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Research Article

GENE CLONING, EXPRESSION AND HOMOLOGY MODELING OF E6 GENE FROM HUMAN PAPILLOMAVIRUS 16

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ABSTRACT

These high risk HPV types, usually HPV-16 can be found in over 80% of cervical carcinomas. E6 is a significant toxin secreted by HPV-16, which contributes pathogenicity of to women. The complete ORF of E6 gene (2482 bp) was amplified using PCR. It was cloned in TA and sub-cloned in pET28a vector then transformed into Escherichia coli BL21(DE3) codon plus RP cells expressed by the induction with 1.0 mM of IPTG. The expected size of expressed protein was 68.0 kDa estimated by migration in 12% SDS–PAGE. Anti-His monoclonal antibodies were used to sub- stantiate the recombinant protein by Western blotting. The percent similarity between E6 of HPV-16 with other HPVs E6 toxins revealed that the E6 sequence varied from 99.35 to 50.40%. Homology modeling was used to construct 3-D structure of E6 of HPV-16 with the known crystal 3-D structure (PDB: 1PRE). This protein can be used for immunoassays and it is suitable for vaccine candidate against HPV-16 infection.

INTRODUCTION

The HPV E6 proteins are small polypeptides of approximately 2500 nucleotide sequence and contain two zinc-finger motifs (Barbosa *et al*., 1989), whose integrity is essential for E6 function (Sherman and Schlegel, 1996). The first indirect evidence that E6 was a viral oncoprotein came from studies on cervical tumours and derived cell lines, where E6 was found to be retained and expressed many years after the initial transforming events (Banks *et al*., 1987). Although E6 may dimerize at high salt and protein concentrations, it is thought to be monomeric at physiologic conditions (Lipari *et al*., 2001). It has been difficult to study the expression of endogenous E6 proteins as they are expressed at low levels and few sensitive antibodies exist, however E6 is thought to be largely nuclear, though some fraction of E6 may also be cytoplasmic (Lowy and Howley, 2001). No enzymatic activities have been reported for E6, and although the HR E6 proteins have been reported to bind specifically to four-way DNA junctions (Ristriani *et al*., 2001), most of the activities of E6 are thought to be mediated by protein–protein interactions.The first protein that was shown to interact with E6 was E6 associated protein (E6AP), E6AP is the founding member of the HECT-domain family of ubiquitin ligases, a group of related proteins with homology to E6AP C-terminal (HECT) domain involved in ubiquitination of bound substrates, and divergent N-termini that mediate substrate specificity (Schwarz *et al*., 1998). E6AP forms a complex with both E6 and target proteins leading to ubiquitination of the target protein and subsequent proteasome mediated degradation (Scheffner *et al*., 1993).

Bacterially expressed fusion proteins and synthetic peptides, however, are not ideal target antigens for serological surveys, because they are often highly insoluble and can present only linear epitopes. It has been established from studies on bovine papillomaviruses that antibodies generated against denatured capsid proteins are broadly cross-reactive with a wide range of papillomavirus types, whereas antibodies raised against intact virus particles tend to be type-specific (Lim *et al*., 1990). Similarly, where bacterial fusion proteins or synthetic peptides have been used to generate antibodies against HPV coat proteins, broadly cross-reactive antisera are obtained (Strike *et al*., 1989; Christensen *et al*,, 1990). This factor has been shown to have practical importance in serological surveys. Strike *et al.* (1990) and Bonnez *et al.* (1990) have found that Western blots using HPV-6b capsid antigen fusion

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proteins cannot discriminate serologically between condylomata acuminata patients and normal controls. However, an ELISA-based method using whole particles of the closely related HPV-11 is able to do so (Bonnez *et al.*, 1991). The use of immobilized bacterial fusion protein antigens might restrict assay sensitivity by not identifying antibodies targeted to conformational epitopes. At the same time, specificity might be reduced by the exposure of potentially cross-reactive epitopes which normally would be hidden. It is not yet known whether these considerations apply to the early proteins of HPV. However, these reports would seem to indicate that the true serological picture might better be revealed using assays which present target antigens in more realistic conformational states. This requires the synthesis of antigens at high levels in a soluble and correctly folded form. To generate improved HPV serological target antigens that fulfil these criteria, we have employed a eukaryotic expression system based on an insect baculovirus (Summers & Smith, 2006). We describe here the construction and characterization of a recombinant baculovirus vector expressing the HPV-16 E6 ORF. The baculovirus-derived E6 protein displayed more authentic antigenic characteristics. The aim of the present study is to clone, express and purify the E6 protein of HPV16 to detect the presence of anti-E6 antibodies in human.

MATERIALS AND METHODS

Designing and synthesis of E6 primers

The E6 gene sequences of *HPV-16* and other viruses were retrieved from the National Centre for Biotechnology Information (www.ncbi.nlm.nih.gov/). These sequences were aligned in ClustalX (Thompson *et al.*, 1997) and verified the conserved region. These regions were selected for designing E6 specific primers with the help of Oligo 4.0, Gene runner and DNAStar software. The restriction site was incorporated in some primers and it has helped for cloning and sub-cloning of E6 gene in the vector. All these designed primers were chemically synthesized from Integrated DNA Technology (IDT, USA)

The E6 specific primers of *HPV-16* used in this study

Primers	Restriction site	Nucleotide sequences
16E6F		5` GAGAACCTCAGTCTGACGACATTG`3
16E6R		5` GCCAAAGGATTTGGCATGCTGTA` 3
F1	NdeI	5` catatg AGCCATCACGTATGCCAAGGATGGC` 3
R1	BamHI	5` ggatcc TCAGTCTGACGACATGAAGCTAGCG` 3

Note: Lower alphabets of nucleotide indicate the restriction site in primers.

Genomic DNA isolation and PCR amplification

The HPV-16 were grown on developed cell line and collected to extract the genomic DNA by previously described methods with slightly modifications (Hiney *et al.*, 1992). The PCR reaction mixture (50 µl) consist of 10 ng of genomic DNA, 2.5 units of Taq DNA polymerase, 5 µl of 10X PCR amplification buffer (100 mM Tris-HCl, 15 mM $MgCl_2$, 500 mM KCl pH 8.3), 200 µM dNTP and 10 pmoles of each primer. Amplification included initial denaturation at 94^0C for 3 minutes, followed by 30 cycles of denaturation at 94^0C for 30 seconds, annealing of primers at 52^0C for 30 seconds and extension at 72^0C for 30 seconds. A final extension at 72^0C for 10 minutes was used. 10µl of the reaction mixture was then analyzed by submarine gel electrophoresis in 1.0% agarose with 10µg/ml of ethidium bromide at 8V/cm. The PCR products were visualized under Gel documentation system.

Construction and identification of E6 gene

The amplified product was purified (QIAGEN gel extraction kit) from agarose gel and the purified product was ligated in pTZ57R-T cloning vector using T4 DNA ligase. This construct (pTZ57R-T-e6) was transformed into competent *E. coli* DH5α cells prepared by the $CaCl_2$ method (Sambrook *et al.*, 1989). Ampicillin antibiotic was used as a selection marker and X-gal (20mg/ml) as well as IPTG (100 mM) were used to screen the recombinant E6 clones. The recombinant E6 plasmid was isolated from the *E. coli.* These constructs were verified with PCR amplification and double enzyme digestion. The recombinant plasmid and pET28a expression plasmid were simultaneously digested with NdeI and BamHI restriction enzymes. The E6 was again ligated in pET28a expression vector with T4 DNA ligase and transformed into *E. coli* BL21(DE3) codon plusRP competent cells. The recombinant E6 gene was identified and characterized by restriction digestion with NdeI/BamHI, PCR amplification and DNA sequencing. The strategy for E6gene construct preparation with vector was performed.

Expression and characterization of E6

A single colony of *E. coli* BL21(DE3) codon plusRP positive clone having the (pET28a-e6) plasmid was inoculated into 5 ml Luria Bertani (10 g/litre tryptone, 5 g/litre yeast extract, 10 g/litre NaCl) broth containing 30 µg/ml kanamycin and 50 µg/ml chloramphenicol antibiotics. The culture was incubated at 37^0C overnight on a shaker incubator (200 RPM). The next day, 200 µl of overnight grown culture was inoculated into flasks each containing 100 ml of LB broth and again incubated until absorbance (A_{600}) of culture reached up to 0.6. Bacterial culture was induced by different concentrations of IPTG (0.25 mM to 1.0 mM) and the culture harvested at every hour up to 16 hours. Similarly, the *E. coli* BL21(DE3) codon plusRP containing pET28a(+) was induced up to 16 hours as a negative control.

The 1.5 ml bacterial cultures were centrifuged at 13,000 X g for 10 min at 4^0C. The bacteria were homogenized in 80 µl phosphate buffer saline (PBS pH7.2) and 20 µl of 5 X SDS sample loading buffer (0.225 M Tris·Cl, pH 6.8; 50% glycerol; 5% SDS; 0.05% bromophenol blue; 0.25 M DTT) was added, mixed properly boiled at 90^0C for 5 min and centrifuged at 13,000 X g for 10 min. The 10 µl lysates were loaded directly onto 12% SDS-PAGE following the procedure described earlier (Laemmli, 1970; Sambrook *et al.*, 1989). The proteins bands were visualized by staining with commassie brilliant blue R250 dye.

Protein purification under denaturing conditions

The expression of recombinant E6 in *E. coli* can lead to the formation of an insoluble inclusion body. Strong denaturants such as 8M urea or 6M guanidine-HCl were used to solubilize

the inclusion bodies. The induced *E. coli* cells pellet was thawed for 15 minutes on ice and resuspended in buffer B (100 mM NaH_2PO_4, 10 mM Tris Cl, 8 M urea pH 8.0) at 5 ml per gram weight. Cells were stirred for 60 minutes at 25^0C and the lysate centrifuged at 10,000 X g for 30 minutes at room temperature and the cellular debris discarded 1 ml of the 50% Ni-NTA slurry was added to 4 ml protein lysate and mixed gently by shaking 200 rpm for 60 minutes at room temperature. The lysate-resin mixture was loaded carefully in an empty chromatography column (Genei, Bangalore). The bottom cap was removed from column and the protein flow-through collected. The column was washed with 4 ml buffer C (100 mM NaH_2PO_4, 10 mM Tris·Cl, 8 M urea, pH 6.3) and the recombinant E6 eluted 4 times with 0.5 ml buffer D (100 mM NaH_2PO_4, 10 mM Tris Cl, 8 M urea pH 5.9), followed by 4 times with 0.5 ml buffer E (100 mM NaH_2PO_4, 10 mM Tris·Cl, 8 M urea, pH 4.5). These fractions were analyzed by 12% SDS-PAGE and visualized by staining with Commassie brilliant blue R250 dye.

Western blot analysis

The proteins separated by SDS-PAGE were transferred onto nitrocellulose membrane (Millipore) by Amersham Pharmacia Western blotting system following the procedure described (Sambrook *et al*., 1989). The membrane was checked by transferring protein onto a membrane with 1X Ponseau S solution. The membrane was properly washed with deionized distilled water and air dried. It was blocked by 3% bovine serum albumin (BSA) then dipped into 1:2000 BSA diluted Anti-His antibody (QIAGEN, Germany). The horseradish peroxidase (HRP) conjugated rabbit antimouse antibody was diluted 1:4000 and used as the secondary antibody. The membrane was washed 4 times and detected by 3, 3', 5, 5'-tetrametylbenzidine (TMB) membrane peroxidase substrate (KPL) until dark purple bands appeared. The reaction was stopped using distilled water and membrane was air-dried.

2.7. Homology modeling and validation of the 3-D structure

The protein sequence of E6 of *HPV-16* was searched for 3-D homology with the protein data bank (PDB: www.rcsb.org/pdb/home/home.do) BLASTP. High score and minimum E-value were considered. The E6 of *HPV-16* resembled the 3-D crystal structure of preE6 on 2.8 Å resolutions (1PRE) of *HPV-16*. The comparative homology modeling was used to generate the 3-D structure of E6 through Modeller (Sali and Blundell, 1993). The 3-D structure of protein was visualized in Pymol program (Delano and Palo Alto, 2002). The method implied for the evaluation of the 3D model using the parameters such as minimum model score and dope score of model and template. The 3-D structure was evaluated and validated with the help of PROCHECK, favoured amino acids residue in Ramachandran plot, accuracy and G- factor (Laskowski *et al*., 1993).

RESULTS

PCR amplification and cloning of E6 gene

The new primers were used to target the full length amplification of E6 gene by PCR in *HPV-16*. The amplification of E6 gene (2482 bp) was obtained at 52^0C annealing temperature of primers with 1.5 mM $MgCl_2$ (**Fig. 1**). The two bands such as 2494 bp of E6 and 2.8 kb of plasmid were observed on the agarose gel (**Fig. 2**).

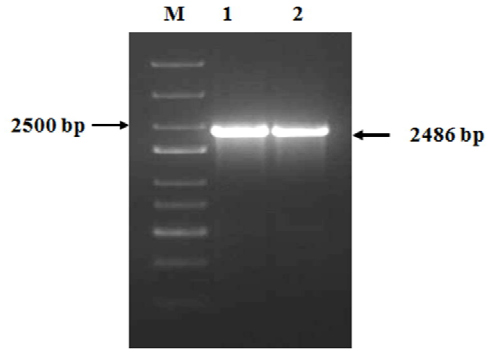


Fig.1: PCR amplification of e6 gene (2486 bp) of HPV-16. Lane M: expressed DNA ladder (Fermentas); Lane 1, HPV purified sample-1 and Lane 2: HPV purified sample-2

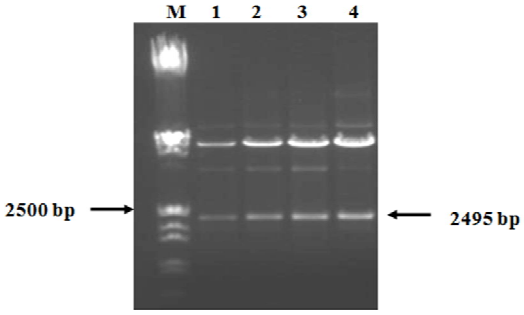


Fig. 2: Double restriction digestion (NdeI and BamHI) of different e6 clones of HPV in pTZ57RT cloning vector. Lane M: Lambda EcoRI/HindIII digested DNA marker; Lane 1-2: Clones containing e6 gene; Lane 3-4; Clones containing e6 gene

E6 gene and verified from the existing database. The E6 gene of *HPV-16* showed homology with the E6 gene available in the NCBI Genbank. The G+C content of full length E6 gene was 56.27%. The E6 encoded the 865 amino acids that were rich with glycine (9.35%) and serine (8.33%) amino acids in the peptide chain. The cysteine present in 0.81% could help sulphide bond formation in the tertiary structure of E6. Histidine and methionine presented in E6 were 1.22% and 1.42%, respectively. The theoretical isoelectric point of E6 was 5.74 that could provide the stability of structure. The percentage identity and positivity of E6 of HPV-16 with other E6 that was present in other *HPV*.

Induction and characterization of recombinant E6

The pET28a-aer clones were successfully constructed and used for expression in *E. coli* BL21 (DE3) codon plus RP cells. Constructs were confirmed by digestion with NdeI and BamHI restriction enzymes.

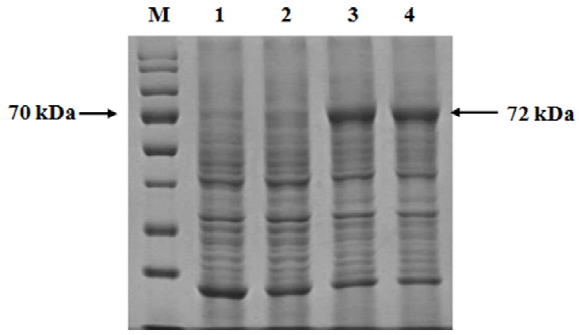


Fig. 3: SDS-PAGE for expression of recombinant e6 in *E. coli*. Lane M: Pre-stain protein marker; Lane 1: BL21(DE3) codon plus RP cells; Lane 2: BL21(DE3) codon plus RP cells with pET28a-e6 Uninduced); Lane 3: BL21(DE3) codon plus RP cells with pET28a-e6 (induced by 0.25mM IPTG) and Lane 4: BL21(DE3) codon plus RP cells with pET28a-e6 (induced by 1.0mM IPTG).

Two bands, one of E6 (2494 bp) and the other pET28a expression (5200 bp) were obtained (**Fig. 2**). The optimum expression of E6 was found using 0.5 mM concentration of IPTG at 25^0C within the 16 hours incubation of culture. The characterization of recombinant E6 was observed on 12% SDS-PAGE and found the 72 kDa of protein band in the induced lysate (**Fig. 3**).

Protein purification and Western blot

The expression of E6 was not seen in the native condition. Hundred ml of induced and uninduced cells were dissolved in 8M urea solution. The lysate was directly used for purification through Ni-NTA affinity chromatography. The monomer of E6 was eluted in the buffer E (pH 4.5). Characterization of purified E6 was on 12% SDS-PAGE and the 72 kDa recombinant protein band was obtained (**Fig. 4**). The Western blot analysis showed the 72 kDa band on the nitrocellulose membrane (**Fig. 5**).

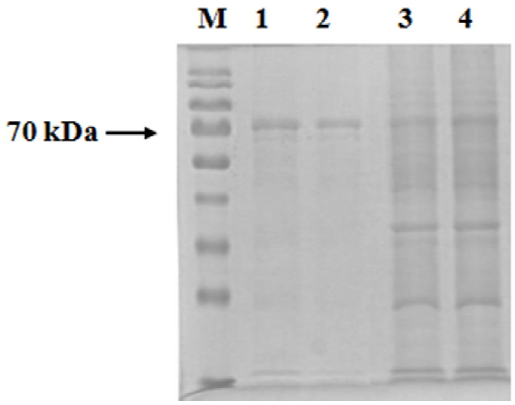
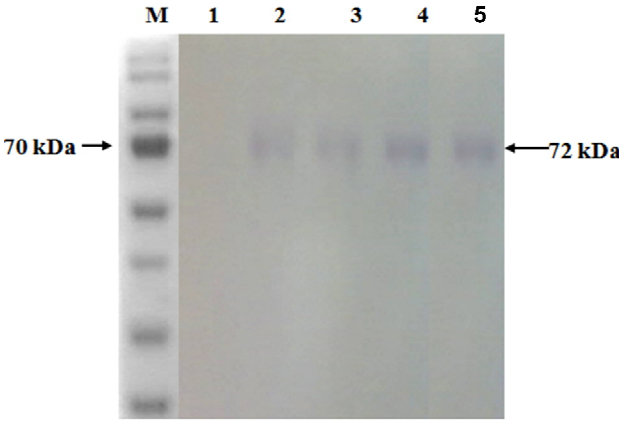


Fig. 4: Ni-NTA affinity chromatography used for purification recombinant e6. Lane M: Pre-stain protein marker; Lane 1-2: purified e6; Lane 3-4: flow through.



Analysis of the 3-D structure of E6

The complete ORF of E6 in *HPV-16* encodes 866 amino acids; the 3-D structure shows the homology with 1PRE in PDB. The amino acid sequence of E6 was 80% identical. The positives of amino acid of E6 were similar by 82%. A total of six α-helix and 13 β-sheets were observed in the 3-D structure of E6 as indicated in the toxin rich in β-sheet. The 3-D structure of E6 of *HPV-16* was given (**Fig. 6**); and evaluated on the basis of minimum free energy -48139.53 kcal/mol. The free energy of template (1PRE) 3-D structure was -47407.34 kcal/mol.

Fig. 6 Three-dimensional structure of e6 of *HPV-16.*

The free energy of E6 resembled 1PRE indicating the favored quality of structure. The validation of the structure was done on the basis of spatial arrangement of amino acids residue in the most favoured region of Ramachandran plot. The torsion angles of 90.9% amino acid residues found in most favoured region and only 0.7% amino acid residues found in the disallowed region. The overall G-factors were 2.2 at resolution of 2.80 Å.

DISCUSSION

During HPV infection E6 plays multiple roles, interfering with several cellular pathways in order to create a favorable environment for viral replication, and neutralizing the cellular surveillance controls that are turned on as the infected cell is unnaturally forced to restart DNA replication.

In the present study we found that both optimized Western blotting and bE6 RIPA could be used in the identification of anti-E6 serum antibodies. Soluble native HPV-16 E6 protein produced using a baculovirus vector was readily recognized by antibodies present in some cervical carcinoma patients' sera, suggesting that the baculovirus- derived E6 protein closely resembles the original immunogen, i.e. E6 protein encountered during the course of a natural infection.

The PCR product size (2482 bp) was observed at an optimal concentration of 1.5 mM $MgCl_2$ and primer annealing at 52^0C (**Fig. 1**). The primer targeting the complete ORF amplified the E6 gene of HPV-16. The size of the PCR product was 2458 bp excluding the signal peptide sequence region. The E6 gene was cloned in pET32a vector (Zhu *et al*., 2007). The full length ORF was amplified and cloned into the pGEM-T vector encoding for E6 protein in HPV-16. It has been verified by DNA sequencing which showed similar homology (Xia *et al*., 2004).

The new E6 gene was cloned, sequenced and overexpressed in *E. coli*. The E6 gene exhibited 96% identity with its homolog found in a recently annotated genome sequence of an environmental isolate, namely the type strain ATCC® 45113D of *HPV-16*. The E6 gene did not exhibit any homology with other known E7 & E6 genes detected in *HPV-16* (Erova *et al*., 2007).

E6 has been expressed in *E. coli* under the control of the bacteriophage T7 promoter. The coding region of the E6 gene of *HPV-16* was cloned and expressed in *E. coli* BL21 cells, excluding the signal peptide. The size of E6 was 72 kDa on SDS- PAGE and Western blot analysis. The Western blot analysis demonstrated good antigenicity (Zhu, *et al*., 2007). However, in the present study, a high level of expression of E6 gene was found at 1.0 mM IPTG at 25^0C. The 72 kDa size of the recombinant expressed protein was seen on 12% SDS-PAGE (**Fig. 4**). The monomer of the E6 protein was eluted in buffer E (pH 4.5). The eluted fraction of E6, uninduced and induced protein was used for the characterization by Western blotting. The Western blot analysis showed the 72-kDa band on a nitrocellulose membrane (**Fig. 5**). This indicates that E6 may be major virulence factor of *HPV-16*. In this study, we expressed E6 gene and purified the protein by using dialysis membrane and centriplus column (Zhu *et al*., 2007).

The 3-D structure of E6 of *HPV-16* fulfilled the basis of energy and most amino acid resides present within the most favoured region in Ramachandran plot. The stereo chemical quality of theE6 was favoured due to fewer amino acid residues lying within the disallowed region. Since, the studied E6 model has only 0.7% amino acid residues in the disallowed region, this demonstrates that this structure satisfies the parameters of a good model. The various steps that allow E6 to form a pore in the plasma membrane of a target eukaryotic cell have now been identified: secretion, activation, receptor binding, heptamerization and membrane insertion, but the mechanism is not fully understood. Since, solving the pro-E6 structure (Parker *et al*., 1994), the structure function relationship has considerably increased. However, pro-E6 is composed of 470 amino acids. Homology searches with other proteins in the database reveal only two other protein alpha-toxins from *C. septicum* (Ballard *et al*., 1995) and a plant enterolobin (Sousa *et al*., 1994). The crystal structure of proE6 reveals an L-shaped molecule which can be divided into a small N-terminal globular domain (Domain I) and a long elongated domain. E6 is rich in beta-sheet but contains a significant amount of helical structure.

The β-ketoacyl acyl carrier protein synthase (KAS) III plays an important role in the initiation of fatty acid in *Enterococcus faecalis*. The homology modeling method has been used to generate a 3-D structure of the KASIII protein. The generation of 3-D structure was done using Modeller and validated its structure using PROCHECK. The identification of active site residue in this protein has been targeted with two viral drugs (Jeong *et al*., 2007**).** Multidrug resistance protein 1 (MRR1/ABCC1) is a 190 kDa member of the ATP binding cassette (ABC) superfamily of transmembrane protein. The molecular modeling method has been reported to generate 3-D structure of protein by Modeller and validation of 3D structure by PROCHECK and WHAT IF tools (De Gorter *et al*., 2008**)**. Modeller6v2 has been used to generate the 3-D structure of NAD+ dependent DNA ligase of *Mycobacterium tuberculosis* and validated the 3D structure by PROCHECK. This study reports several compounds that could block the pathway of the ligase synthesis in the *M. tuberculosis* (Srivastava *et al*., 2005).

In conclusion, the present study reports the cloning, expression, purification and molecular modeling of E6 of *HPV-16*. The recombinant E6 of *HPV-16* could be produced and purified in adequate amounts in the future and used as an antigen in developing a convenient and economical diagnostic method. It will reduce the cost, transport and reproducibility problems associated with the present diagnostic tests which require growth and purification. It can be used as diagnosis of *HPV-16* infection in women to control and prevent the spreading and mortality. E6 can be used as a future vaccine candidate against HPV-16. The 3-D structure of E6 of *HPV-16* may help to understand the nature for potential drug targets against the active amino acid residue.

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