the 5´end of F primer of VP1 coding sequence to add ATG initiation codon and stop codon and EcoRI restriction site, GGAATTC, was used in the R1 primer for cloning the VP1 sequence into pEt-23a+.

Reverse transcription. First strand cDNA synthesis was performed for 60min at 42°C in a 20µl reaction mix containing a total of 5µl denatured RNA, 4µl of 5×RT Buffer, 2µl DTT (BRL), 2µl of 10mM dNTP mixture, 1µl oligo dT (12-18) and /or 1µl of 20pmol R1 primer, and/or 1ul of oligo dT (12-18) and 1µl of superscripts II RNase H- reverse transcriptase (BRL) and 5µl of sterile RNase free ddH_2O. This was followed by a 3min heating step at 95°C in order to denature RNA-cDNA hybrids and to inactivate the reverse transcriptase The cDNA samples were chilled on ice and stored at -20°C.

Polymerase Chain Reaction. A PCR reaction mix consisted of the following: 5µl of 10×PCR Buffer (200mM Tris-HCl, pH8.4; 500mM KCl), 20pmol of each primer, 1.5mM $MgCl_2$, 0.2mM dNTP, 2.5 unit of Taq polymerase (BRL), 10% of the first strand reaction mixture and ddH_2O to a final volume of 50µl. This was heated to 94°C for 2min followed by 35 cycles of denaturation for 1min at 94°C, annealing for 1min at 67°C and extension for 1min at 72°C in a thermal cycler. Analysis of PCR products were carried out on 1.2% agarose gels containing 0.5µg/ml EtBr and electrophoresed in TBE buffer (100mM Tris-HCl, 50mM boric acid, 2mM EDTA,). DNA molecular weight marker type 100bp DNA ladder (BRL) was applied to identify the size of the PCR products.

Dot blot hybridization. Dot blot hybridization was carried out using PCR products of primer pair F-R2 as probe. The probe was labeled with the hapten digoxigenin by random primed labeling method (Templeton *et al* 1992). The DNA samples were denatured by heating for 10min in boiling water bath chilled on ice and adsorbed to nylon membranes (Boehringe-Manheim) according to Darling & Brickell (1994). After heating at 80°C for 2h the membrane were prehybridized for 1h at 42°C in prehybridization solution containing 5×SSC, 0.02% SDS, 0.1% N-Lauroylsarcosine and 0.1% Blocking reagent (Boehringer-Mannheim). Hybridization was carried out for 16h at 42°C in the same solution containing 25ng/ml of labeled probs. After

hybridization, the membrane were washed 2×5min at room temperature in 2×SSC containing 0.1% SDS and 2×15 min in 0.1×SSC containing 0.1%SDS at 42°C and then rinsed in washing buffer (Maleic acid buffer [0.1M maleic acid, 0.15M NaCl; pH7.5], 3% Tween20 v/v). Membrane was incubated in 1×blocking solution [Blocking reagent in Maleic acid buffer] for 30min at room temperature. Followed by incubate for 30min at RT with anti-DIG-AP conjugate. The membrane were washed 2×15 in washing buffer and equilibrate 2-5min in detection buffer (0.1M Tris-HCl, 0.1M NaCl, 50mM $MgCl_2$, pH9.5). Then the membrane was incubated for 5min in freshly prepared color-substrate solution (NTB/BCIP Boehringer-Mannheim) in dark.

Restriction enzyme analysis. Confirmation of specificity of the PCR product was performed by restriction enzyme digestion with BstEII endonuclease. Briefly 10µl of the PCR sample was digested at 60°C for 2-3h in a reaction mixture containing 10-20 of restriction enzyme, 2µl of 10×reaction buffer and ddH_2O in a total volume of 20µl. Aliquot of 10µl of reaction mixture was loaded on a 1% agarose gel in TBE buffer.

Cloning of VP1 cDNA into pEt-23a+. All basic recombinant techniques were performed according to Sambrook *et al* (1989).

Plasmid isolation. Plasmid pEt-23a+ with or without inset was isolated from overnight culture of harboring *E.coli* in Luria–Bertani (LB) medium containing 100µg/ml ampicillin by alkali lysis method with some modification (Sambrook *et al* 1989, Vanden Heuvel 1998). In brief, the cells were palliated by centrifugation at 9000×*g* for 5min at 4°C. The pellet was washed once with 20ml of ice-cold STE buffer (10mM Tris-HCl pH8.0, 0.1m NaCl, 1mM EDTA), resuspended in 2ml of solution I (25mM Tris-HCl pH8.0, 10mM EDTA, 50mM glucose), kept at room temperature for 5min. Then 4ml of freshly prepared solution II (0.2N NaOH, 1% SDS) was added and the mixture kept on ice for 5min. 3ml ice cold potassium acetate solution pH4.8 was added, kept on ice for 5min, centrifugation at 10000×*g* for 5min at 4°C. The crud plasmid DNA in the supernatant was extracted once with phenol/chloroform/isoamyl alcohol, then once with chloroform/isoamyl alcohol and precipitated with 2.5 vol. 100% ethanol at 80°C for 30min. The DNA pellet was washed with pre-chilled 70% ethanol, dried at room temperature, dissolved in 200µl

of TE buffer (10mM Tris-HCl, 1mM EDTA) pH8.0 and treated with RNase A (20μg/ml) for 2h at 37°C and stored at -20°C.

Preparation of competent E.coli cells. 0.5ml of night culture of DH5α was added to 50ml of LB medium and incubated at 37°C in a shaking incubator until OD550 reached to 0.5. Then culture was chilled on ice for 60min, and spun at 3000 rpm at 4°C for 10min. The cells were resuspended in 1/3 volume of FB buffer (KCl, $CaCl_2 \times 2H_2O$, Glycerol, 0.5M MOPS pH6.5) and incubated on ice for further 60min. The cells were spun at 3000rpm for 10min at 4°C, and the pellet was resuspended in 1/12.5 volume of FB buffer. The competent cells were dispensed in 200μl aliquots in to cryoproteceiv tubes and immediately freeze at –70°C.

Construction of recombinant DNA. Digestion of PCR product and vector was carried out at 37°C for 2-3h or for overnight with NdeI and EcoRI restriction enzymes. Digested DNAs were run on a 1% low melting point agarose (BRL) in TAE buffer (10×TAE buffer: 0.4M Tris-acetate, 10mM EDTA, pH8.0) and the desired segments were excised and purified from the gels according to Sambrook *et al* 1989 with some modification. Briefly, the excised gels were melted at 65°C. Then quickly Tris-HCl saturated phenol pH8.0 was added to the melted gel, vortexed for 1min, centrifuged and the crud DNA in the supernatant was extracted as described for plasmid DNA and dissolved in 25μl of sterile ddH_2O. Legation of digested and purified PCR product and vector were carried out in 20μl reaction mixture containing 4μl 5×ligase buffer 100ng of vector, 56ng of PCR product, 0.5μl of T4 Ligase and sterile ddH_2O at room temperature for 1–2h.

Transformation. The recombinant DNA molecules were transformed into *E.coli* strain DH5α according to Sambrook *et al* (1989) with some modification. 5μl of ligation mixture was mixed with 50μl competent E.coli cells, kept on ice for 30min and then at 42°C for 90sec, diluted with 200μl of LB medium and incubated at 37°C for 1h. Then the cells were plated on LB agar containing 100μg/ml ampicillin and incubated at 37°C overnight.

Identification of recombinant colons. Three methods were used for identification of recombinant clones. 1) Restriction enzyme analysis. All of the colonies which were grown on LB plate after transformation were picked and cultured simultaneously on 5ml of LB medium and LB plate containing 100μg/ml ampicillin. The cultures were incubated at 37°C overnight for plasmid isolation and further analysis with restriction

enzymes. 2) PCR amplification of insert. Enzymatic amplification was carried out on extracted plasmid as DNA template using the primer pair F-R1 and F-R2. The PCR condition was the same as befor. 3) Dot blot hybridization. The purified plasmid pEt-VP1® (pEt-VP1 Razi), pEt-23a+, and the PCR products resulting from enzymatic amplification of VP1 sequence in plasmid pEt-VP1® were blotted on nylon membrane and hybridized. The procedure was basically the same as described above for the detection of PCR products.

Results

RNA isolation. RNA was successfully extracted using Chomczynski & Sacchi (1987) method. Figure 1 shows the integrity of extracted RNA. Formation of the 28S and 18S ribosomal RNA shows that the isolated RNA is intact. FMDV causes lysis of BHK-21 cell, therefore we control the infected cells during incubation and at onset of appearance of CPE, the cells were collected by trypsinization, washing with ice cold PBS pH7.2 and centrifuged. Harvesting the cells before complete CPE would increase the yield of isolated RNA and gives us a mean for assessment of integrity of RNA samples.

RT-PCR Amplification. We selected conserved region which flanking sequences coding for VP1 protein on viral genome for designing primer pairs. The primer F and R1were used to introduce start and stop codon into the sequence of VP1 along with NdeI and EcoRI restriction enzymes, respectively. Analysis of the PCR products by electrophoresis on 1.2% agarose gel using 100bp DNA ladder and subsequently autoradiography showed a dark band corresponding to about 640bp (Figure 2). The nucleotide sequence analysis of FMDV genome showed that the VP1 sequence lie within the nucleotide positions 2886 and 3524 (Fross *et al* 1984). Therefore the length of about 640bp indicate the near full-length cDNA of the VP1 sequence that was obtained by our RT-PCR.

Restriction enzyme analysis. The sequence coding for structural VP1 protein of FMDV contains a unique restriction site for the endonuclease BstEII (Locher *et al* 1995). Digestion of PCR products with this enzyme yielded two fragments of about 436 and 204bp in length (Figure 3), which confirms specific PCR products.

Cloning of VP1 into pEt-23a+. For constructing the recombinant pEt-VP1®, the pEt-23a+ plasmid (a gift from Dr. F. S. Mozafari, The Pasture Institute of Tehran)

was used. This vector is developed for cloning and expression of recombinant proteins in *E.coli.* For cloning the VP1 cDNA into the pEt-23a+ vector, the NdeI restriction site was used in the F primer to introduced ATG initiation codon for translation and EcoRI restriction site and stop codon were used in the R1 primer for cloning of the gene. The amplified DNA was then inserted into the NdeI and EcoRI restriction enzyme sites in the polylinker sequence of the pEt-23a+ vector. The resulting construct was designated pEt-VP1®. For initial cloning and maintaning DNA fragment the *E.coli* strain DH5α was used. The recombinant clones containing VP1 gene were screened on LB plate containing 100μg /ml ampicillin.

Identification of recombinant clones. Twenty colonies were obtained after overnight incubation. Three methods were used for identification of recombinant clones. 1) correct constructs were initially identified by restriction endonuclease analysis of miniprep plasmid purification. Briefly, all of the colonies were grown on both LB plate and LB medium containing 100μg/ml ampicillin. Plasmid isolation and purification were carried out. Extracted plasmids were digested with NdeI or EcoR restriction enzymes. Digested plasmids were loaded on 1% agarose gel for plasmid size estimation (Figures4 and 5). Then the plasmids containing the insert were digested with NdeI and EcoRI restriction enzymes (Figure 6). We found eight recombinant clones that contained the insert at about 640bp. 2) PCR amplification of extracted plasmid showed the band of about 640bp that correspond to VP1 sequence (Figure7). Identity of the PCR product was verified by digestion of the PCR product with BstEII restriction enzymes (Figure 8). 3) Confirmation of PCR products obtained from enzymatic amplification of FMDV type O1, plasmid pEt-VP1® was carried out by dot blot hybridization using the internal probe as described in materials and methods. Plasmid pEt-23a+ was used as negative control. The entire blotted DNA showed strong signals on hybridization except negative control (Figure 9). Analysis of hybridization data confirmed cloning of VP1 gene of FMDV type O1/Iran.

Discussion

The advent of recombinant DNA technology has made it possible to clone viral genomes and thereby to produce viral antigens in microorganism such as *E.coli.* To clone genomic sequences from RNA viruses like FMDV it is necessary to transcribe

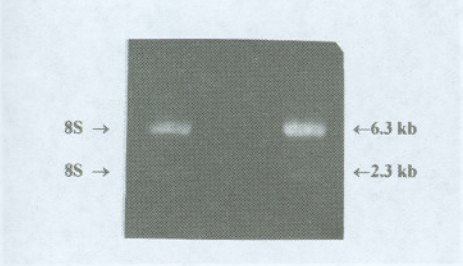

Figure 1. *Total RNA were formaldehyde-denatured and electrophoresed in 1% agarose gel at constant 30vol. Overnight. The sharp definition of the 28S and 18S rRNA species demonstrates the integrity of the sample, with minimum smearing above, between, and below the 28S and 18S rRNAs*

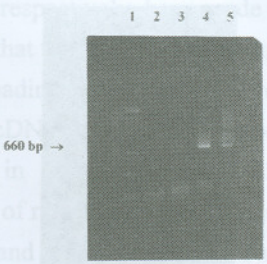

Figure 2. *Serial dilution of $MgCl_2$ were added to the reaction mixture, then the PCR was run at 35 cycles. Lane 1: 100bp DNA ladder. Lane 2: 0.5mM; lane3: 1mM; lane 4: 1.5mM and lane5: 2mM of $MgCl_2$*

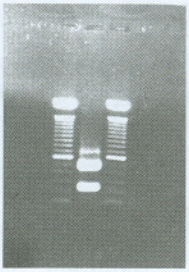

Figure 3. *Confirmation of specific PCR product by BstEII digestion. Lane 1 and 3: 100bp DNA ladder; lane2:BstEII digested FMDV-PCR product*

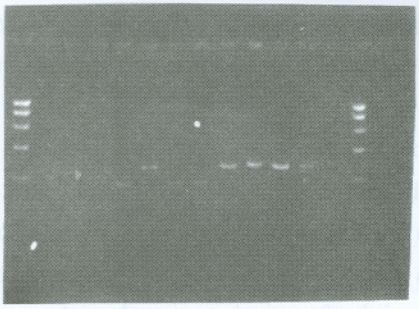

Figure 4. *Digestion of plasmid purified after transformation of DH5α with ligation mixture. Lane 1 and 14: size marker VII; lane 2-13: plasmid digested with EcoRI*

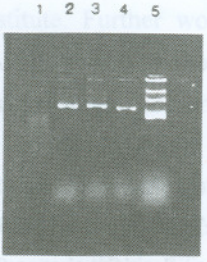

Figure 5. *Comparison of pEt-VP1 and pEt-23a+ digested with NdeI. Lane 1: size marker VII. Lane 2 and 3: pEt-VP1; lane 4: pEt-23a+; lane 5: uncut plasmid*

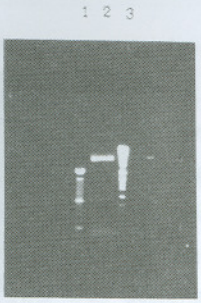

Figure 6. *Digestion of plasmid pEt-VP1® with NdeI and EcoRI restriction enzymes. Lane 1: 100bp DNA ladder; lane2: digested plasmid; lane 3: size marker VII.*

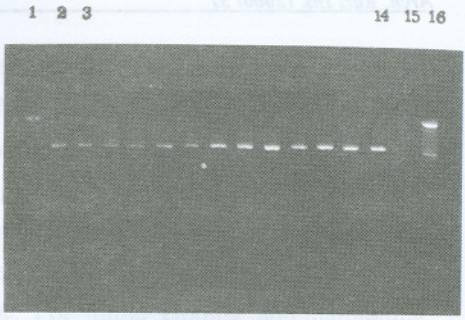

Figure 7. *The PCR products resulting from enzymatic amplification of plasmid pEt-VP1 with primer pair F-R1. Fragment sizes (in base pair) are indicated on the right. Lane 1 and 16: 100bp DNA ladder; lane 2: VP1; lane 3-14: pEt-VP1; lane 15: negative control*

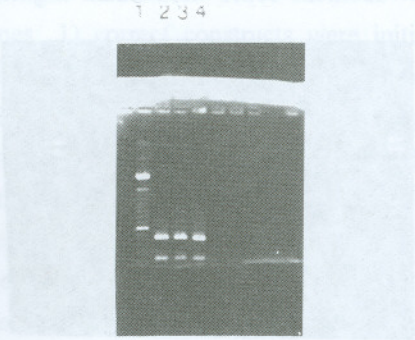

Figure 8. *Digestion of PCR products results from enzymatic amplification of plasmid pEt-VP1® with BstEII. Lane 1: 100 bp DNA ladder; lane 2-4: BstEII digested PCR products*

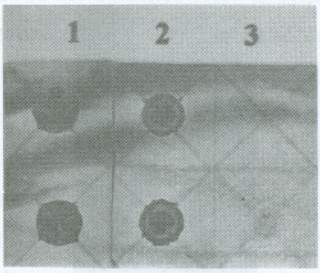

Figure 9. *Dot blot hybridization of PCR products and pEt-VP1. Lane 1: PCR products; lane 2: pEt-VP1; lane 3: pEt-23a+*

the RNA into ds cDNA. To clone of this ds cDNA into *E.coli*, several groups have used the oligo (dC/dG) tailing method (e.g. King *et al* 1981). This method can result in cloning small fragments for which it can be difficult to identify their genomic localization and sometimes they have to analyze more than one thousands of transformant to find the desired clone. We used PCR method, which provides an easy way for cloning and subsequent identification of the cloned gene, for preparing ds cDNA and constructing VP1 operon. This ds cDNA molecule encodes an open reading frame for full length VP1 protein. The restriction sites of NdeI and EcoRI,

which were added to 5´ end of F and R1 primers respectively, have made cloning of VP1 ds cDNA much simpler. Also it is essential that the VP1 ds cDNA inserted into the cloning vehicle can be read in the correct reading frame. The NdeI recognition sequence that was added to 5´end of VP1 ds cDNA fragment to introduce ATG initiation codon achieves this. The vector used in this study was pEt-23a+ vector (Novagen) developed for cloning and expression of recombinant protein in *E.coli*. It carrying strong bacteriophage T7 transcription and trnslation signals. Target genes are initially cloned using hosts that do not contain the T7 RNA polymerase gene, so they are virtually "off" and cannot cause plasmid instability due to the production of proteins potentially toxic to the host cells. Once established, recombinant plasmids are transferred into expression hosts. Therefore subcloning of the desired gene isn't required. The pEt-23a+ vector carries unique restriction sites for NdeI and EcoRI restriction enzymes at its polylinker sequence. Using these two sites, the constructed VP1 operon was inserted into the vector such that only one orientation of insertion is possible.

Recombinant plasmid was constructed as described in materials and methods, and transformed into an *E.coli* strain *DH5α* host, which is $recA^-$ and does not contain the T7 RNA polymerase gene. The presence of a known restriction site at each end of inserted VP1 operon offers a first important means for initially identification of recombinant plasmids. Therefor restriction endonuclease analysis of miniprep plasmid purification was carried out for identification of correct constructs (Figures 4 and 5). PCR amplification of recombinant plasmid (Figure 7) and dot blot hybridization of pEt-VP1® was also used for confirmation of identity of cloned gene. The pEt-VP1® plasmid is being maintained in the Biotechnology departmentat, Razi Vaccine & Serum Research Institute. Further work is required for expression of cloned gene.

Acknowledgments

The Biotechnology department of Razi Vaccine & Serum Research Institute supported this work. We are indebted to Dr. M. P. Ashtiani and Dr. A. T. Shoshtari for their supports. Dr. A. Khorasani is thanked for his kindly help in culturing cells and FMDV. We are grateful to M. Esmaelizad, A. Aghaie and Dr. Z. Fayazi for their enlightening conversations on the PCR and cloning technique.

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