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Designing of a novel and potent HPV66 L1 major capsid protein-epitope based therapeutic vaccine against Human Papillomavirus (HPV): A bioinformatics approach

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Abstract

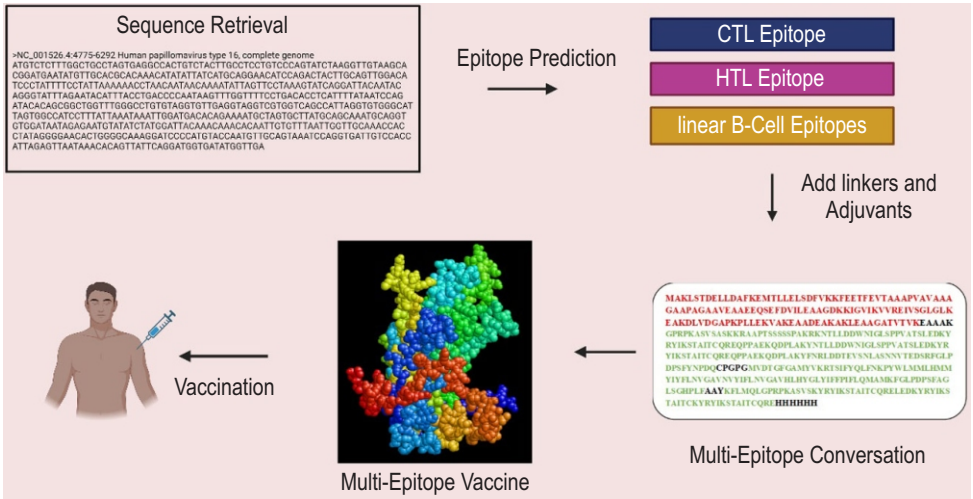
Aim: Oncogenic human papillomaviruses (HPV) cause various types of cancer, including cervical cancer. The main protein of HPV is capsid, targeted in many vaccine attempts. However, these vaccines do not cover enough high-risk HPV serotypes. Therefore, a low-cost potential HPV vaccine to protect against all serovars of the α-papillomaviruses family would be promising in the future. Our study aimed to develop a therapeutic epitope vaccine for HPV using bioinformatics methods.

Methodology: Bioinformatics approach was followed to analyze and identify potential T-cell and B-cell dominant epitopes of HPV-66 L1 major capsid protein. Additionally, various aspects of this protein were examined, including its physico-chemical properties, and secondary and tertiary structures. These analyses helped us to design an effective HPV infection therapeutic vaccine.

Results: The findings revealed that the L1 major capsid protein was unstable and hydrophilic. The secondary structure of this protein composed 39% α-helices, 17.97% β sheets and 31.80% loops. Our study demonstrated 19 dominant epitopes of HPV-66 L1 major capsid protein including 5 B-cell epitopes and 14 T-cell epitopes (10 HTL epitopes, and 4 CTL epitopes) for a novel vaccine candidate.

Interpretation: This study provides a comprehensive biological information about the HPV-66 L1 major capsid protein, which will serve as a theoretical foundation for developing a multi-epitope vaccine against HPV infection.

Key words: B-cell epitope, Cervical Cancer, Human papillomavirus, HPV66 L1 major capsid protein, T-cell epitope.



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Introduction

Cervical cancer is the fourth most common cancer among women worldwide, with about 0.6 million cases and 0.3 million deaths per year (Arbyn *et al.,* 2020). Human papillomavirus (HPV) causes infections in the human reproductive tract. In many cases, HPV infections have no symptoms and are usually cleared by the system. It is a non-enveloped virus with a circular double-stranded DNA genome that is approximately 8 kb in length (Doorbar *et al.,* 2012). However, if the infection persists, it may lead to the development of warts in the cervical, anogenital, or oropharyngeal regions in both men and women. Chronic HPV infection can lead to cervical cancer, which is the most common HPV-related disease. While many pre-cancerous lesions caused by HPV may disappear without intervention, women with HPV infection are at risk of developing persistent and pre-cancerous lesions that can progress to invasive cervical cancer (WHO, 2023).

Cervical cancer is the primary reason for death among women, with a worldwide incidence rate of 15.6% and a mortality rate of 8.8%. According to the Global Cancer Observatory (GLOBOCAN) 2020 report, cervical cancer has an age-specific standardized incidence rate of 13.3%. The prevalence of cervical cancer in Asia has reached 58.2%, with a five-year prevalence of 59.5%. In India, 679,421 men and 712,758 women are expected to receive cancer diagnoses in 2020. In women, the cancer incidence rate was 104 per 100,000, while in men it was 94 per 100,000. Only 22% of women in India undergo cervical screening examinations, as per the National Family Health Survey (NFHS) report (Sen *et al.,* 2022). According to WHO, high-risk human papillomavirus (HR-HPV) is responsible for 99%of occurrences of cervical cancer (WHO, 2023). Among the Indian population, sex workers in Mumbai's urban slums and HIV-positive women have a higher prevalence of cervical cancer. About 56% of cases in the West Indian region have been reported to test positive for HPV 16 and 18 (Sreedevi et al., 2015; Thobias *et al.,* 2019).

The clinical study demonstrated that in the brothel-based sex workers in West Bengal, India, 25% were infected with HR-HPV and 1% had a pre-cancerous lesion along with HR- HPV. Similar to AIDS, young sex workers appear to be more at risk of acquiring oncogenic HPV infection as a secondary infection in HIV-positive patients. The study observed older sex workers have a relatively lesser incidence of HPV infection with acquired immunity against HPV (Sarkar *et al.,* 2008). There are different types of papillomaviruses, including alpha, beta, gamma, delta, zeta and theta. Human papillomaviruses are classified into two categories: High-risk HPV (HR-HPV) and Low-risk HPV (LR-HPV). Based on molecular epidemiological evidence, persistent infection with high-risk HPV is the main cause of invasive cervical cancer and condyloma acuminate. More than 99% of all tumors in the uterine cervix are detected with HPV DNA. HR-HPV types include HPV-16, HPV-18, HPV-31, HPV-35, HPV-39, HPV-45, HPV-51, HPV-52, HPV-56, HPV-58, HPV-59, HPV-68, HPV-73, HPV-53, HPV-30, HPV-66 and HPV-82, which are known for their malignant properties. A study shows that HPV-66 is one of the highly prevalent HPV serotype types in India, among the high-risk type viruses (Senapati *et al.,* 2017).

For the development of a novel HPV vaccine, the main focus should be to target the HPV-66 serotype. Current research on high-risk (HR-HPV) types has revealed that these vaccinations can prevent majority of high-risk HPV infections and lessen the severity of HPV-related disorders. However, these vaccines are only effective in people who have never been exposed to HPV. As a result, there is an urgent need for a therapeutic vaccine for HPV-infected females (Yousefi *et al.,* 2022). The HPV therapeutic vaccine mainly targets the HPV L1 capsid protein, which is responsible for binding the viral genome. During infection, the HPV interacts with the host through endosomal fusion and dissociates the L1 and L2 proteins. The L1 region remains bound to the DNA, facilitating the binding of viral genome (Stephen *et al.,* 2017). Vaccines are widely classified as prophylactic and therapeutic vaccines. The major types of vaccines are attenuated vaccines, live attenuated vaccines, sub-unit vaccines, DNA vaccines and RNA vaccines. Eliciting long-term immunity against pathogen is crucial for the development of the potential vaccine candidate (Saravanan *et al.,* 2024).

The role of HPV prophylactic vaccines is to produce humoral immunity by neutralizing antibodies to prevent HPV infection. However, this does not protect an already infected organism because several genes [early genes (E1, E2, E4, E5) and late genes (L1, L2)] are missing in the existing vaccine, which are related to the integration of virus genome into the host genome. Hence, HPV 66-based subunit therapeutic vaccine may give protection against the existing infection (Zur Hausen, 2002).The HPV-66 L1 major capsid protein is crucial in fighting HPV infection. A potential therapeutic vaccine can be produced by using the correct antigenic peptides of the protein. To identify the best candidate for this vaccine, a bioinformatics approach was used to predict and evaluate the B-cell and T-cell antigenic sites of L1 major capsid protein of HPV-66. The dominant T-cell and B-cell epitopes were determined using docking analysis with physio-chemical characteristics, as well as secondary and tertiary structures. In view of the above, the objective of this research was to find the best peptide for producing therapeutic vaccination against HPV-66.

Materials and Methods

Amino acid sequence retrieval: The complete amino acid sequences of HPV-66 L1 major capsid proteins were obtained from the UniProt database. The antigenicity and allergenicity of the retrieved sequence were determined by using Vaxijen v2.0 and AllerTop v2.0. Web Portal. The previous tool selected "virus" as the target organism based on the input epitope sequence, with a specified threshold score of 0.4.

Prediction of physio-chemical parameters: To analyze the molecular mass, theoretical isoelectric point, amino acid

composition, extinction coefficient, instability coefficient and total average hydrophobicity of HPV-66 L1 major capsid proteins, ProtParam tools from Expasy (https://web.expasy.org/cgi-bin/protparam/protparam) was used (Wilkins *et al.,* 1999). Additionally, Prot Scale tool (https://web.expasy.org/protscale/) was used to predict the hydrophilic and hydrophobic properties of proteins.

Secondary structure prediction: After using the SOPMA online analysis software, the secondary structure of HPV-66 L1 major capsid protein was analyzed.

Tertiary structure prediction: HPV-66 L1 major capsid protein sequences were submitted by using the Phyre online service (Kelley *et al.,* 2015) (http://www.sbg.bio.ic.ac.uk/phyre 2/html) and templates were selected according to the similarity of protein amino acid sequences. The 3D models of the tertiary structure of HPV-66 L1 major capsid protein were carried out by combining multiple templates. After modeling, RasMol Software was used to analyze the 3D models of the protein.

Linear B-cell epitope prediction: The development of a flawless peptide vaccine can trigger a sustained immune response similar to the human natural defense mechanism against pathogenic microorganisms. The B-cell epitopes induce the humoral immune response, which helps the immune system combat infections by producing antibodies that target the exposed antigens. To identify the B-cell linear epitope, IEDB server (http://tools.iedb.org/main/) was used (Larsen *et al.,* 2006).

T-cell Epitope prediction

HTL epitope prediction: To create a multi-epitope-based vaccine, it is crucial to ensure it can trigger effective immunological responses. The protein sequences obtained were utilized as building blocks for the immunogenic epitopes. To maximize accuracy, the NetMHCIIv.2.3 (https://services.healthtech.dtu. dk/services/NetMHCII2.3) approach was employed (Jensen et al., 2018), which involves training individual molecules from complex experiments. All predictions were grouped for each allele and were filtered by <2% rank to narrow down probable binders with more predictions with <IC50 affinities. The predictions that remained after filtering were considered potential HLA-II epitopes that could be recognized by HTL Cd4+.

CTL epitope prediction: Next, NetMHCpan 4.0 server (https://services.healthtech.d-tu.dk/cgi-bin/sw_request) was used to predict the cytotoxic T-lymphocytes (CTL) and their epitopes (Reynisson *et al.,* 2020). To ensure safety, all predicted epitopes were checked and their corresponding antigenicity, toxicity, and allergenicity using Vaxijen, ToxinPred and AllerTop. Only antigens with no toxicity or allergenicity were selected for further studies.

Results and Discussion

Human Papillomavirus (HPV) is a pathogenic virus that is closely linked to cervical cancer (Koliopoulos *et al.,* 2017). Currently, HPV infection is primarily treated by recombinant

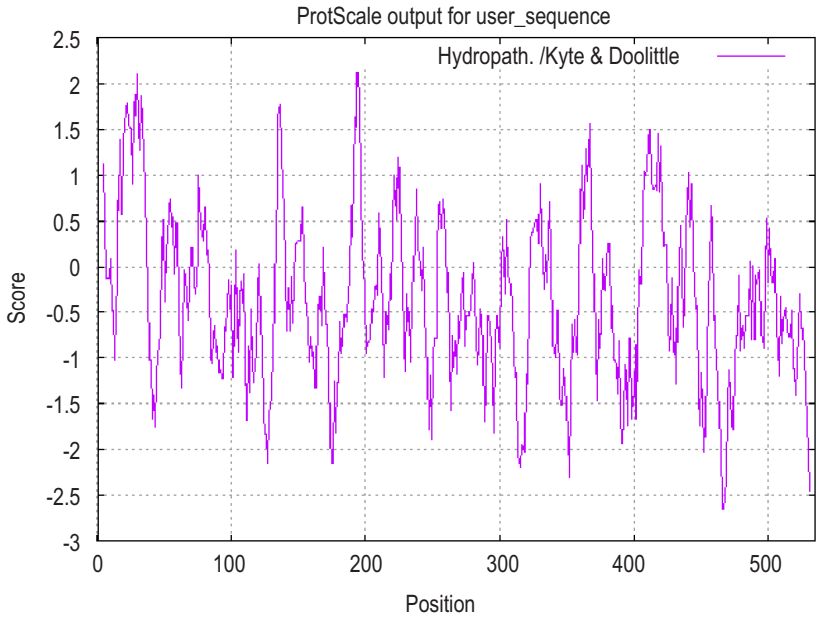


Fig. 1: Protein hydrophilicity of HPV-66 L1 major capsid protein predicated by ProtScale server.

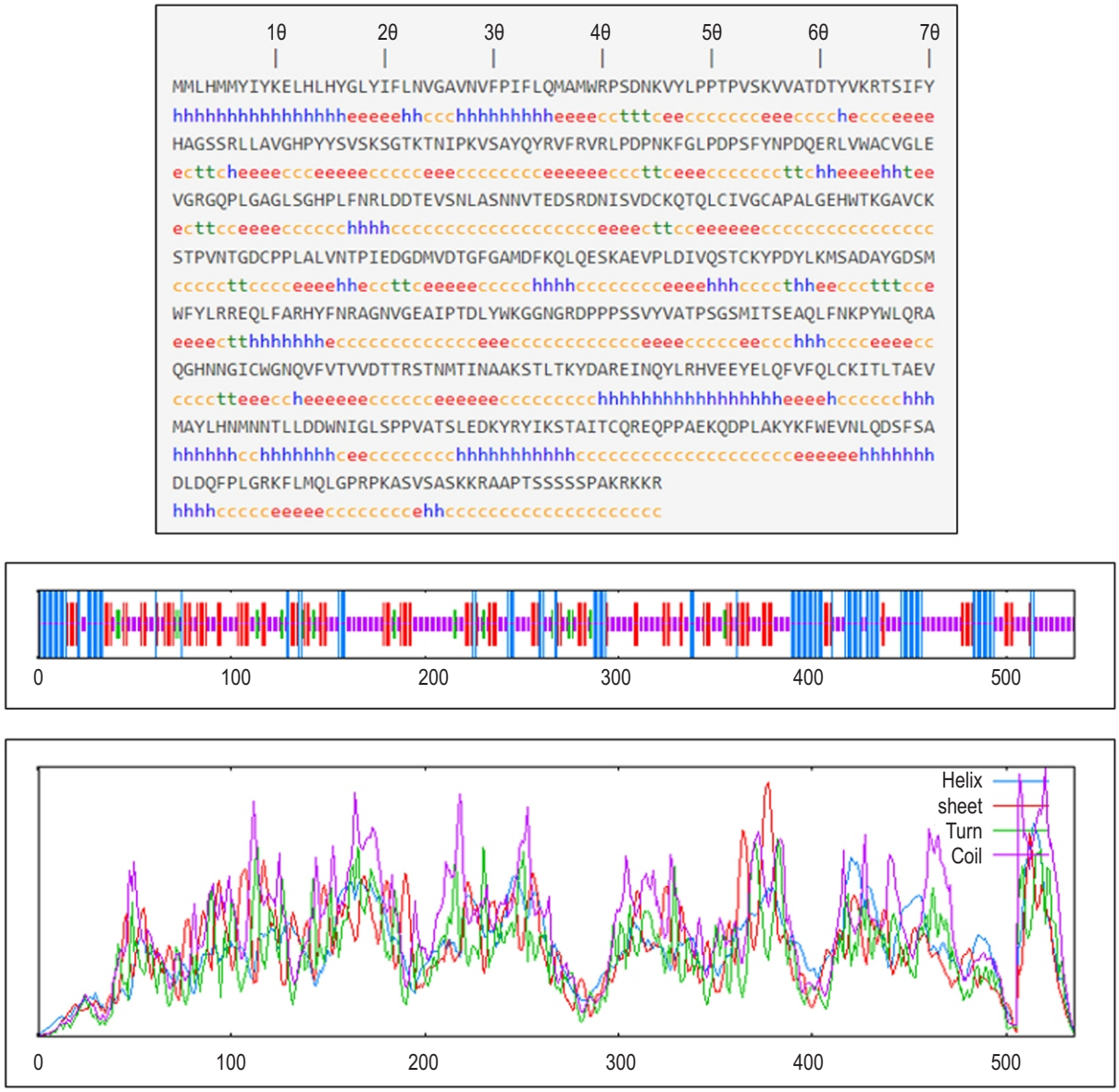
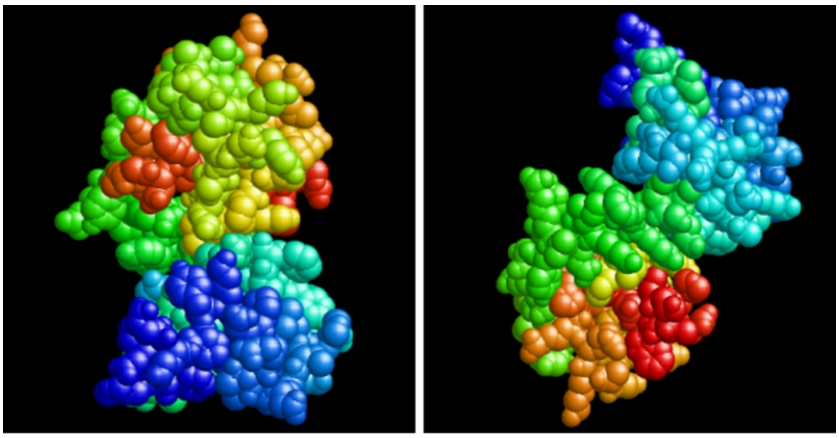


Fig. 2: Secondary structure of HPV-66 L1 major capsid protein predicated by SOPMA software.

interferon therapy. However, this method is inefficient and expensive. To address this, it is essential to develop a prophylactic based on the biological properties of HPV L1 capsid protein. This protein is responsible for binding the viral genome and is critical during infection. The HPV interacts with the host through endosomal fusion, which separates the L1 and L2 proteins. The L1 protein remains bound to the DNA and facilitates the binding of viral genome (Stephen *et al.,* 2017).

Therefore, the primary capsid proteins of HPV L1 make excellent targets for both therapeutic vaccine development and diagnostic testing. By supplying antigenic protein that controls the body's immune response, human leukocyte antigen (HLA) can regulate HPV infection and virus clearance (Hu *et al.,* 2017). Epitope analysis has gained importance as a method for vaccine development and it is crucial for developing therapeutic HPV vaccination for those who have been infected with the virus. Variations in immune response and carcinogenic potential can result from viral mutation (Dong *et al.,* 2020). According to epidemiological studies, different geographic locations and populations have varied prevalences of HPV types and variants (de Martel *et al.,* 2017; Modibbo *et al.,* 2017) and different populations have distinct mutations in the same HPV types (Andersson *et al.,* 2000). For HPV-16 and HPV-18, E6 and E7 oncoprotein antigens, results and epitope information are currently accessible, but the database has no data on the structure and epitope of HPV-66 L1



(A) (B)

Fig. 3: HPV-66 L1 major capsid protein's predicted tertiary structures: (A) front view and (B) back view.

main capsid protein antigens. Therefore, analysis of HPV-66 L1 main capsid protein's antigenic structure and epitope is required.

The structure of proteins can be categorized into four levels: primary structure, secondary structure, tertiary structure and quaternary structure. The primary structure refers to the sequence of amino acids, while the secondary structure pertains to the local arrangements of main chain atoms, such as α-helix and β-fold. The tertiary structure on other hand is three-dimensional spatial configuration of protein. Finally, the quaternary structure is the arrangement of multiple protein subunits, which can vary in number and form complex structures. The study was initiated by retrieving the protein sequence of human papillomavirus (HPV) 66 L1 Major Capsid Protein. (https://www.uniprot.org/uniprot/) accessed on August 26 2023, A0A7G2A8N7 fasta format. Vaxijen v2.0 and AllerTop v2.0 were used to determine the antigenicity and allergenicity of the obtained sequence. Following examination, the protein sequence (A0A7G2A8N7) was discovered to have an antigenicity score of 0.5509 and was selected for further investigation. Similarly using VaxiJen v2.0 and AllerTOP v2.0, the antigenicity and allergenicity of each recovered sequence were assessed. Following analysis of these protein sequences, the sequence (AAV91682.1) with the highest antigenicity score (0.5540) was selected for further investigation (Shahab *et al.,* 2023).

HPV-66 L1 major capsid protein has 535 amino acids with a molecular mass of 60113.50, The theoretical pI value is 8.69 and it contains strong basic (+) amino acids (R, K); 17 strong acidic (-) amino acids (D, E) the atomic composition is $C_{2692}H_{4155}N_{727}O_{788}S_{25}$; the instability coefficient is 41.79, indicating that the protein is stable (predicted protein instability when greater than 40); average hydrophilicity coefficient GRAVY: -0.380 (GRAVY value ranges from -2 to 2). A negative value indicates a hydrophilic protein and is classified as a hydrophilic protein (Fig. 1). These values indicate that the proposed HPV-66L1 can be considered as a potential vaccine candidate due to its structural stability. The L1 main capsid protein's secondary structure was obtained using the SOPMA online server. The SOPMA web server's capacity to simultaneously display the secondary structure of the chart in multiple ways is beneficial. Hydrogen bonds are not readily distorted and the majority of them are found inside proteins that are challenging to recognize and bind by antibodies. According to the properties of spatial conformation, α-helix and β-sheet structure of protein secondary structures are maintained by hydrogen bonds. The β-turn and random coil are primarily found on the protein surface as a noticeable structure. The SOPMA online software examined the distribution of different protein secondary structures. The SOPMA internet server was used to conduct the analysis. It is known that the extended chain accounts for 24.30%, the random coil accounts for 48.97%, the β-turn angle accounts for 4.86% and the α-helix accounts for 21.87% of secondary structure of L1 main capsid protein. There are also numerous possible epitope sites (Fig. 2).

The Phyre server, which employs fold recognition modelling (protein penetration) to generate a tertiary structure protein, was then used to model L1 main capsid proteins in a tertiary structure. Proteins are created utilizing the same folds as existing protein structures using the folding recognition modelling technique. Using known protein folding as a template, the target amino acid sequence is searched for and potential folding models are identified. A series of folding models are scored, with the highest-scoring model being the most likely tertiary structure (Zhang *et al.,* 2011). The L1 main capsid protein's template amino acid confidence in our analysis was 100.0%. The confidence scale, ranging from 0 to 100, showed the likelihood that the target sequence and the template would match. Multiple random coil configurations in the L1 main capsid protein exhibited considerable antigenic potential, according to the secondary structure prediction and tertiary structure simulation. The secondary structure labelling on the three-dimensional design

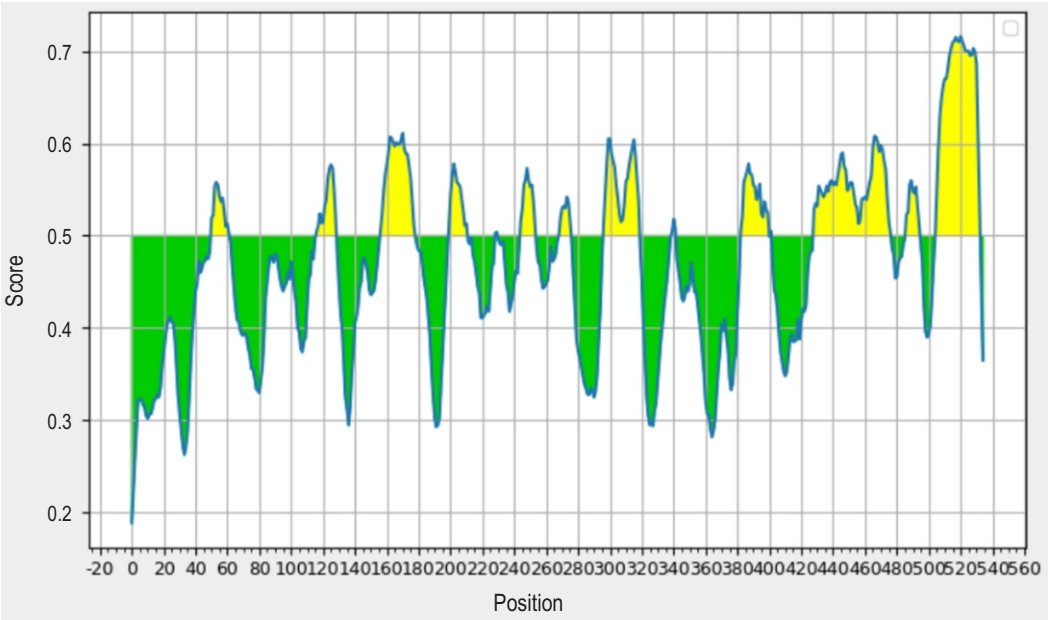


Fig. 4: Illustration of two regions of B-cell, the non-epitomic selection (shown in green) and the epitomic region (shown in yellow).

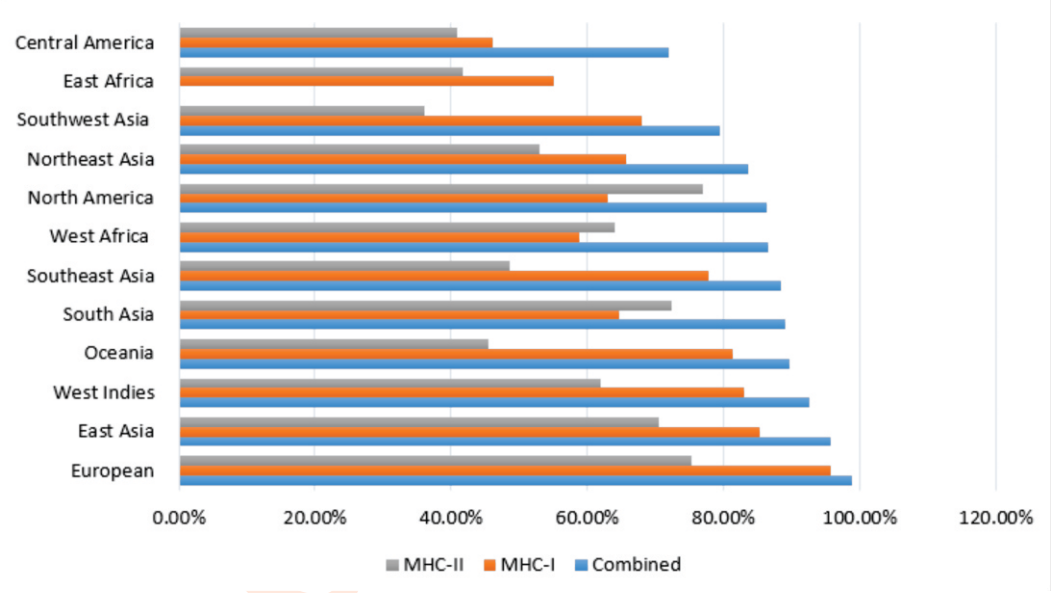


Fig. 5: Population coverage of the selected epitopes estimated, based on MHC-I and MHC-II restriction data from all continents.

was then completed using RasMol software (Fig. 3). The helix and fold structural constraints of the protein's secondary structure were maintained by hydrogen bonds, which were not easily distorted and were frequently found inside the protein, by the spatial conformation characteristic. While β-turn and irregular crimped sites are primarily found on the surface of proteins, protruding structures, which are favorable for binding to antibodies and are more likely to be epitopes, are difficult to bind with antibodies and to form epitopes through secondary structure prediction and tertiary structure construction. The irregular coil

Table 1: The extracted linear epitopes include their peptide sequence, antigenic score and properties related to allergenicity and toxicity

Peptide	Antigenicity	Allergenicity	Toxicity
FGLPDPSFYNPDQ	0.4168	NO	NO
FNRLDDTEVSNLASNNVTEDSR	0.5795	NO	NO
RAGNVGEAIPTDLYWKGGNGRDP	0.7728	NO	NO
NTLLDDWNIGLSPPVATSLEDKYRYIKSTAITCQREQPPAEKQDPLAKY	0.6421	NO	NO
GPRPKASVSASKKRAAPTSSSSSPAKRK	0.7987	NO	NO

Table 2: Predicted ten HTLs with their antigenic score and allergenic and toxic properties

Epitopes	Interacting alleles	Antigenicity	Allergenicity	Toxicity
MVDTGFGAM	HLA-A*01:01, HLA-A*26:01, HLA-B*07:02, HLA-B*39:01	1.6337	NO	NO
YVKRTSIFY	HLA-A*01:01, HLA-A*03:01, HLA-A*26:01, HLA-B*15:01	0.7799	NO	NO
QLFNKPYWL	HLA-A*01:01, HLA-B*39:01, HLA-B*08:01	0.1842	NO	NO
MMLHMMYIY	HLA-A*01:01, HLA-A*03:01, HLA-B*15:01	0.5708	NO	NO
HLHYGLYIF	HLA-A*26:01, HLA-B*58:01, HLA-A*24:02	1.1302	NO	NO
FPIFLQMAM	HLA-B*39:01, HLA-B*08:01, HLA-B*07:02, HLA-A*26:01	0.8036	NO	NO
FLNVGAVNV	HLA-A*02:01	1.5035	NO	NO
YIFLNVGAV	HLA-A*02:01, HLA-A*26:01	1.0847	NO	NO
KFGLPDPSF	HLA-A*24:02	0.8496	NO	NO
AGLSGHPLF	HLA-A*24:02	0.7016	NO	NO

Table 3: CTL epitopes extracted along with their antigenicity, toxicity and allergenicity

Epitopes	Interacting alleles	Antigenicity	Allergenicity	Toxicity
LEDKYRYIKSTAITC	HLA-DRB1*04:01, HLA-DRB1*04:05, HLA-DRB1*07:01, HLA-DRB1*08:02, HLA-DRB1*09:01, HLA-DRB1*15:01, HLA-DRB1*16:02, DRB3_0202	0.5695	NO	NO
KYRYIKSTAITCQRE	HLA-DRB1*01:01, HLA-DRB1*04:01, HLA-DRB1*04:05, HLA-DRB1*07:01, HLA-DRB1*08:02, HLA-DRB1*01:02, DRB3_0202	0.6721	NO	NO
YRYIKSTAITCQREQ	HLA-DRB1*04:01, HLA-DRB1*07:01, HLA-DRB1*08:02, HLA-DRB1*16:02, DRB3_0202	0.7366	NO	NO
KFLMQLGPRPKASVS	HLA-DRB1*01:01, HLA-DRB1*13:01, HLA-DRB5*01:01, HLA-DRB4*01:03	0.8680	NO	NO

configurations and strong antigenic potential of synthesized proteins are visible in the mold. Typically, eliminating a virus from the body involves both cellular and humoral immunity. For this reason, using the IEDB web server, B-cell epitopes against HPV were identified. Eleven epitopes in total were anticipated and their antigenicity and allergenicity were calculated. After careful examination, they were narrowed to five epitopes that were suitable for vaccine development. Fig. 4 illustrates the epitope and non-epitope regions, with a cut-off value of 0.5. Five epitopes were selected based on their sequence, position, length, favorable antigenic scores and non-allergenic and non-toxic behavior. Table 1 presents the selected epitopes and their respective scores, with the minimum score being 0.4168 and the maximum score being 0.7982. The minimum value and highest value of antigenicity of the epitopes were also measured. Nevertheless, we found that the mean value was 0.64. In the host immunological surveillance of viruses, the antigen-presenting molecule human leukocyte antigen (HLA) is crucial.

In the course of the host's immune reaction to pathogens like viruses, it can present foreign antigens to immune cells (Ohl and Tenbrock, 2015). Importantly, the three forms of HLA antigens—HLA-I, HLA-II and HLA-III—that are disseminated throughout nucleated cells have numerous distinct allele frequencies. Cytotoxic T lymphocytes (CTL) that are CD8 + are recognized and stimulated by HLA-I. HLA-II activates CD4+ helper T lymphocytes (HTLs) and aids in the recognition of foreign antigens. Growing interest has also been paid to the connection between cervical cancer and HLA gene polymorphism. Consequently, the experiment included software for Net CTL and

Net MH Cpan analysis. By using NetMHCpan 4.0 search tools, out of 207 peptides 27 peptides were selected immunogenic epitopes based on the interaction of the epitope with at least ten alleles. Out of these 27 epitopes ten epitopes were selected to develop a vaccine candidate based on its immunogenicity, toxicity and allergenicity. Table 2 shows ten non-toxic, non-allergic and most antigenic epitopes. Cytotoxic T-lymphocyte (CTL) epitopes were predicted using the NetCTL4.0 web server. Four CTL epitopes were identified from the HPV-66 L1 major capsid protein based on their IC50 values. These epitopes, namely LEDKYRYIKSTAITC, KYRYIKSTAITCQRE, YRYIKSTAITCQREQ and KFLMQLGPRPKASVS, are non-allergenic and non-toxic and have been found to strongly bind with alleles HLA-DRB1*04:01, HLA-DRB1*04:05, HLA-DRB1*07:01, HLA-DRB1*08:02, HLA-DRB1*09:01, HLA-DRB1*15:01, HLA-DRB1*16:02, DRB3_0202. The comb score of these prediction epitopes and their corresponding antigenicity were found to be significantly high enough to make them lead candidates.

Prediction of leading T-cell and B-cell epitope of HPV-66 L1 major capsid protein: In this study, 19 dominant epitopes of HPV-66 L1 major capsid protein, including 5 B-cell epitopes and 14 T-cell epitopes (10 HTL epitopes and 4 CTL epitopes) were identified. Population coverage analysis: Allelic population coverage refers to the proportion of individuals within a specific population that exhibit a certain set of alleles. According to IEDB server, MHCI and MHCII T-cell epitopes have a global population coverage of 88.42% and 75.26%, respectively (Shahab *et al.,* 2023). When combined, the coverage is estimated to be 96.78%.

European populations have the highest collective epitope coverage (98.94%), followed by East Asia (95.62%), West Indies (92.67%), Oceania (89.76%), South Asia (89.08%), Southeast Asia (88.48%), West Africa (86.54%), North America (86.32%), Northeast Asia (83.72%), Southwest Asia (79.37%), East Africa (78.13%), Central America (71.93%) and South America (64%) respectively. The population of Europe is the highest, while South America has the lowest population. The MHC-I epitopes that are most important for binding are YVKRTSIFY, YIFLNVGAV and HLHYGLYIF. Three MHC-II alle epitopes (LEDKYRYIKSTAITC, KYRYIKSTAITCQRE and YRYIKSTAITCQREQ) provide significant coverage when compared to the world's total population. Globally, European populations are estimated to have the highest coverage of collective epitopes (CAIYYKAREMGFKHI, AIYYKAREMGFKHIN and EKWTLQDVSLEVYLT) (95%), followed by North America (92%), East Asia (81%), South Asia (89%), Southeast Asia (79%), Southwest Asia (78%), East Africa (77%), West Africa (79%), Central Africa (66%), North Africa (84%), South Africa (71%), West Indies (88%), Central America (21%), South America (74%) and Oceania (72%), respectively (Shahab *et al.,* 2023).

Although there are several therapeutic HPV vaccines currently in the market, including DNA vaccines, polypeptide/ protein vaccines and vector vaccinations, several subtypes of infections frequently develop due to wide variety of HPV infections. One therapeutic vaccine's efficacy cannot be increased at all costs (Lopalco, 2017). Given the specific characteristics of HPV infection, producing an HPV therapeutic vaccination demands the use of a variety of multivalent composite vaccines, the identification of effective antigenic components and the use of molecular biotechnology to allow the carrier to express several antigenic components at the same time. Another area to investigate is how the body can develop a stronger immune response. Currently, vaccination is the most effective prevention against viruses. The failure rate of conventional vaccine development is higher and it comes with time and cost constraints. Numerous studies highlight the chimeric vaccine construct's potency, efficacy, safety and potential as a novel approach to control pandemics. Numerous earlier studies have documented the use of immunoinformatics techniques in vaccine design and potency testing against a wide range of targets (Hayat *et al.,* 2023; Shahab *et al.,* 2023).

In conclusion, the HPV-66 L1 major capsid proteins have high potential as antigens for building immunity. By analyzing biological information of dominant epitopes for T-cells and B-cells, we can lay the preliminaries for developing HPV therapeutic vaccines and detection tools. The proposed vaccine candidate is checked for allergic activity by AllerTop v2.0 and its toxicity by ToxinPred to ensure biocompatibility and environmental friendliness.

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