C-TERMINAL REGION OF VP1 OF SELECTED FOOT-AND-MOUTH DISEASE VIRUS SEROTYPES: EXPRESSION IN *E. COLI* AND AFFINITY PURIFICATION

G. RATISH[1], S. VISWANATHAN, V.V.S. SURYANARAYANA*

Indian Veterinary Research Institute, Hebbal, Bangalore 560 024, India

Received December 21, 1998; accepted February 25, 1999

Summary. – Foot-and-mouth disease (FMD), one of the most contagious and economically important diseases of farm animals, is caused by a FMD virus (FMDV) which belongs to the family of *Picornaviridae*. The virus occurs as seven serotypes of which four (A, O, C and Asia 1) are prevalent in India. Immunoprophylaxis supported by precise diagnosis is the prime requirement for achieving the success in controlling the disease. Recently, recombinant DNA technology is gaining importance for the production of cost-effective and safer diagnostics and immunogens. Based on this approach, cDNA of a part of gene for major variable immunogenic region, VP1, of FMDV of four serotypes (A22, O, C and Asia 1) was amplified by PCR and cloned into expression vector. The expression of the 16 K protein gene from the clones was induced with IPTG and analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) and [^{35}S]-methionine labeling. The immunoreactivity of the labeled proteins was assayed by immunoprecipitation with anti-FMDV type-specific sera. Since the proteins contain 6 His residues at the N-terminal end, their affinity purification was carried out using nickel nitrilo-tri-acetic acid (Ni-NTA) agarose matrix. The proteins were found to be immunoreactive and the useful in the FMD diagnosis.

Key words: PCR; diagnostics; FMDV; expressed protein; affinity purification

Introduction

FMD is one of highly contagious and economically important diseases of cloven hoofed animals such as cattle, buffaloes, sheep, goats and pigs (Bachrach, 1968). FMD is endemic to several countries including India. The causative agent of FMD is an RNA virus, FMDV, that belongs to the family of *Picornaviridae* (Murphy *et al.*, 1995) having an icosahedral capsid structure (Acharya *et al.*, 1989), composed of 60 copies of each of the four proteins, 1A, 1B, 1C and 1D (VP1-VP4). The genome consists of an infectious positive-sense RNA of 8.5 kb which comprises a small virus-coded protein VPg (3 K) bound covalently at the 5'-end, followed by poly(C)-tract and poly(A)-tail at the 3'-end (Grubman, 1980). The genome has a single open reading frame (ORF) flanked on both ends by untranslated regions (UTRs). The virus occurs as seven serotypes, namely A, O, C, Asia 1, SAT 1, SAT 2 and SAT 3, and India harbors four of them (A, O, C and Asia 1). Several molecular studies on FMD directed towards the development of better vaccines had a limited success. However, limited efforts were made to utilize the recombinant DNA technology for the development of FMD diagnostics. Detection of FMDV was previously done by virus isolation in host animals (Skinner and

[1]Part of the work reported is from Master's Dissertation of the author.
*Corresponding author. E-mail: bngivri@kar.nic.in.

Abbreviations: CBB = Coomassie Brilliant Blue; CFT = complement fixation test; ELISA = enzyme-linked immunosorbent assay; FMD = foot-and-mouth disease; FMDV = FMD virus; IPTG = isopropyl-(-D-thiogalactopyranoside); ME = mercaptoethanol; NiNTA = nickel nitrito-tri-acetic acid; ORF = open reading frame; PMSF =; PCR = polymerase chain reaction; SAB = sample application buffer; SDS-PAGE = polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; UTRs = untranslated regions

Knight, 1964) or tissue culture (Snowdown, 1966). Subsequently, complement fixation test (CFT) (Casey, 1965) and enzyme-linked immunosorbent assay (ELISA) (Abu Elzein and Crowther, 1978) have come into existence for the FMDV detection and typing. Of late nucleic acid-based tests polymerase chain reaction (PCR), nucleic acid hybridization (Reddy and Suryanarayana, 1993), and nucleotide sequencing are being used for the detection of virus type and strain variation (Tulasiram, 1994; Suryanarayana, 1998). Though the nucleic acid-based tests are highly sensitive, their use at the field level is still limited. Subsequently, the recombinant proteins have found their way in diagnostics (Banks *et al.*, 1995). In case of FMDV, initial attempts have been made in our laboratory to produce type-specific antigens in *Escherichia coli* (Tyagi, 1993) for using as diagnostic reagents. However, due to limitations in achieving high level expression and efficient purification of the cloned gene product, further progress could not be made. With the availability of vectors with T7 promoter (Studier *et al.*, 1990) and His-tag affinity purification techniques (Hochuli, 1990) the production of proteins in *E. coli* or any other host and their purification have become easier. The current work presented here deals with the overexpression in *E. coli* of type-specific immunoreactive proteins of FMDV of serotypes A22, O,C and Asia 1 circulating in India. The cloned genes were expressed as His-tag fusion proteins, which were purified by using Ni-NTA agarose matrix and characterized.

Materials and Methods

Recombinant DNAs. M13 phage single-stranded DNAs carrying 276 nt of C-terminus of VP1 gene along with 51 nt of 2A gene of FMDV vaccine viruses (A22, O, C and Asia 1) were used as source of genes. These were available in the Protein and Nucleic Acid Laboratory of Indian Veterinary Research Institute (Suryanarayana *et al.*, 1991).

Escherichia coli strains DH 5α and BL21(DE)3pLys S were used.

Oligonucleotide primers. 5'-end (SV1) and 3'-end (P1) primers were used for amplification as described by Locher *et al.* (1995) with suitable restriction sites in the expression vector.

Cloning in expression vector. Amplification of a 330 bp cDNA corresponding to C-terminal VP1 and 2A was done by PCR with primers SV1 and P1 following the methodology described by Locher *et al.* (1995) with few modifications. The amplified 330 bp products were purified from Low Melting Point (LMP) agarose gel and cloned into pRSET expression vector (Invitrogen) at *Bam*HI-*Eco*RI sites (Maniatis *et al.*, 1989). The bacterial colonies containing the insert were individually screened by PCR using an upstream (T7) primer corresponding to the vector sequence and a downstream primer (P1) corresponding to the 3'-end of the insert.

Screening for protein expression. Protein expression from each single *E. coli* clone was monitored after induction as described by Studier *et al.* (1990). The bacteria carrying the plasmid with or without an insert were grown separately in LB broth overnight in the presence of ampicillin (50 μg/ml) and chloramphenicol (50 μg/ml). The overnight culture was inoculated into fresh LB broth and further grown till A_{540} reached 0.8. The cells were then pelleted, suspended in fresh LB broth, isopropyl-(-D-thiogalactopyranoside) (IPTG) and rifampicin were added to a final concentration of 0.8 mmol/l and 200 μg/ml, respectively, to induce expression and to reduce the background.

In vivo labeling and immunoprecipitation of proteins was carried out as described by Suryanarayana *et al.* (1992). The bacteria carrying plasmid with or without insert were grown overnight. The bacteria from 1 ml of overnight culture were pelleted, washed thrice with chilled M9 medium and grown in M9 medium containing 10 μCi/ml [^{35}S]-methionine (800 Ci/mmol) and 1 nmole each of other amino acids in the presence of IPTG and rifampicin as described above. The labeled bacteria from 1 ml of culture were lysed in 200 μl of an immunoprecipitation buffer (25 mmol/l Tris-HCl pH 7.4, 5 mmol/l EDTA pH 8.0, 10% glycerol, 1% Triton X-100, 0.05% SDS, 0.5% NP-40 and 5 mmol/l PMSF). Clarified supernatant (50 μl) from each lysate was subjected to immunoprecipitation. The specific guinea pig antiserum (against A, O, C and Asia 1, respectively) was diluted 2000- to 4000-fold in the immunoprecipitation buffer, mixed with an equal volume of lysate obtained from *E. coli* carrying pRSET plasmid without insert, and incubated at 37°C for 1 hr to adsorb antibodies against *E. coli*. The adsorbed antiserum was centrifuged and added to the labeled protein, so as to get the desired final dilution. The mixture was incubated at 37°C for 3 hrs, protein A-activated cells (30 μl) were added to the antigen-antibody complex and further incubated for 30 mins at room temperature in a shaker. The pellet after centrifugation was washed 5 times with the immunoprecipitation buffer, resuspended in 30 μl of a loading solution (15% mercaptoethanol (ME), 15% SDS, 1.5% Bromophenol Blue and 50% glycerol) (Laemmlli, 1970), boiled and subjected to SDS-PAGE (15% gel) followed by fluorography.

Affinity purification of expressed proteins. Affinity purification of His-tagged expressed proteins was done by the method recommended by the manufacturer (Qiagen, Germany) with modifications. Two ml of 50% Ni-NTA agarose slurry was packed into a column and allowed to settle for 1 hr. Initially, the Ni-NTA agarose column was washed with 5 volumes of water before equilibrating with 10 ml of sample application buffer (SAB) consisting (8 mol/l urea, 1 mol/l NaCl, 20% glycerol, 0.25% Triton X-100 and 20 mmol/l imidazole in 10 mmol/l Tris-HCl pH 8.0). The pellet obtained from 50 ml of the bacterial culture was resuspended in 4 ml of SAB and the lysate was passed 5 times through the column. In order to standardize the optimum conditions of washing for removing the non-specific proteins, different buffer systems were tested. Wash buffer A (20% glycerol, 100 mmol/l NaCl, and 20 mmol/l imidazole in 10 mmol/l Tris-HCl pH 5.9), wash buffer B (20% glycerol, 1 mol/l NaCl, and 20 mmol/l imidazole in 10 mmol/l Tris-HCl pH 5.9), wash buffer C (20% glycerol, 1 mol/l NaCl, 20 mmol/l imidazole, and 5 mmol/l ME in 10 mmol/l Tris-HCl pH 5.9), wash buffer D (20% glycerol, 100 mmol/l NaCl, 0.5 % Triton X-100, and 20 mmol/l imidazole in 10 mmol/l Tris-HCl pH 5.9), and wash buffer E (20% glycerol, 500 mmol/l NaCl, 20 mmol/l imidazole, and 5 mmol/l ME in 10 mmol/l Tris-HCl pH 5.9). The bound protein was eluted with 120 mmol/l imidazole

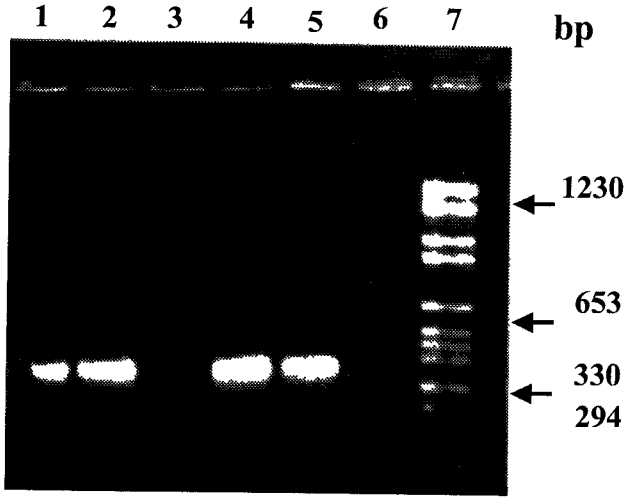

Fig. 1
Agarose gel electrophoresis of PCR products
Plasmids with A22, O, C and Asia 1 inserts (lanes 1, 2, 4 and 5); negative control (lane 6); DNA size markers (lane 7).

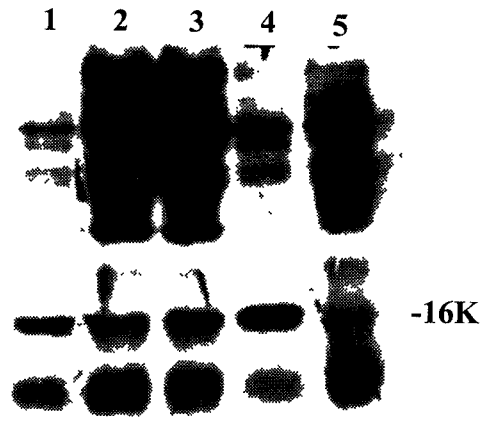

Fig. 2
Autoradiograph of SDS-PAGE of ^{35}S-labeled proteins
Plasmids with A22, O, C or Asia 1 inserts (lanes 1–4); protein size markers (lane 5).

in SAB. Approximately 10 fractions of 1 ml each were collected, and A_{280} of each fraction was monitored spectrophotometrically. The peak protein fractions were pooled and the concentration of the protein was estimated. A_{280} of 1.4 corresponded to 1 mg of protein per ml.

Results

Cloning of FMDV-specific 330 bp DNA into pRSET vector

The DNA regions corresponding to the C-terminal half of VP1 and 2A of FMDV serotypes A22, O, C, and Asia 1 were amplified by PCR and the products were analyzed by agarose gel electrophoresis (Fig. 1). Intense bands corresponding to 330 bp were observed; this band was absent in negative control. The amplified products were cloned and some of the positive clones were further analyzed by restriction digestion with *Bam*HI and *Eco*RI, followed by agarose gel electrophoresis.

Expression of the cloned gene

The pRSET plasmids carrying the insert from FMDV DNA were transferred into *E. coli* BL21 (DE3) pLysS and expression of the protein was induced. The bacteria from 400 µl of culture were pelleted and lysed by freeze-thawing. The total labeled proteins were analyzed by SDS-PAGE (15% gel) (Fig. 2). More than 3 well separated protein bands were seen in the flourograph. Moreover, an intense band of about 16 K could be seen in case of plasmids with A22, O, C, and Asia inserts. Protein bands of a little lower intensity and size were seen also in case of plasmid without an insert. The intense band of about 16 K might be due to the presence of two overlapping proteins of which one having lower M_r could originate from the host without insert and the other from the host with insert. Since the bacteria were induced in the presence of rifampicin, the majority of the host-specific proteins were inhibited, leading to the expression of genes recognized by T7 polymerase alone. The accurate size of the extra protein as determined from the mobility of standard proteins was 15.8 K. In accordance, the calculated M_r of the protein based on amino acid composition was also 15.8 K. Other protein bands in case of the hosts without insert and with O or C inserts might be of host or plasmid origin. The intense protein bands of about 30 K in case of the hosts with O or C inserts might not be due to the presence of double inserts as the directional cloning was done in these experiments.

Immunoreactivity of the expressed proteins

In order to confirm their specificity and size, the labeled proteins were subjected to immunoprecipitation using FMDV type-specific antisera raised against whole virus. The antigen-antibody complexes were separated, analyzed by SDS-PAGE and fluorographed. Well separated radioactive protein bands could be seen in fluorograph (Fig. 3) in all samples in which labeled proteins from each clone having either A22, O, C or Asia1 insert were immunoprecipitated with specific antisera. The intense protein band of 16 K corresponded to the virus-specific protein. The other less intense protein band might correspond to the host or plasmid, which might have been precipitated non-specifically.

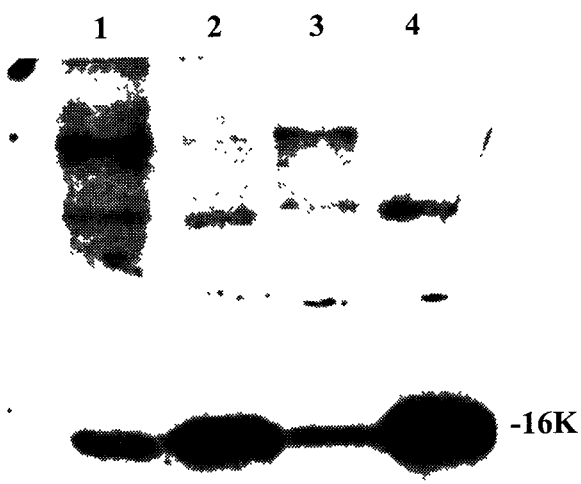

Fig. 3
Autoradiograph of immunoprecipitated ^{35}S-labeled proteins subjected to SDS-PAGE
Plasmids with C, A22, Asia 1 or O inserts (lanes 1–4).

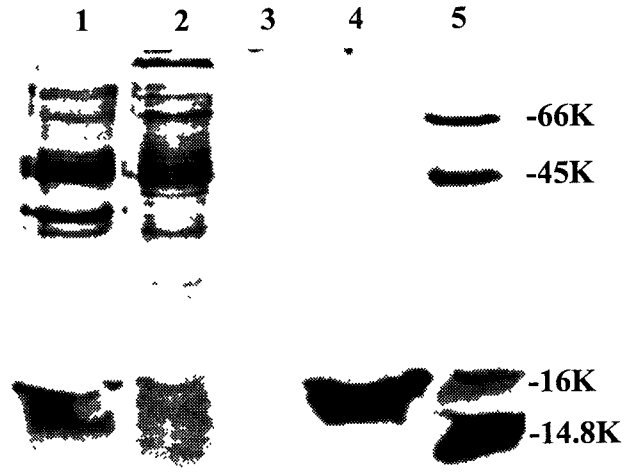

Fig. 4
SDS-PAGE of urea-solubilized proteins
Induced bacteria were pelleted, lysed and centrifuged. Clear lysate and urea-solubilized pellet were fractionated through Ni-NTA column. Various samples were subjected to SDS-PAGE and CBB staining. Whole lysate before centrifugation (lane 1), supernatant after centrifugation (lane 2), column-eluted proteins from supernatant (lane 3), column-eluted proteins from urea-solubilized pellet (lane 4), and protein size markers (lane 5).

Nature and quantity of proteins produced in the clone

The *E. coli* BL21 (DE3) pLysS host (Goldberg *et al.*, 1988), used in expression, allowed an overexpression of the inserted gene (Figs. 2 and 3). This is evident from the presence of an additional protein band in the Coomassie Brilliant Blue-stained gels even when the protein from 1 ml of culture was analyzed. Since there was tight regulation of the host expression due to the presence of lysozyme coded by pLys, the expression of the foreign gene could be stable. The pLys has also facilitated the lysis of the host by a single freeze-thaw cycle. It has been observed that most of the protein was present as insoluble aggregates necessitating the use of 8 mol/l urea for their solubulization (Fig. 4) for SDS-PAGE or purification. When the bacteria from 1 ml of culture were suspended in 50 µl of water, lysed and centrifuged, and both the pellet and the supernatant were analyzed by SDS-PAGE (Fig. 4) it was observed that the majority of the expressed 16 K protein was in the pellet (lane 1) as compared to the supernatant (lane 2). Further, to confirm the aggregated nature of the expressed product, the bacterial pellet from 50 ml of culture was suspended in 4 ml water, lysed and centrifuged at 10,000 x g for 5 mins, and the supernatant as well as the pellet were subjected to affinity purification using Ni-NTA agarose column followed by SDS-PAGE (Fig. 4). No band of 16 K was seen in case of the eluate from supernatant fractions (lane 3), while an intense band of 16 K was seen in case of the eluate from the aggregate fractions (lane 4) indicating that the protein was in the form of aggregates. The presence of a single band during purification and isolation indicated that the protein was stable in the bacteria. The amount of protein produced in the clone as determined spectrophotometrically was 5 mg per 50 ml of culture. In order to achieve homogeneity of the product of affinity purification optimum conditions for column washing and elution were sought. Buffers containing different concentrations of salt, reducing agent, detergent etc. were tested. The bacteria in 250 ml of culture were induced with IPTG for 6 hrs at mid log phase, pelleted and resuspended in 15 ml of SAB. The lysate was divided into five equal portions of 3 ml each, which were and passed through a 2 ml Ni-NTA agarose column. The columns were washed with different wash buffers to

Table 1. Amount of protein eluted from Ni-NTA agarose column with different wash buffers

Wash buffer	Yield of protein (mg/50 ml)
A	5
B	6.4
C	6 8
D	4.4
E	2 06

Composition of buffers A, B, C, D, and E are given in Materials and Methods.

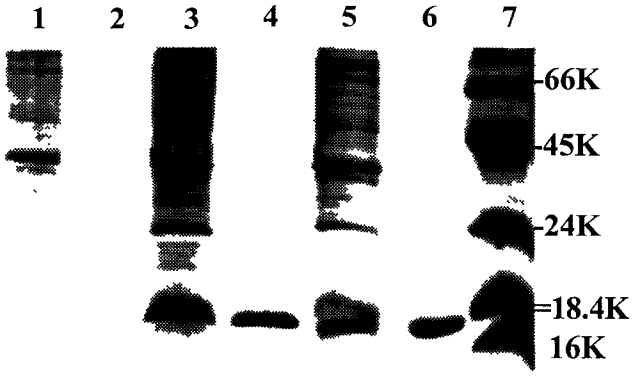

Fig. 5
SDS-PAGE of purified proteins
Lysate from induced bacteria were fractionated through Ni-NTA column and the obtained fractions were subjected to SDS-PAGE and CBB staining. Lysate of bacteria without insert (negative control, lane 1), column-bound proteins from negative control (lane 2), lysate and column-bound proteins from bacteria with A22 insert (lanes 3 and 4), lysate and column-bound proteins from bacteria with O insert (lanes 5 and 6), and protein size markers (lane 7).

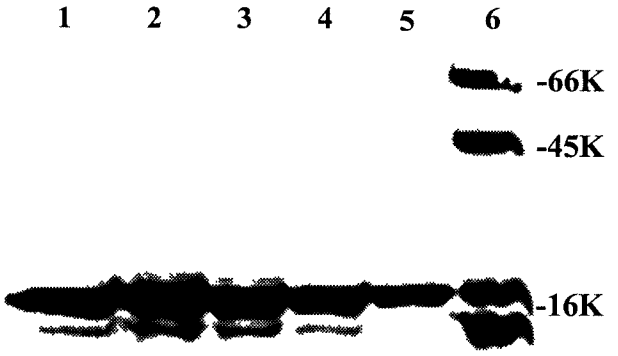

Fig. 6
SDS-PAGE of column-purified proteins after washing the column with different buffers
Proteins from the lysate were bound to the column, washed with different wash buffers (A-E), eluted with SAB-imidazole solution and subjected to SDS-PAGE with CBB staining. Wash buffers A-E (lanes 1–5) and protein size markers (lane 6).

remove bound contaminants, and the column-bound proteins were eluted with 120 mmol/l imidazole in SAB and analyzed by SDS-PAGE (Fig. 4). The analysis showed the presence of a single highly intense band of 16 K along with a minor band below 16 K (lanes 1-5) corresponding to wash buffers A to E, respectively. The intensity of the 16 K band was maximum in case of buffer B (lane 2). In addition to the intense 16 K band a less intense minor band of lower size was seen, which may correspond to the processed protein, as later confirmed by Western blot analysis. Therefore, the optimum buffer was B. The amount of protein, eluted after washing with the different wash buffers, is shown in Table 1. The other minor bands of higher M_r may correspond to co-purified host proteins.

Discussion

Most of the diagnostic techniques used in case of FMD are directed towards the detection of virus type as there is no cross-protection among different serotypes of FMDV. The nucleic acid-based techniques are aimed at amplification of major antigenic protein VP1 gene using type-specific primers (Rodriguez and Schudel, 1993) or specific immunocapture and amplification (Suryanarayana *et al.*, 1990), or at nucleic acid hybridization using gene probes corresponding to VP1 (Mc Farlane *et al.*, 1990; Reddy and Suryanarayana, 1993). The protein-based techniques depend on the use of antibody raised against the whole virus. The former techniques are highly sensitive, but their use is limited due to the requirements of high cost materials and trained manpower; the latter techniques are cheaper and of wider use, however, their application in large scale is constrained due to the requirement of necessary containment facilities. Nevertheless, taking the feasibility into account, the present day requirement for endemic countries is the production of safe and easily available protein-based diagnostic tests. In order to produce these, the immediate approach one can think of is the expression of specific genes in heterologus systems. VP1 of FMDV has been identified as a major immunogen, and it has been shown that the expressed VP1 elicits immune response in vaccinated animals (Kleid *et al.*, 1981). However, only the C-terminal half of the VP1 has the necessary neutralizing epitopes (Strohmaier *et al.*, 1982) and carries variable, type-specific region (Suryanarayana *et al.*, 1993). Hence, the expression of this sequence may result in the production of type-specific antigens, which may replace the whole virus antigens in the diagnostics. As a first attempt in this direction we have amplified the C-terminal 276 nt region of all four serotypes (vaccine strains) of VP1 using primers corresponding to conserved regions of 2A and VP1. The genes were overexpressed in *E. coli* using T7 promoter-driven vectors. The quantity of the protein produced was up to 100 mg per liter of culture. The protein formed aggregates which could be sedimented by centrifugation. The pellet contained exclusively the expressed protein as observed by SDS-PAGE. It was reported that the *E. coli*-produced proteins form aggregates when expressed at higher levels (Tabor and Richardson, 1985). In our experiments we found that the formation of aggregates increased in the stability of the protein. There was no loss of the protein during its purification or storage of the cells.

We have used rifampicin for the enrichment of the immunoreactive protein in the culture. Its addition at a concentration of 200 μg/ml increased the expression of the specific gene while reducing the background. Rifampicin is known to inhibit *E. coli* RNA polymerase by binding to a subunit of RNA polymerase but not T7 RNA polymerase which is a single polypeptide.

Protein purification to homogeneity is important for diagnostic purpose. The expressed protein showed maximum affinity towards the Ni-NTA matrix. However, some host proteins, which may have affinity to metals tend to bind non-specifically to the column, and in addition, if the expressed protein is highly basic as in this case (Tyagi, 1993), the recombinant protein may bind to the host proteins due to ionic interactions. In either case the co-purification of host proteins with the recombinant protein is possible. It has been found that at least three other *E. coli* proteins might contaminate the column, most abundant of which is a superoxide dismutase (Stuber *et al.*, 1990). To remove these contaminating proteins effectively, optimum conditions for protein binding, column washing and elution of the bound protein need be worked out. In our studies, the addition of 1 mol/l NaCl to the sample application and wash buffers increased the protein yield by 36%, i.e. from 5 mg to 6.8 mg from 50 ml of culture. The presence of high concentration of salt might help in solubilizing the protein and thereby enhancing the recovery of the protein in purification (Catherine, 1990). We have also observed that the addition of glycerol up to 20% reduced the non-specific binding, probably, by reducing hydrophobic interactions between proteins (Catherine, 1990). In addition, inclusion of imidazole up to 20 mmol/l in the application and wash buffers reduced the non-specific binding of host proteins. However, further increase of imidazole concentration (from 20 mmol/l to 60 mmol/l) resulted in reduction in protein yield, though, there was an increase in purity of the eluted protein. We have not calculated the dissociation constant of the His-tagged 16 K protein for the Ni-NTA column. Other reagents like ME, which was found to reduce the background even at 5 mmol/l concentration probably by reducing cysteine-cysteine interactions, also reduced the protein yield by 60% (to 2 mg/50 ml). The addition of ME might also reduce the Ni-NTA column and therefore we do not recommend its use in protein purification. However, it has been suggested that ME may be used to solubilize the protein (Stuber *et al.*, 1990) in the case it remains insoluble even in the presence of other solubilizing agents. The addition of Triton X-100 (0.25%) also helps to solubilize the protein and reduces the protein-protein interactions as reported by Catherine (1990). However, the increase in Triton X-100 concentration above 0.5% reduced the protein yield. We found that the bound protein was resistant to high salt (1 mol/l NaCl) and detergent (0.25% Triton X-100) washing. The protein bound to column could be washed even with buffer containing salt concentration as high as 2 mol/l (result not shown). Similar results were also obtained in purification of other His-tagged proteins (Hoffman and Roeder, 1991; Gu *et al.*, 1994). The expressed proteins were analyzed for their reactivity with antibodies produced against the whole virus. The virus-specific antibodies could recognize the major epitopes on the proteins. The major antigenic sites, A and C, are located at aa 20-40 and 80-93, respectively, on the expressed protein (corresponding to aa 140-160 and 200-213 of VP1, respectively) (Domingo *et al.*, 1990), and these sites might be responsible for antibody recognition. The immunoprecipitation of the labeled protein using type-specific homologous sera showed more intense bands in our experiments. The use of antibodies against whole virus for virus typing may cause a cross-reaction due to the presence of antibodies against other common proteins. Since the expressed proteins carry a variable region of the immunoreactive protein gene (VP1), the antibodies raised against them may be highly specific. The production of antibodies against purified proteins for using in the diagnostic tests is in progress. Our approach here is to use the recombinant antigens for FMDV typing which may avoid the risk of using whole virus for raising antibodies or as standard antigen. The purified antigen may be used in a competitive ELISA-based antigen detection. In addition, the antibodies raised against these proteins may detect even the denatured virus, which in turn helps in safe handling of field samples. A truncated protein produced by the clones may be used for evaluating the vaccine potency *in vitro* by assaying serum neutralization titers before and after adsorption of the sera to the specific proteins.

Acknowledgements. This research was supported by Department of Biotechnology, Government of India and Swiss Development Cooperation under the Indo-Swiss Collaborative Project. The authors wish to thank the Director of the Indian Veterinary Research Institute for providing necessary facilities for carrying out this work.

References

Abu Elzein EME, Crowther JR (1978): Enzyme linked immunosorbent techniques in FMD research. *J. Hyg. Camb.* **80**, 391.

Acharya R, Fry F, Stuart D, Rowlands D, Brown F (1989): The three dimensional structure of FMDV at 2.9 A resolution. *Nature* **337**, 709–716.

Bachrach HL (1968): Foot-and-mouth disease. *Annu. Rev. Microbiol.* **22**, 201.

Banks M, Tehranchi B, Weightman S, Schwyzer M, Ackermann M (1995): Aujeszky's disease ELISA using glycoprotein gE (gI) expressed in baculovirus. 3rd Congr. Eur. Soc. Vet. Virol., Interlaken, Switzerland.

Casey HL (1965): Standardised diagnostic complement fixation method and adaptation to micro test. I. Laboratory bench complement fixation method II. Adaptation of LBCF method to microtechnique. *Public Health Monogr.* **74**, 1228.

Catherine Schiene H (1990): Solubility as a function of protein structure and solvent components. *Biotechnology* **8**, 308.

Domingo E, Mateu MG, Martinez MA, Dopazo, J Moya A, Sobrino F (1990): Genetic variability and antigenic diversity of foot-and-mouth disease virus. In *Applied Virology Research.* Vol. 2. Plenum Press, New York, pp. 233–266.

Goldberg AI, Goff SA, Casson LP (1988): Hosts and methods for producing recombinant proteins in high yields. *U.S. Patent* **4**, 512.

Gu J, Stephenson CG, Landorola MJ (1994): Recombinant proteins attached to a nickel column; use in affinity purification of antibodies. *Biotechniques* **17**, 257.

Grubman MJ (1980): The 5' end of foot-and-mouth disease virus RNA contains a protein covalently linked to the nucleotide pUp. *Arch. Virol.* **63**, 311.

Hochuli E (1990): Purification of recombinant proteins with metal chelate adsorbent . Vol. 12., 87. In Setlow JK: *Genetic Engineering, Principle and Methods.* Vol. 12. Plenum Press, New York, p. 87.

Hoffman A, Roeder RG (1991): Purification of His tagged proteins in non-denaturing conditions suggests a convenient method for protein interaction studies. *Nucleic Acids Res.* **22**, 6337.

Kleid DG, Yansura D, Small B, Dowbenko D, Moore DM, Grubman MJ, McKercher PD, Morgan DO, Robertson BH, Bachrach HL (1981): Cloned viral protein vaccine for foot and mouth disease , response in cattle and swine. *Science* **214**, 1125.

Laemmli UK (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680.

Locher F, Suryanarayana, VVS, Tratschin JD (1995): Rapid detection and characterisation of foot-and-mouth disease virus by restriction enzyme and nucleotide sequence analysis of PCR products. *J. Clin. Microbiol.* **33**, 440.

Maniatis T, Fritsch EF, Sambrook J (1989): *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Mc Farlane RG, Molitor TW, Vakharia VN (1990): The detection and differentiation of foot and mouth disease viruses using solid-phase nucleic acid hybridisation. *J. Virol. Methods* **27**, 175.

Murphy FA, Fauquet CM, Bishop DHL, Ghabrial SA, Jarvis AW, Martelli GP, Mayo MA, Summers MD (1995): *Virus Taxonomy. Sixth Report of the International Committee on Taxonomy of Viruses.* Springer Verlag, Wien – New York.

Reddy GR, Suryanarayana VVS (1993): A sensitive non radio-isotopic hybridisation method for the detection of specific type of foot-and-mouth disease virus. *International Symposium on Virus-Cell Interaction: Cellular and Molecular Responses*, Bangalore, India.

Rodriguez M, Schudel AA (1993): Nucleicacid hybridisation and polymerase chain reaction in the diagnosis of the infectious animal diseases. *Rev. Sci. Tech. Int. Epizoot.* **12**(2), 405.

Rossi MS, Sadir AM, Schudel AA, Palma EL (1988): Detection of foot-and-mouth disease virus with DNA probes in oesophageal-pharyngeal fluids. *Arch. Virol.* **99**, 67.

Skinner HH, Knight EH (1964): Environmental factors influencing the response guineapigs to modified strains of foot-and-mouth disease virus. *Bull. Int. Off. Epizoot.* **61**, 1523.

Snowdown WA (1966): Growth of foot-and-mouth disease virus in monolayer cultures of calf thyroid cells. *Nature* **210**, 1097.

Stuber D, Matile H, Garolta G (1990): System for high level production in *E. coli* and rapid purification of recombinant proteins: Application to epitope mapping, preparation of antibodies and structure function analysis. In Lefkovits I, Pernis B (Eds): *Immunological Methods.* Vol. 4. Booknews Inc., Portland, p. 121.

Studier FW, Rosenberg AH, Dunn JJ, Dubendorf JW (1990): Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol.* **185**, 60.

Suryanarayana VVS, Bhanumathi N, Reddy GR (1991): The hypervariable portion of VP1 gene of Foot and Mouth Disease virus amplified through RT/PCR as a tool for the detection of type specificity of virus. *International Symposium on Virology in Tropics*, Lucknow, India.

Suryanarayana VVS, Venkataramanan R, Rao BU Padayatty JD (1992): Characterisation and immune response of a protein produced by a cDNA clone of foot and mouth disease virus, Type Asia 1 63/72. *Biochem. Int.* **26**, 1003.

Tabor S, Richardson CC (1985): A bacteriophage T7 RNA Polymerase/promoter system for controlled exclusive expression of specific genes. *Proc. Natl. Acad. Sci. USA* **84**, 4767.

Tulasiram P (1994): Molecular basis of antigenic variation in foot-and-mouth disease virus type Asia-I. *M.V.Sc. Thesis*, Indian Veterinary Research Institute, Izatnagar.

Tyagi M (1993): Characterisation of immunogenic protein gene of foot-and-mouth disease virus type Asia-I 63/72. *M.Sc. Thesis*, Indian Veterinary Research Institute, Izatnagar.

Von Heijne G (1990): The signal peptide. *J. Membr. Biol.* **115**, 195.