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# Unveiling the Molecular Landscape of Bovine Papillomavirus E6 Gene in Iraqi Cattle: Implications for Genetic Variations, 3D Structure, and Immunogenic Epitopes

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***Abstract*** The E6 gene is encodes a minor oncoprotein that highly expressed in PV-infected host. Antibodies targeting E6 may have the potential to eliminate PV infection. This study focused on molecular and bioinformatics analysis of the E6 gene, encompassing identification of genetic variations, 3D structure prediction, and epitope prediction. 50 tumor samples were collected from cattle with papilloma like lesions from Al-Qadisiyah, province, Iraq. Samples submitted to PCR based on amplifying the complete E6 gene. Various bioinformatics tools were utilized to analyze physicochemical properties, tertiary structures of deduced protein, and predict immunodominant epitopes for B and T cells. 42 out of 50 (84%) cattle were found to be infected with BPV. Sequence analysis of ten samples identified BPV-1 as the predominant type in the region. Phylogenetic analysis revealed a close genetic relationship with Deltapapillomavirus 4. Genetic analysis identified seven single nucleotide polymorphisms (SNPs), including four synonymous mutations at nucleotides residues of 48,303, 210, 345, and three non-synonymous mutations (His38/Arg, Lys99/Gln, and Asp126/Asn). The E6 protein was found to contain at least four continuous B-cell epitopes and four T-cell epitopes, all of which exhibited strong immunogenicity and high sequence conservation. The mutations are located within loop regions of the predicted structure. This study provides valuable insights into the molecular characteristics of the E6 gene of BPV circulating in Al-Qadisiyah, Iraq. The predicted B-cell and T-cell epitopes characterized by high immunogenicity and sequence conservation, offer promising targets for the development of diagnostic tests and potential vaccine candidates.

**Keywords**: Bovine papillomavirus, Epitopes, in silica analysis, Iraq, Mutation

## *Introduction*

Bovine papillomaviruses (BPVs) encompass approximately 44 distinct genotypes organized into five genera. Among these, BPV-1 stands out as a high-risk type and most prevalent globally in cattle populations (1, 2). BPV-1 infection can lead to the development of hyperproliferative tumors, carrying the potential for cancer transformation (3). This carcinogenic process is primarily driven by the actions of nonstructural viral oncoproteins like E5, E6, and E7, in conjunction with other contributing factors. Additionally, BPVs constructed a highly significant threat not only to cattle, but to wide host range, including horses, sheep, cats, and numerous wild ungulates (4,5,6,7). This broad infection spectrum results in substantial economic losses, as treatment cost and infected animals culling to prevent disease spread within herds (8).

Several factors make BPV difficult disease to control, these including the existence of different genotypes with varying degrees of carcinogenic potential (9,10), latency nature of virus and the high transmissibility throughout animal papulations (11, 12). This complexity hinders the development of effective control measures that can address all aspects of the disease. Interestingly, the classical treatment approaches, such as surgery and chemotherapy, are often costly and limited therapeutic efficacy (13, 14). While autogenous vaccines are currently the primary preventive measure, they not without limitations (15). These include the risk of viral reversion or recombination, risk of blood-borne pathogen transmission, and the requirement for substantial

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wart tissue amounts for vaccine preparation (14. 16). To address these limitations, innovative vaccine approaches utilizing subunits viral partitions, like proteins, mRNA, or plasmid DNA are being explored. Genetically engineered viruses expressing full-length viral antigens have shown promise in vaccine development, with their effectiveness evaluated through both humoral and cellular immune responses (17). The advancements in bioinformatics and virtual molecular biology tools have significantly improved vaccines designing and refinement.

BPV produces a variety of proteins, some of which can stimulate the immune system, The E6 gene, present in most BPV types (18). The E6 protein approximately 135 amino acids contain a two spaced zinc-like fingers joined by an interdomain linker (19). In spite of, the E6 targets a number of cellular proteins Like p53, MAGI-1, and the pro-apoptotic Bak proteins to prevent cellular apoptosis (20,21), it may have a limited role in advanced cancer development, it elevated centrosome copy number and eliminated detectable p53 protein but did not produce neoplasia or cancer (22; 23). In addition, obviously cancer risk from BPV appears significantly elevated in tissues with a transforming activity, like the cervix, anus or nailbed, then in those without. (24). E6 oncoprotein is a frequent target for HPV therapeutic vaccines due to several key factors, E6 is primarily expressed in precancerous and cancerous lesions, hence, that minimizing the risk of targeting healthy tissues, it is crucial for preventing programing cell death (apoptosis) and maintaining the transformed of infected cells, often in corporation with E7 oncoprotein (25). E6 elicits a robust immune response, with no documented cases of immune escape or immune tolerance. and the immune response against E6 has been well-characterized in both preclinical and clinical studies (26, 27). So, these characteristics make E6 an attractive target for developing effective prophylactic and therapeutic vaccines against PV-related infections (28). The research aims to comprehensively characterize the E6 gene from Iraqi cattle samples. throughout, preliminary study of molecular characterization of the E6 gene, in silico prediction of the E6 protein's 3D structure, predict of its immunological properties. All that are important to trigger an immune response. The findings from this study will be instrumental in informing the design and execution of in vitro and in vivo experiments to evaluate the E6 protein's suitability as a target for immunotherapy.

## Materials and methods

### Ethical Statement

Animal experiments were performed in accordance were in line with the ethical standards of College of Veterinary Medicine/University of Al-Qadisiyah, Iraq under 1890 on August 28, 2023, which complies with all relevant Iraqi rules and regulations

### Clinical presentation and samples collection

An investigated study conducted to collect 50 skin samples from 50 papillomatosis suspected cows of different ages and sex were performed at different areas of three provinces from Iraq are Babylon, Wasit, and Diwaniyah. Study extended from during august 2023 to July 2024. In sampling errand, Morphology was used to identify papilloma-like warts (plane or cauliflower). The samples were transported in a cooler box, and upon reception at the laboratory, the hair was removed, and the samples were washed with sterile phosphate-buffered saline (PBS) and stored at −80°C until used.

### DNA extraction and molecular detection

Tumor samples were grinded with liquid nitrogen. Two gram of grinding tumor was used for DNA extraction. DNA extraction was performed using the gSYNC™ viral DNA/RNA Extraction Kit (Geneaid, Taiwan) according to the manufacturer's instructions. The extracted DNA was quantified and stored at -20°C until further use. PCR reaction was conducted with specific primer was designed for E6 forward primer, 5’- tgacaGGATCCgaccccggttttcaccatggacctgaaacc-3’; and E6 reverse primer, 5’- aggtcTCGAGaatcatccaagtttctatgg -3’. duple underlined letters indicate BamHI and XhoI restriction sites and forward primer was optimized to adding kozak consensus sequence (underlined sequence) by inserted (ACC) used APE- A plasmid editor software (APE- A plasmid editor v3.1.3) at forward and reverse primers respectively. The primers were designed based on complete genomes sequences of BPV deposited in GenBank (accession numbers X02346, NC_001522, AB626705, and JX678969).

The PCR reactions were carried out using a 2X PCR MasterMix kit (ABM, Canada) in a 50 µl reaction volume, consisting of 25 µl 2X PCR MasterMix, 1.5 µl of each primer (10 pmol), 5 µl of DNA template, and 17 µl of nuclease-free water. The reaction condition was started with initial denaturation step at 95°C for 5 min., followed by 35 cycles of denaturation at 95°C

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for 30 sec., annealing at 55°C 30 sec., extension at 72°C for 90 sec., and a final extension at 72°C for 7 min. The PCR products were electrophoresed using a 1% ethidium bromide-stained agarose gel, and then visualized with UV light.

**Preparation and cloning of E6 gene**

Amplicons of 10 PCR product were cleaned up used DNA PCR Purification Kit (BioRad, USA), and then E6 gene were cloned in pcDNA3.1-E6 vector (purchased from Gene script company, USA) used T4 DNA ligase (Invitrogen, USA) for the ligation reaction (then called: pcDNA3.1-E6) (Figure 1). The vectors were transformed by heat shock method into E. coli BL31 (DE3) competent bacterial cells according to (29). The grown positive clones were selected from LB agar plates supplemented with 100 µg/ml Kanamycin. Touch clone PCR was performed to chick inserts presence. Then pcDNA3.1-E6 vectors were extracted from overnight grown broths with Mini Prep Plasmid Extraction Kit (Geneaid, Taiwan) according to the manufacturer's recommendations. Vectors were digested with BamHI and XhoI to confirm E6 insert presence.

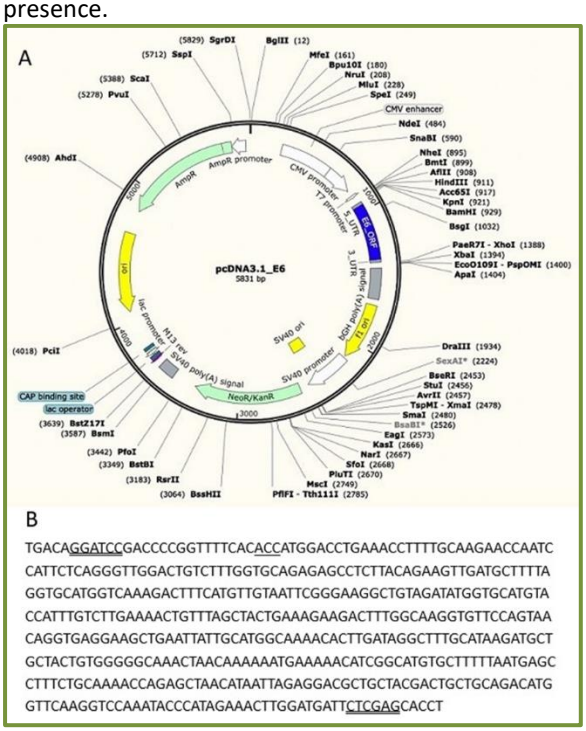


**Figure 1**: showed, A DNA vaccine construct pcDNA3.1_E6 gene. full length, Bovine codon optimized BPV-1 E6 oncogenic gene cloned into expression vector pcDNA3.1 with significant vector features indicated. B: Full length E6 gene sequence flanked with cloning primers. the duple underlined letters indicate BamHI and XhoI restriction sites in forward and reverse primers respectively. forward primer was optimized to adding kozak consensus sequence (underlined sequence) by inserted (ACC) sequence.

**Sequences acquisition and phylogenetic analysis**

The positive insertion plasmids (pcDNA3.1-E6) were submitted to bi-directional sequencing using the same primers employed for amplification, through the Sanger sequencing method (Macrogen, Seoul, Korea). The Mega XI program (Version 11.0.13) (30) was utilized for the characteristics of the complete E6 gene sequences including multiple sequence alignment, predictions of putative ORF, and translating to amino acids. Nucleotide similarity searches were conducted using BLAST analysis (http://blast.ncbi.nlm.nih.gov). Genetic distances were estimated using the maximum composite likelihood method (31). Phylogenetic reconstruction was performed using pooststrip method, integrated within the same software. the confidence values of internal branch nodes were assessed with A 1,000 bootstrap replicates. Notably, this analysis encompassed 20 complete E6 gene sequences related to BPV genotypes related to PV genera that were obtained from GenBank. Phylogenetic trees were constructed from the alignment of E6 sequences using the Neighbor-joining method. Lastly, the complete ten E6 sequences obtained from BPV Iraqi strain were deposited in GenBank, where they can be accessed under the accession numbers PP067762. to PP067771.

**In silico analysis of E6 protein**

The nucleotide of E6 gene sequences was translated to amino acid sequences by utilizing ExPASy Translate Tool (https://web.expasy.org/translate/) (32). The protein characteristics and the Physicochemical properties such as the number of amino acids, molecular weight, isoelectric points, and percentage of strongly basic, acidic, hydrophobic, polar amino acids, solubility, estimated half-time, instability index, and antigenicity were determined by ExPASy tool (https:// web.expasy.org/protparam) (33) tool. To predicate antigenicity default parameters were applied to the server, with a threshold of 0.5 used to identify the antigenic protein. allergenicity was evaluated to the proposed epitope for vaccine development, the web-based AllerCatPro (version 2.0) server was utilized (https://www.ddg-

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pharmfac.net/AllerTOP/data.html) (34). Protein solubility was assessed with Protein-Sol online server (https://protein-sol.manchester.ac.uk/) (35).

Prediction of 3D structure of E6 protein:

Secondary structure prediction of E6 protein was implemented by I-TASSER online server-based algorithms for generating high-quality model predictions of the 3D structure and function of protein molecules from E6 amino acid sequences (http://zhanglab.ccmb.med.umich.edu/I-TASSER/)(36). I-TASSER predicted a secondary structure from RCSB Protein Data Bank (RCSB PDB) based on Z-score. The highest Z-score of model hit was selected and then downloaded from RCSB PDB server under (ID: 3py7) (37). The E6 protein was manipulated, and the SNPs of study strains were drop-on E6 model using a ChemiraX server program (http://www.rbvi.ucsf.edu/chimerax). Following that, the quality of E6 the crystal structure was verified using a Ramachandran plot and Klash. The Ramachandran plot assesses the backbone conformation of protein structures by analyzing φ (Phi) and ψ (Psi) dihedral angles for each residue.

B cell and T cell epitopes identification:

B cell epitope prediction is performed to identify potential E6 protein capable of interacting with B lymphocytes and inducing an immune response. Two types of predicting B cell epitopes were identified. The linear and discontinuous B cell epitopes were employed in both the ABCpred server (http://www.imtech.res.in/raghava/abcpred/) (38). The predicted B cell epitopes were ranked based on it score obtained by the recurrent neural network using with 0.5 threshold value. The higher score of the peptide referred to the higher probability of being as epitope. and the Bepipred server (http://tools.immuneepitope.org/bcell/) (39). Epitopes that were common between these two prediction tools were selected. Additionally, for the determination of conformational (discontinuous) epitopes, the E6 protein sequences were submitted to both the CBTope server (http://www.imtech.res.in/raghava/cbtope/) (33) and the BepiPred 2.0 server (40). Common epitopes predicted by these two programs were considered. To further assess the antigenicity of the predicted B cell epitopes, we utilized the VaxiJen 2.0 server, accessible at http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html (41). Further, the predicted B cell epitopes were checked for toxicity, antigenicity, and allergenicity using the ToxinPred, VaxiJen v 2.0, and the AllerTOP v.2.0 servers respectively.

To prediction of cytotoxic T-Lymphocyte (CTL) Epitopes that binding to major histocompatibility complex (MHC) class I molecules. The NetMHCpan-4.1 server, accessible at (https://services.healthtech.dtu.dk/services/NetMHCpan-4.1/) was conducted with default settings and utilized the binding affinity of peptides was focused on cow MHC alleles. For predicting Major Histocompatibility Complex MHC class II (MHC class II) T cell epitopes, we utilized the IEDB server,http://tools.immuneepitope.org/mhcii/. The IEDB Recommended approach employed the Consensus method (41). To assess the antigenicity of the predicted T cell epitopes, we employed the Kolaskar and Tongaonkar antigenicity method (42), which is part of the IEDB analysis resource available at http://tools.immuneepitope.org/bcell/. The standard threshold value of 1.120 was used in this analysis.

Epitope conservancy analysis:

The conservation of predicted epitopes among different strains was assessed using the IEDB epitope conservancy analysis tool, accessible at http://tools.immuneepitope.org/tools/conservancy/iedb_input.

**Results**

**Molecular detection**

A total of fifty skin wart samples were collected from fifty cows from the provinces of Babylon, Wasit and Al-Qadisiyah provinces of Iraq. The cows were having normal temperature, respiration, and appetite. The majority of these wart lesions appeared in the abdomen, udder, neck, and face. It was around 1-5 cm in diameter, with grey and hyperkeratotic epidermis. According to PCR results showed 84% samples were positive for BPV, with E6 specific primers used and the band size was 449 bp that showing in (Figure 2), whereas 16 % of samples were given negative results.

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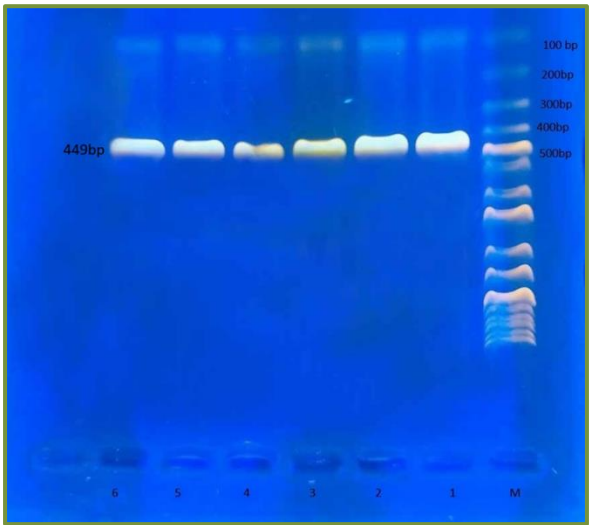


**Figure 2:** Bovine papillomaviruses-E6 gene specific primers were used to amplify whole length E6 gene (449bp). The amplicons were visualized on an 1% ethidium bromide impregnated 1% agarose.

Genetic diversity and phylogenetic analysis

Ten positive PCR of E6 gene send for sequence. A sequence analysis revealed that the E6 gene of the BPV identified in current study matched with Dulta-papillomavirus-4/BPV type 1, and have distance value of 0.021 – 1.768 with globally Deltapapillomavirus 4, and the high distance values were among our strains and others papillomavirus genera demonstrated in. E6 gene nucleotides analysis revealed distance value between 0.021with Deltapapillomavirus 4 and 1.768 with others globally papillomaviridae strains. In other hand, E6 gene appeared have 7 single nucleotides polymorphism consist of 4 synonymous mutations in 48, 210, 303, and 345 positions. And three non-synonymous mutations were reported are His38/Arg, Lys99/Gln, and Asp126/ Asn, as it is shown in (Table 1).

phylogenetic analysis was used to determine the evolutionary links between the current BPV-1 strain and other world strains. When all 10 sequence samples aligned with 20 strains of various papillomaviruses, which had been downloaded from GenBank, 12 primary clusters emerged with node robustness percentages equal to or exceeding 70%. The first cluster primarily consisted of BPV-1 types and included our 10 identified genotypes. Intriguingly, USA, Switzerland, Brazil and China (Figure 3) exclusively represented these genotypes.

**Amino acid sequence analysis, antigenicity and allergenicity of BPV-E6 protein**

Evaluation of amino acid sequence was next target. First, the results of all ten samples of E6 protein showed 100% homogeneity between them, where PP082031.1 BPV E6 sequence has been used as reprehensive current sequences. Second, the physiochemical properties of E6 protein were assessment by employed the ExPASy ProtParam tool and Compute pI/Mw-https://web.expasy.org/compute_pi/. E6 protein has molecular weight (15869.49 Da and 15698.24 Da for PP067771.1 and PP067766.1 respectively) and has 135 aa residues. The computed isoelectric point (pI) value was above 7 (8.75), which indicates the alkaline nature of final product and confirmed that the protein carries a positive charge at neutral pH Third, the half-life of the L1 protein was evaluated to be 30 hours in mammalian reticulocytes (in vitro), and more than 20 hours in yeast (in vivo), and more than10 hours in Escherichia coli (in vivo) for all ten samples. According to the instability index (45.72/ unstable for PP067771.1, whereas 38.55/ stable for and PP067766.1), the vaccine was classified as a stable protein. The aliphatic index was estimated as (70.44 and 67.01 for PP067771.1 and PP067766.1 respectively), The high aliphatic index indicate that the designed vaccine is stable in different temperature ranges. the estimated grand average hydropathy (GRAVY) values was (-0.471 and -0.317 for PP067771.1 and PP067766.1 respectively), the negative GRAVY value showing that the protein vaccine is hydrophilic and interacts well with the surrounding water molecules (Table 2). Finally, analysis of antigenicity and allergenicity for all proteins were conducted using the VaxiJen 2.0 server, available at http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html. The E6 protein was non-allergen, and the antigenicity value were 0.5468 and 0.5105 for PP067771.1 and PP067766.1 respectively. All these parameters of proteins were similar to E6 proteins sequences of reference strains (X02346.1 and M20219.1).

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**Table 1**: The nucleotides sequences alignment and amino acid characterization of E6 gene indicated 7 novel mutations, included 3 nonsynonymous SNP and 3 synonymous with locations represented with 2 amino acids changes in the major antigenicity regions compared to other available reference strain (R*).

| Strains (GenBank accession number) | Nucleotide Mutation positions | | | | | | | Amino acid positions | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Synonymous | | | | nonsynonymous | | | | | |
| | 48 | 303 | 210 | 345 | 113 | 295 | 376 | 38 | 99 | 126 |
| X02346.1 BPV 4 E6 (R)* | T | A | A | C | A | A | G | His | Lys | Asp |
| PP067762.1 (Iraq1) | C | G | C | T | G | C | G | Arg | Gln | Asp |
| PP067763.1 (Iraq2) | C | G | C | T | G | C | A | Arg | Gln | Asn |
| PP067764.1 (Iraq3) | C | G | C | T | G | C | A | Arg | Gln | Asn |
| PP067765.1 / Iraq4 | C | G | C | T | G | C | A | Arg | Gln | Asn |
| PP067766.1 / Iraq5 | C | G | C | T | G | C | A | Arg | Gln | Asn |
| PP067767.1 / Iraq6 | C | G | C | T | G | C | A | Arg | Gln | Asn |
| PP067768.1 / Iraq7 | C | G | C | T | G | C | A | Arg | Gln | Asn |
| PP067769.1 / Iraq8 | C | G | C | T | G | C | G | Arg | Gln | Asp |
| PP067770.1 /Iraq9 | C | G | C | T | G | C | G | Arg | Gln | Asp |
| PP067771.1 / Iraq10 | C | G | C | T | G | C | G | Arg | Gln | Asp |

*The amino acid characterization of E6 indicated novel mutations, some in the region of highest antigenicity compared to other available reference strain.

**Prediction of secondary, tertiary and refinement of the E6 protein**

To prediction the protein structure model of BPV-1 E6 Iraqi strain, the I-Tasser was employed for this purpose. The BPV-1 protein sequence was used as input and keep the default threshold values selected model of high Z-score when comparing with selected Model and confidence 0f 100%. Secondary structure of our E6 protein demonstrates in (Figure 4, A). The tertiary structure prediction, which resulted in the generation of distinct structural models' protein orthologs. the highest C-score model was selected as the best that used to next step. PDB model file (3py7) Bovine papillomavirus type 1 oncogenic protein PDB. SWISS-MODEL tool was used to modeling and refinement of subjected crystal structure. This refined homology model was then validated via a structure validation based on the Ramachandran plot of Chimera tool (Figure 4, B). Importantly, the Lys99/ Gln and Asp126/ Asp mutations located within the region (Loops structure) that may be associated with immunological activity.

The secondary structure of E6 protein was including 35 % alpha helix, 18% beta strand and loops. E6 protein is structure has 135 aa residues, the first ten and the last seven residues are not represented in the three-dimensional structure. It composed of two zinc-binding domains with a linker helix antigenic loop (Figure 5). 3D allowed the clear visualization of the amino-acid differences within BPV-1 protein from Iraqi strain compered to control reference strain. It is clear that the three mutations (His 38/ Arg, Lys99/ Gln and Asp126/ Asp) which were recorded in (Table 1), and which resulted in aa substitution, Lys99/ Gln and Asp126/ Asp located in loops while, one mutation His38/Arg located within alpha helix 1 which results to slightly affect the protein structure on a 3D level (Figure 6).

**Predicted B cell epitopes of E6 protein**

The B cell epitopes of E6 protein. Both B cell epitopes, discontinuous (linear) and continuous (conformational) types were predicted. the continuous B cell, out of 9 overlapping predicted linear B cell epitopes only 4 linear epitopes (LE1-LE4) within the E6 protein were selected based on high antigenic scores, nonallergenic, non-toxicity, and positive immunogenicity scores. The conservancy linear epitopes were assessed using the IEDB conservancy analysis tool, with the results presented in (Table 4-6). The conservation rates were 100% for

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all B cell epitopes, ensuring their preservation across all BPV-1 protein orthologues. However, we applied the linear B cell epitopes on the basis of the protein antigen's 3-D structure employed SWESS MODDEL. which makes it a more reliable method for B cell epitopes prediction visualize as it is shown in (Figure 7). The data analysis showed the His 38/ Arg, Lys99/ Gln and Asp126/ Asp residues were located out immune active epitopes.

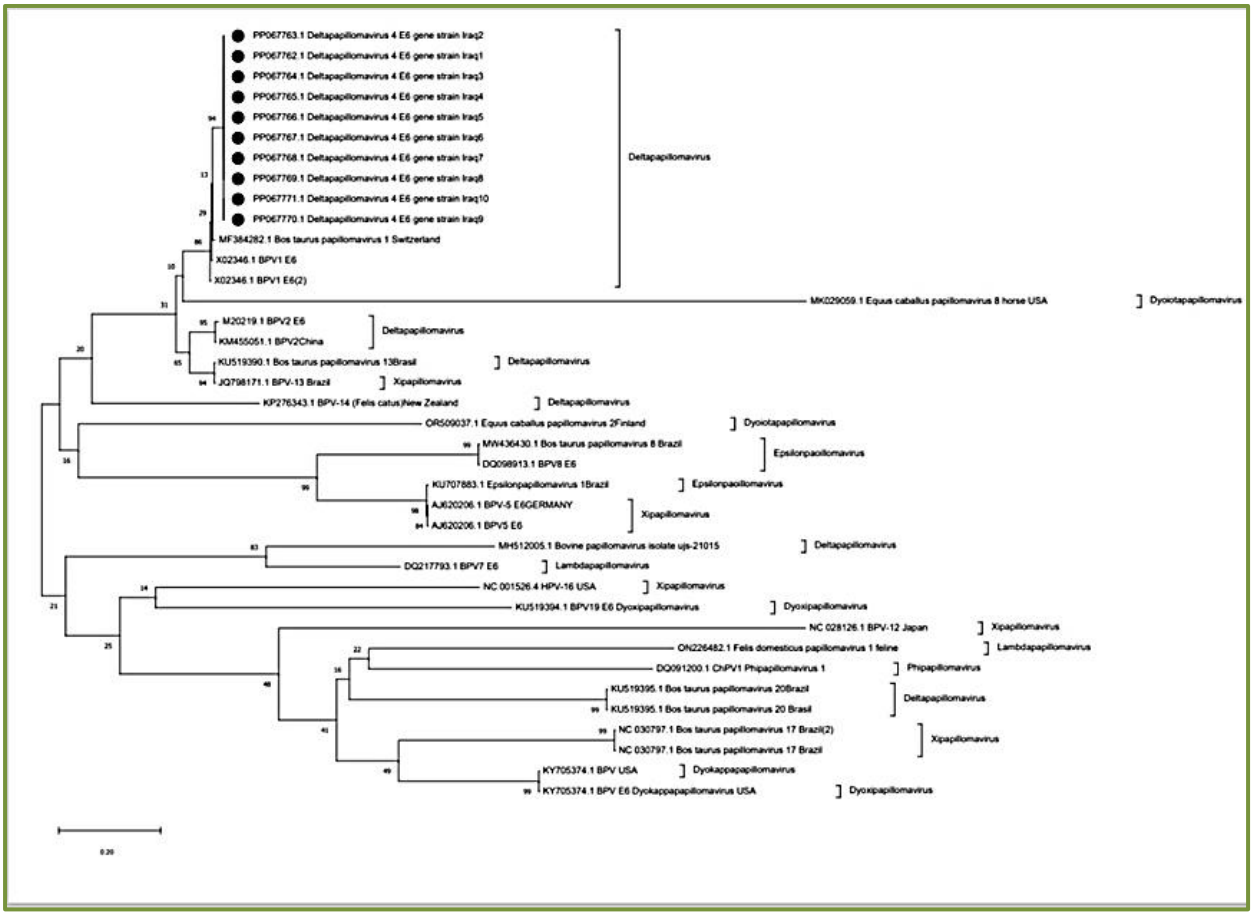


**Figure 3:** Phylogenetic tree of the Iraqi BPV-1 variants based on E6 gene. Phylogenetic study was constructed based on 10 whole length E6 gene sequences (indicted with light cycle) alignment with 20 sequences from different papillomavirus genera, which were constructed by the Maximum likelihood method and the Kimura 2-Parameter model by MEGA 11. Bootstrap proportions were calculated with 1000 replicates.

**Prediction of conformational B cell epitopes in the 3D structure of BPV-1 E6 protein**

conformational B-cell epitopes on E6 protein were identified by the Ellipro server (accessible via the link http://tools.iedb.org/ellipro/). The server predicts the discontinuous antibody epitopes on the basis of the protein antigen's 3-D structure which makes it a more reliable method for B cell epitopes prediction. The server associates each predicted epitope with a score, defined as a PI (protrusion index) value averaged over epitope residues. The discontinuous epitopes are defined based on the PI values and clustered based on the distance R between the residue's centers of mass. A total of 5 B-cell discontinuous epitopes were predicted, (DE1-DE8). The representations of the predicted discontinuous residues on L1 protein were shown in (Supplemental file 1). The Predicted residues were arranged according to the number of residues and their score which could a ranging from 0.59 to 0.817.

**Table 2**: BPV E6 proteins physiochemical properties.

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| Protein | PP067771.1 BPV E6 protein Iraq strain | PP067766.1 | X02346.1 BPV1 E6 protein Reference strain | M20219.1 BPV2 E6, protein Reference strain |
|---|---|---|---|---|
| **Molecular Weight** | 15869.49 | 15698.24 | 15850.48 | 16213.71 |
| **Theoretical pI** | 8.75 | 8.65 | 8.75 | 8.87 |
| **Half-Life** | >30 h. (MR, IV). >20 h. (YIV). >10 h. (E. coli, IV). * | >30 h. (MR, IV). >20 h. (YIV). >10 h. (E. coli, IV). * | >30 h. (MR, IV). >20 h. (YIV). >10 h. (E. coli, IV). * | >30 h. (MR, IV). >20 h. (YIV). >10 h. (E. coli, IV). * |
| **Instability index (II)** | 45.72 | 38.55 | 46.28 | 53.11 |
| **Stability** | Unstable | Stable | Unstable | Unstable |
| **Aliphatic Index** | 70.44 | 67.01 | 70.44 | 63.55 |
| **Number of amino acids** | 137 | 137 | 137 | 141 |
| **Grand Average of Hydropathy** | -0.471 | -0.317 | -0.366 | -0.493 |
| **Antigenicity prediction** | 0.5468 | 0.5105 | 0.5386 | 0.4236 |
| **Allergenicity prediction (Y/N)** | N | N | N | N |

* >30 hours (mammalian reticulocytes, in vitro), >20 hours (yeast, in vivo). >10 hours (E. coli, in vivo).

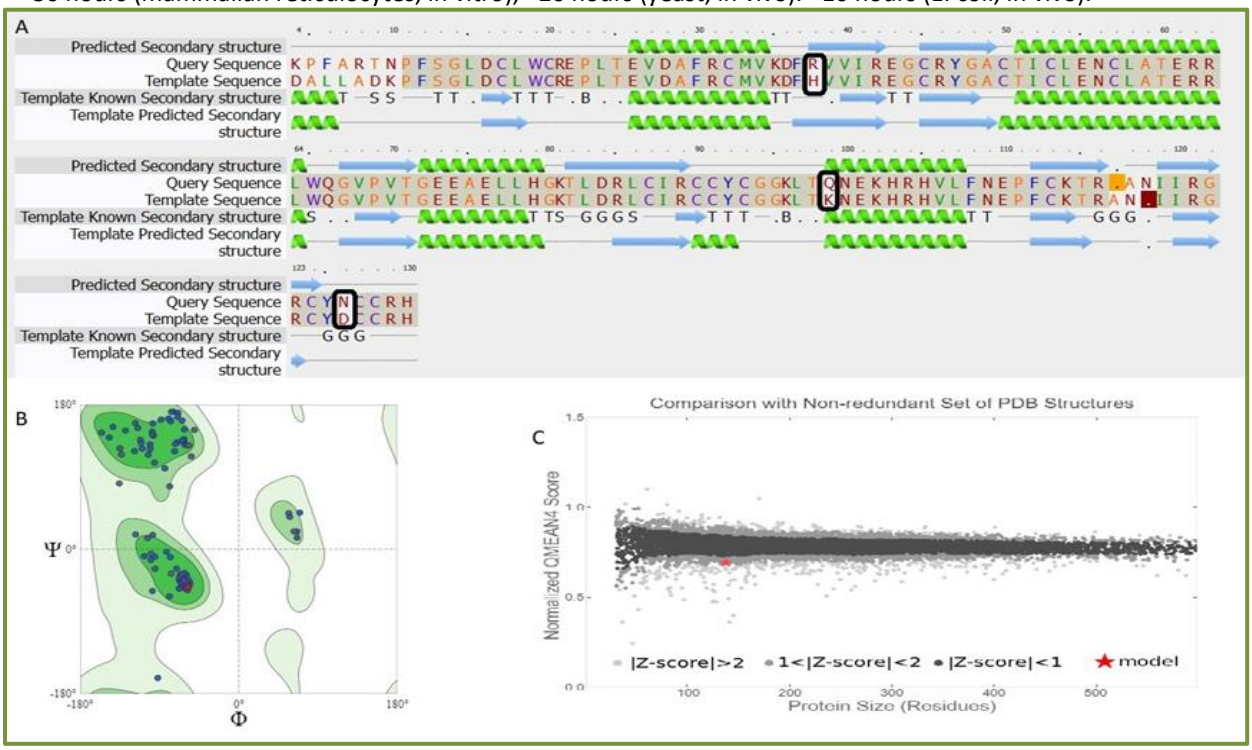


**Figure 4:** Secondary structure of the BPV-1 E6 protein. Demonstrate (A) alpha helix (green), beta sheet (blue arrow) , and coil with its percentages perdition. Each mutation located at coils and alpha helix represented in (box), B: (B) Ramachandran plot showed all residues within right regions particularly the two amino acid mutations Lys99/ Gln and Asp126/ Asp. (red dot). (C): Showing verify of 3D interpretation of L1 protein.

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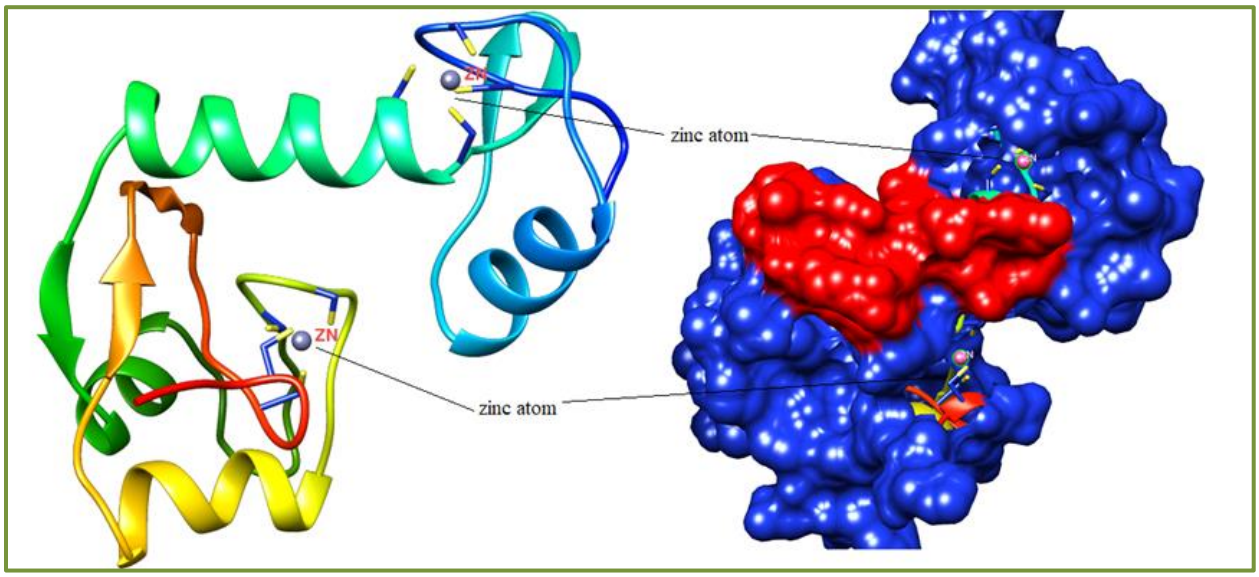


**Figure 5:** The 3 D cartoon structure of BPV type 1 E6 protein that composed of two zinc-binding domains with a linker helix antigenic loops.

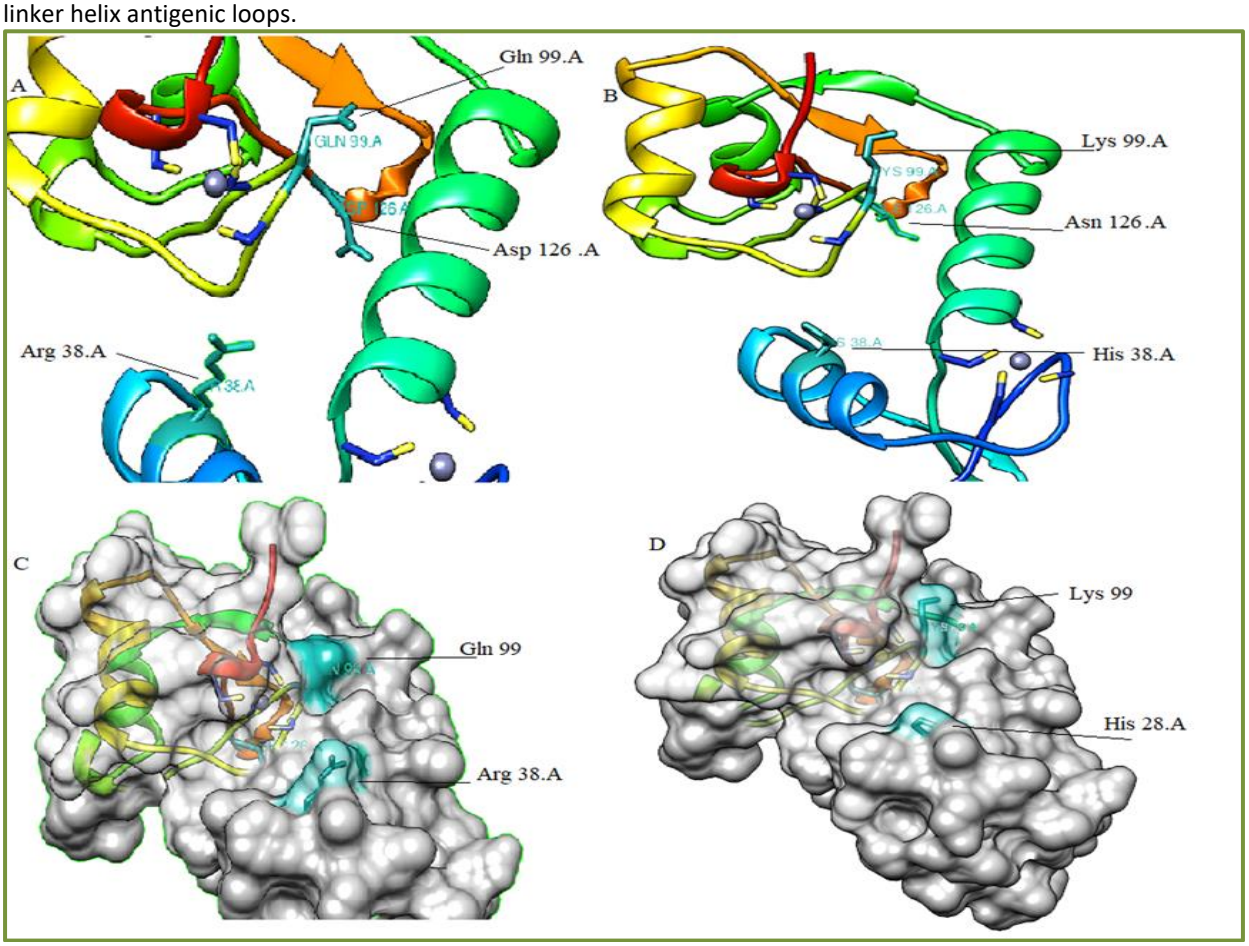


**Figure 6**: 3D carton structures of E6 protein of BPV-1 Iraqi strain, visualization of the amino-acid differences within BPV-1 protein from Iraqi strain compered to control reference strain (A and C= control and B and C study protein).

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**Table 3**: B-cell linear epitopes of the E6 protein predicted with epitope conservancy results (0.5) Threshold.

| Name | Start | End | Epitope sequences | Length | Antigenicity | Epitope conservancy (conserved sequence/total) |
|---|---|---|---|---|---|---|
| **LE1** | 5 | 15 | SFSRGNPFSG | 10 | 0.73 | 100.00% (7/7) |
| **LE2** | 29 | 34 | EPLTEV | 6 | 0.78 | 100.00% (7/7) |
| **LE3** | 69 | 77 | RRLWKGVPV | 9 | 0.79 | 100.00% (7/7) |
| **LE5** | 127 | 135 | IRGRCYNCC | 9 | 0.85 | 100.00% (7/7) |

**Notes**: The reference sequence considered in this analysis is deduced from the BPV-1 (GenBank accession number X02346.1). The letters found in parentheses represent the amino acids substituted in at least one of the proteins deduced from current study other than the reference variant the.

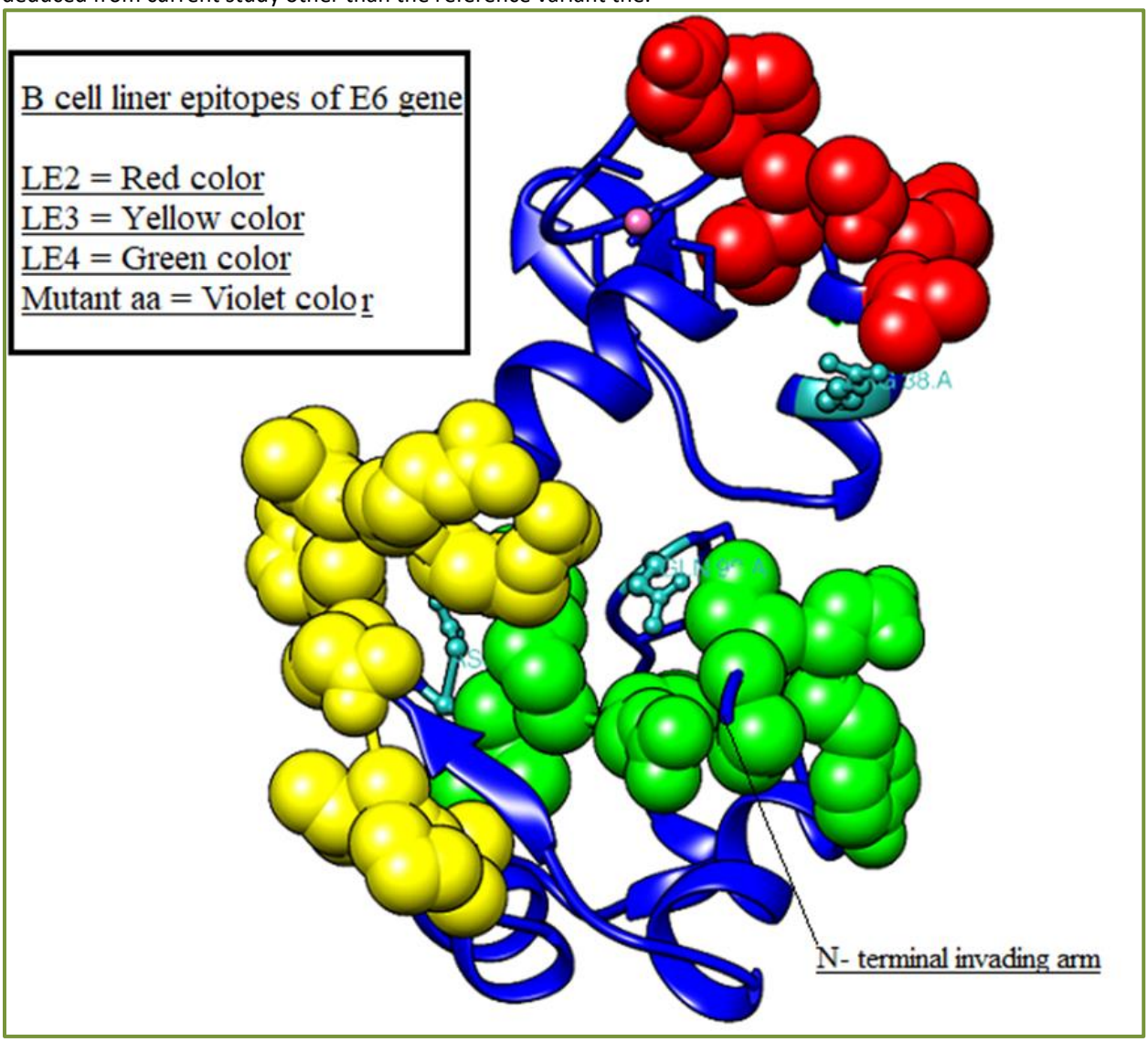


**Figure 7**: B cell liner epitopes in the cartoon of BPV type1 E6 protein, each epitope is highlighted specific color and labeled with specific name in all chains of protein.

**Predicted T cell epitopes of BPV-1 protein**

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The prediction of MHC class I T cell epitopes was performed using the NetMHCpan - 4.1 server. The default parameters were kept and the primary protein sequence was provided as input. Interestingly, T cell epitopes MHC-1 of E6 protein prediction for MHC class I T cell epitopes Interestingly, 9-12 mers epitopes were predicted each with an associated binding affinity score (nM) which display in (Table 4). More, all these 6 peptides were corresponding to BoLA-1:00901, BoLA-1:06101, BoLA-1:00902 and BoLA-T2b haplotypes presented a highest binding affinity scores which were <100nM, which makes them a promiscuous MHCI T-cell epitopes.

**Table 4**: Six potential T-cell epitopes of the BPV-1 E6 protein binding to MHC class I alleles were selected based on the basis of their Binding score rank predicted by the NetMHCIIpan - 4.1 server and epitope conservancy results.

| Allele | # | Start | End | Length | Peptide | Core | Score | Percentile Rank |
|---|---|---|---|---|---|---|---|---|
| **BoLA-1:00901** | 4 | 11 | 19 | 9 | TGEEAQLLH | TGEEAQLLH | 0.268036 | 0.62 |
| **BoLA-1:00901** | 4 | 10 | 19 | 10 | VTGEEAQLLH | VTEEAQLLH | 0.148921 | 1.2 |
| **BoLA-1:06101** | 2 | 11 | 20 | 10 | RCMIKDFHVV | RMIKDFHVV | 0.042745 | 4.0 |
| **BoLA-1:00902** | 7 | 5 | 16 | 12 | YDCCRHGSRSNY | YRHGSRSNY | 0.020824 | 4.4 |
| **BoLA-1:00901** | 1 | 5 | 12 | 8 | SFSRGNPF | S-FSRGNPF | 0.011034 | 7.0 |
| **BoLA-T2b** | 6 | 2 | 13 | 12 | KQRHVLYNEPFC | KQRHNEPFC | 6.6e-05 | 38 |

Whereas, the prediction of epitopes binding to MHC class II alleles of E6 protein, the NetMHCIIpan - 4.1 server was utilized and a total of 15-mers epitopes were predicted each with an associate which bind to MHC class II predominant allele H2-IEk, H2-IAb, H2-IAd, H2-IAu, exhibits a notably high binding affinity score (0.0308 -0.0003nM) making it a potential good target for subsequent investigations. The conservancy of the predicted T cell epitopes, of both MHC class II, was assessed using the IEDB conservancy analysis tool, with the results presented in (Table 5). The conservation rate was 28 - 100% for all T cell epitopes, ensuring their preservation across all BPV-1 protein orthologues.

**Table 5:** Four potential T-cell epitopes of the BPV-1 E6 protein binding to MHC class II alleles were selected based on the basis of their Binding score rank predicted by the NetMHCIIpan - 4.1 server and epitope conservancy results.

| Allele | # | Start | End | Length | Core sequence | Peptide Sequence | Score | Percental rank |
|---|---|---|---|---|---|---|---|---|
| **H2-IEk** | 1 | 1 | 15 | 15 | LQSFSRGNP | MDLQSFSRGNPFSGL | 0.0308 | 52 |
| **H2-IAb** | 6 | 5 | 19 | 15 | YNEPFCKTR | HVLYNEPFCKTRSNI | 0.0223 | 28 |
| **H2-IAd** | 7 | 1 | 15 | 15 | RCYDCCRHG | RGRCYDCCRHGSRSN | 0.0003 | 100 |
| **H2-IAu** | 7 | 3 | 17 | 15 | RHGSRSNYP | RCYDCCRHGSRSNYP | 0.0052 | 95 |

Prediction method: netmhciipan_el 4.1 | High score = good binders

**Discussion**

The many structural (SP) and non-structural (NSP) papillomavirus coding genes have been potentially candidates as DNA vaccines against papillomavirus infection in human and animals (43, 44). This study is preliminary work focused on a molecular and computational analysis of the full-length E6 gene of BPV-1 isolates from middle part of Iraq. Previous research has demonstrated that the E6 protein possesses potent antigenic properties and retains its native immunogenic characteristics during natural infection (45). Based on our findings, the identified strains were classified as Deltapapillomavirus/BPV type 1 due to their close genetic relationship with global BPV-1 strains. This classification supports the hypothesis that papillomaviruses are considered

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closely related when they share greater than 90% sequence similarity (46). (Deltapapillomavirus 4 is mostly prevalent type in most world countries (47). Prevalence of BPV-1 was reported in the Iraqi literatures with used anther targeted viral gene in Iraqi cattle which was up to 86.42% (48). In addition, additionally, the in middle and southern provinces of Iraq reported that the mostly bovine papilloma infection were linked with BPV type 1 (49,50). Immunodominant epitopes within the E6 protein play a crucial role in generating effective immune responses. These epitopes strongly stimulate E6-specific T-cell responses, making them valuable targets for the development of DNA vaccines against diseases caused by viruses expressing the E6 protein (51,52). Experimental studies in mice demonstrated that a plasmid DNA vaccine incorporating the HPV E6 gene effectively protected against tumor development. Mice vaccinated with the E6-DNA vaccine exhibited 100% protection against viral challenge (45).

The E6 gene appeared have 7 single nucleotides polymorphism care consist of 4 synonymous mutations in nucleotides at positions 48, 210, 303, and 345. The observed genotypic diversity among BPV type1 in Iraq, aligns with the existing understanding of the evolutionary relationships within BPV. The presence of this diversity in BPV-1 isolates from Iraq highlights the virus's capacity for genetic evolution (53). The 3D modeling E6 protein was implemented to assess the impact of non-synonymous mutations on the protein structure, therefore its impact on vaccines. This influence come from alteration in continuous and discontinuous epitopes. most b-cell epitopes are discontinuous epitopes, therefor, any mutation that impact the protein structure may impact B cell affinity to epitopes. This finding has significant evidence for emphasizing for employing characteristics of the prevalent local viral strains in construction of subunits vaccines. Such knowledge can profoundly impact local and vaccine development and global cattle welfare (54). we optimized and cloned a BPV-1 E6 gene using into E. coli BL31 by inserting it after the CMV promoter (pcDNA3.1-E6). E6 is expressed early in the viral life cycle, which is crucial for the virus's ability to manipulate host cellular processes and establish infection (55). Therefore; it is most targeted protein as the basis for highly successful recombinant vaccines against cancer-causing papillomaviruses (56). The E6 protein plays a critical role in viral pathogenesis and oncogenesis (57). the immunodominant epitopes of E6 are immunologically important because its well induce E6-specific T-cell immunological responses that amid it incorporating in development of PV DNA vaccines (58). confirmed that incorporated HPV E6 gene in plasmid DNA vaccine have good tumor protection experimentally in mice, showing that mice vaccinated with E6-DNA vaccine exhibit 100% protection against the virus challenge (59, 60).

**Conclusions**

In conclusions, the study demonstrates serviceable insights into the genetic diversity of BPV-1 in Iraq and provide a principle predicting immunoinformatic data. Moreover, the study offers a comprehensive preview of gene diversity, with emphasizing mutations that the potential impacts of immunodeterminant sites.

Acknowledgments

Not applicable.

Conflicts of Interest

The authors declare there is no conflict of interest.

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