HAYATI
Journal of Biosciences

Vol. 33 No. 3, May 2026 556-565
DOI:10.4308/hjb.33.3.556-565
ISSN: 1978-3019
EISSN: 2086-4094

Research Article

Check for updates

OPEN ACCESS

Preclinical Evaluation of HPV Type 52 L1L2 Chimeric Protein as a Cervical Cancer Vaccine Candidate

Isti Kartika Sari[1,2], Joko Pamungkas[3], Apon Zaenal Mustopa[4*], I Wayan Teguh Wibawan[3], Jendri Mamangkey[5], Sheila Chairunnisa[4], Herman Irawan[4], Ai Hertati[4], Nurlaili Ekawati[4], Rifqiyah Nur Umami[4], Ela Novianti[4], Maritsa Nurfatwa[4], Huda Shalahudin Darusman[1,2,3]

[1]*Primatology Study Program, Graduate School of IPB University, Bogor, Indonesia*
[2]*Primate Research Center, IPB University, Bogor, Indonesia*
[3]*School of Veterinary Medicine and Biomedical Sciences (SVMBS) IPB University, Bogor, Indonesia*
[4]*Research Center for Genetic Engineering, Research Organization for Life Sciences and Environment, National Research and Innovation Agency (BRIN), KST Soekarno, Cibinong, Bogor, Indonesia*
[5]*Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Negeri Makassar, Makassar 90221, Indonesia*

ARTICLE INFO

Article history:
Received September 16, 2025
Received in revised form November 2, 2025
Accepted November 20, 2025
Available Online December 16, 2025

KEYWORDS:
Cervical cancer,
Cross-neutralizing antibodies,
Escherichia coli,
Human papillomavirus type 52,
L1L2 chimeric protein,
Vaccine

ABSTRACT

High-risk human papillomaviruses (HPVs) are the primary etiological agents of cervical cancer, accounting for more than 300,000 deaths annually worldwide. Current prophylactic vaccines based on recombinant L1 major capsid virus-like particles (VLPs) have demonstrated strong efficacy but are restricted to a limited spectrum of HPV types. To address this limitation, the present study evaluated a recombinant L1L2 chimeric protein derived from HPV type 52 as a potential candidate for a broad-spectrum vaccine. The chimeric protein was expressed in *Escherichia coli* strain BL21 (DE3) and purified for immunization studies. Female BALB/c mice (*Mus musculus*, n = 5 groups) were immunized, and immune responses were analyzed by enzyme-linked immunosorbent assay (ELISA) and pseudovirion-based neutralization assays (PBNA). The recombinant L1L2 vaccine candidate induced detectable antibody responses against HPV antigens; however, neutralizing activity remained modest. Histopathological analysis of liver and kidney tissues showed no evidence of toxicity, supporting the safety profile of the candidate. In summary, these results suggest that the HPV type 52 L1L2 chimeric protein represents a promising platform for the development of cervical cancer vaccines, although further optimization is required to achieve robust cross-neutralizing efficacy.

1. Introduction

Human papillomavirus (HPV) -associated cancers remain a major global public health concern, particularly in Asia. In 2022, approximately 831,204 new cases of HPV-related malignancies were reported worldwide, with the Asia-Pacific region accounting for 57.8% of these cases (Zhang *et al.* 2025). The prevalence of HPV and the incidence of cervical cancer exhibit substantial geographic variability across Asia, with HPV-16 and HPV-18 collectively responsible for 67% of cervical cancer cases (Parkin *et al.* 2008). Reported incidence rates per 100,000 person-years in selected Asia-Pacific countries ranged widely, from 15.4 to 252.0 for cervical cancer, 0.2 to 55.5 for head and neck cancers, and 0.2 to 13.7 for anal cancers (Lou *et al.* 2024). These infections contribute to 22.0% of infection-related cancers and 27.3% of infection-related cancer deaths in the region (Huang *et al.* 2015). Despite observed declines in certain HPV-associated malignancies, the absolute number of cases continues to rise, reflecting the ongoing burden of HPV-related diseases.

HPV type 52 is classified as a high-risk HPV genotype and presents ongoing challenges to current cervical cancer prevention strategies. This genotype shows

*Corresponding Author
E-mail Address: azae001@brin.go.id

consistent prevalence worldwide, with particularly high incidence in Asian populations, where it is often among the most frequently detected HPV types. For example, in Shanghai, China, HPV type 52 accounted for 22.47% of HPV infections among women, representing the most common genotype in this cohort (Ruan *et al.* 2023). Existing prophylactic HPV vaccines primarily target HPV types 16 and 18, which are responsible for the majority of cervical cancer cases. However, these vaccines provide limited protection against HPV type 52, creating a notable gap in coverage (Liu *et al.* 2015).

Prophylactic HPV vaccines currently available are based on recombinant proteins that express the major capsid protein L1. The viral capsid is composed of 360 copies of L1 protein, which self-assemble into virus-like particles (VLPs). VLPs structurally and immunologically mimic authentic virions but are non-oncogenic and non-infectious, as they lack viral genetic material. Additionally, these particles are replication-deficient, consisting solely of L1 without any other viral genes (Venkataraman *et al.* 2021). Recent advances in HPV vaccine development have explored the incorporation of the minor capsid protein L2. Although anti-L2 antibodies exhibit lower type specificity compared to anti-L1 antibodies, they have demonstrated the ability to neutralize multiple HPV pseudovirion types *in vitro* (Zhao *et al.* 2024). The L1L2 chimeric protein, which integrate L2 neutralizing epitopes onto the L1 scaffold, have been reported to induce high antibody titers against both capsid proteins (Chabeda *et al.* 2019). Therefore, L1L2 chimeric protein derived from HPV type 52 have been constructed as a potential vaccine candidate aimed at eliciting broadly neutralizing antibodies.

The present study aims to produce the previously constructed L1L2 chimeric protein generated by the research team at BRIN. Protein expression was performed in *Escherichia coli* BL21 (DE3), which offers advantages in terms of increased yield, simplified methodology, and cost-effectiveness. The recombinant protein was fused with SUMO (Small Ubiquitin-related Modifier) and a polyhistidine (His) tag to enhance solubility. Both fusion tags were subsequently removed to obtain the purified protein (Ikramullah *et al.* 2024; Al Adawiah *et al.* 2024). Protein production was carried out using an optimized synthetic medium designed to replace standard Luria-Bertani (LB) medium, modified from M9 medium, and scaled up in a 3-liter fermentor to produce L1L2 chimeric protein. *In vivo* evaluation was conducted using specific pathogen-free (SPF) BALB/c mice. Serum samples were collected to determine antibody titers by ELISA, assess neutralizing activity against HPV pseudoviruses, and perform histopathological analysis of liver and kidney tissues to evaluate the potential toxicity of the chimeric L1L2 vaccine candidate.

2. Materials and Methods

2.1. Ethical Consideration

This study complies with all research ethics, particularly the animal testing research protocol, and has been approved by the Ethics Committee Board of Badan Riset dan Inovasi Nasional (BRIN), the National Research and Innovation Agency of Indonesia, with approval number 164/KE02/5K/08/2023.

2.2. HPV Type 52 L1L2 Chimeric Protein Production

2.2.1. Protein Expression and Optimization

Expression of the L1L2 chimeric protein began with the preparation of a modified M9-based production medium optimized for carbon and nitrogen sources, mineral salts, and bacterial growth support. The recombinant L1L2 chimeric gene was previously constructed as described by Mustopa *et al.* (2022), Ikramullah *et al.* (2024), and Al Adawiah *et al.* (2024). Glucose or glycerol served as the carbon source, while essential salts provided ions necessary for *E. coli* growth (Tripathi and Shrivastava 2019). The medium was further optimized with pH-stabilizing buffers, amino acid supplements, and essential ions, including Zn^{2+} and Fe^{2+}, to enhance protein expression efficiency. The composition of modified media is presented in Table 1.

Table 1. Composition of M9-modified media for the growth of engineered *E. coli*

Ingredients	Amount (g)	Supplementation	Amount	After 2.5-h incubation	Amount
KH_2PO_4	8.58	Vitamin solution	15 µL	0.8% glucose	12 mL
NaCl	1.5	TMS	3 mL	1 mM IPTG	9.3 mL
$CaCl_2•H_20$	0.99	Sterile Milli-Q	Final volume is 3 L	Optimum temperature	25°C
Glucose	12			Agitation	160 rpm
$MgSO_4•7H_2O$	3.6				
$MgCl_2•6H_2O$	1.5				
Yeast extract	36				

2.2.2. HPV Type 52 L1L2 Chimeric Protein Production and Purification

The bacterium *E. coli* expressing the L1L2 chimeric protein was initially cultured in 5 mL of bacterial medium, then scaled up to 500 mL and incubated for 2.5 hours until an optical density (OD) of 0.6–0.7 was reached. A 3-L fermentor containing sterile liquid medium was inoculated with the bacterial culture. During the first 2.5 hours, the fermentor was maintained at 37°C with agitation at 200 rpm. Upon incubation, 15 mL of thiamine solution, 3 mL of trace mineral solution (TMS), 12 mL of 0.8% glucose, and 9.3 mL of IPTG were added. Fermentation was continued at 25°C, 160 rpm, with aeration of 1 vvm and a dissolved oxygen level of 30% for 16 hours. Cultures were harvested by centrifugation at 6,000 rpm for 10 minutes at 4°C. Supernatants were discarded, and cell pellets were resuspended in 1× PBS, followed by lysis using a sonicator (30% amplitude, pulse 02.03) for 15 minutes at 4°C (Ikramullah *et al.* 2024). Cell lysates were pre-filtered using a 0.45 µm Millipore filter before purification. Affinity chromatography was performed using a HisTrap HP column (Cytiva).

2.3. Protein Characterization and Quantification

2.3.1. SDS-PAGE and Western Blot

Protein characterization was performed by SDS PAGE and Western blot. Separating (15%) and stacking (4%) gels were prepared using 30% bis-acrylamide (Biorad, cat.1610158), SDS 10%, Tris-HCl 1.5 M, APS (Biorad, cat.1610700), and TEMED (Biorad, cat.1610400). Protein samples were mixed with sample buffer and electrophoresed at 80 V for 120 minutes. Gels were stained with Coomassie Brilliant Blue (Biorad, cat.610400) and destained using a methanol-acetic acid-water solution. For Western blot, proteins were transferred to nitrocellulose membranes, incubated with anti-6x-His Tag monoclonal antibody as primary antibody for 18 hours followed by incubation with anti-mouse IgG alkaline phosphatase conjugate as secondary antibody and finally the signals were detected using BCIP-NBT substrate (Thermo, cat.34042).

2.3.2. Enzyme-Linked Immunosorbent Assay (ELISA)

Protein concentrations were measured by our in-house sandwich ELISA (Chairunnisa *et al.* 2024). Samples and standards L1 HPV type 52 commercial protein (Creative Diagnostic) were coated onto 96-well plates and incubated at 4°C for 18 hours followed by washing with PBST thrice and blocking with BSA for 1 hour at room temperature. Anti-HPV type 52 L1 monoclonal antibody (1:2000) (Creative Diagnostics) was used as primary antibody followed by HRP-conjugated goat anti-mouse IgG (1:2000) as secondary antibody. Finally, TMB substrate (Sigma, cat.T0440) was added and incubated for 30 minutes followed by the addition of 2 M H_2SO_4 as stop solution. Absorbance was measured at 450 nm.

2.3.3. Transmission Electron Microscopy (TEM)

VLP formation was confirmed by TEM. Samples (1 µg/mL) were applied to carbon-coated grids, excess liquid was removed, and the grids were stained with 2% sodium phosphotungstate (pH 7.0). Imaging was performed using a Talos F200C microscope (Mustopa *et al.* 2022; Ikramullah *et al.* 2024).

2.4. Immunogenicity Assay in Mice

The immunogenicity of the vaccine candidate was evaluated in SPF BALB/c female mice (n = 22), following a two-week acclimatization. Mice were divided into five groups (Table 2) and maintained under ABSL3 conditions (BRIN Approval No. 164/KE02/5K/08/2023). Vaccination schedules and blood collection were planned according to experimental design (Longet *et al.* 2011). The negative control contained adjuvant only, while L1 HPV type 52 commercial protein (Creative Diagnostic) served as a positive control. Gardasil was used as a commercial vaccine control.

2.5. Serum and Organ Examination

2.5.1. ELISA for Antibody Profiling

Serum antibody titers were measured by our in-house ELISA (Chairunnisa *et al.* 2025). The L1 HPV type 52 commercial protein (0.1 µg) (Creative Diagnostic) were diluted in carbonate-bicarbonate buffer and coated onto 96 well plate followed by incubation at 4°C for 18 hours. The coated plate was washed thrice using wash buffer (0.09% NaCl + 0.05% Tween-20) and blocked with BSA. Serum samples were added and serially diluted anti-HPV type 52 L1 monoclonal antibody (Creative Diagnostics)

Table 2. Experimental groups and vaccine doses in mice

Group(s)	Treatment(s)	Number of mice
1	Negative control ($AlPO_4$ 20 µg/200 µL)	4
2	Positive control (Creative Diagnostic L1 HPV type 52)	4
3	Positive control (Gardasil, 2 µg)	4
4	HPV type 52 L1L2 chimeric protein, dose 1 (2 µg)	5
5	HPV type 52 L1L2 chimeric protein, dose 2 (4 µg)	5

were used as standards. The HRP-conjugated goat anti-mouse IgG was used as secondary antibody. Finally, TMB substrate (Sigma, cat.T0440) was added and incubated for 30 minutes followed by the addition of 2 M H_2SO_4 as stop solution. Absorbance was measured at 450 nm.

2.5.2. HPV Type 52 Pseudovirus Production

HPV type 52 pseudovirus (PsV) was generated by co-transfecting HEK293FT cells with p52sheLL and pfwB plasmids containing L1-L2 HPV type 52 and GFP, respectively. After cell extraction and viral maturation, PsV was purified using a stepwise OptiPrep gradient (39%, 33%, 27%) and ultracentrifuged at 35,000 rpm for 4 hours using a SW40Ti rotor. The production of HPV type 52 PsV was confirmed by Western blot and TEM (Buck *et al.* 2005; Chairunnisa *et al*. 2025).

2.5.3. Neutralizing Antibody Assay

Neutralizing antibody responses were evaluated using HPV type 52 pseudovirus (PsV). Serum samples were diluted (1:50) and incubated with PsV (1:100) on ice for 1 hour. The mixtures (100 µL) were added to HEK293FT cells seeded at 1×10^4 cells/well in 96-well plate and incubated at CO_2 incubator at 37°C for 96 hours. Fluorescence microscopy was used for qualitative observation of infection inhibition (Chairunnisa *et al.* 2025).

2.5.4. Histological Analysis

Kidney and liver tissues were collected, fixed in 10% neutral buffered formalin, and processed for histopathology examination following standard procedures (Pang *et al.* 2021). Sections were stained with Harris Hematoxylin and Eosin (H&E) and examined under a light microscope at 400× magnification. Pathological changes were scored based on congestion, hemorrhage, and necrosis. Statistical analysis was performed using the Kruskal-Wallis test followed by Mann-Whitney post hoc comparison.

3. Results

3.1. Optimization of Growth Media for *E. coli* BL21

The L1L2 chimeric protein from HPV type 52 was successfully expressed in the *Escherichia coli* BL21 (DE3) strain. SDS-PAGE analysis of bacterial lysates demonstrated differential protein expression under varying culture conditions, with distinct bands corresponding to the expected molecular weight of 68 kDa for the L1L2 chimeric protein (Figure 1). Western blot analysis confirmed that the level of L1L2 chimeric protein expression was influenced by IPTG concentration, growth media, and incubation temperature. Induction with IPTG at 0.3 and 0.5 mM produced the most intense protein bands, whereas higher concentrations (0.6–0.8 mM) led to a noticeable decrease in band intensity. Among the three media tested (Super Broth, M9, and Terrific Broth), M9 and SB media at 25°C yielded the highest protein expression, as evidenced by stronger Western blot signals.

3.2. Production and Purification of Recombinant HPV Type 52 L1L2 Chimeric Protein

Recombinant HPV type 52 L1L2 chimeric protein was successfully produced and purified using immobilized metal affinity chromatography (IMAC) with a HisTrap column. IMAC exploits the interaction

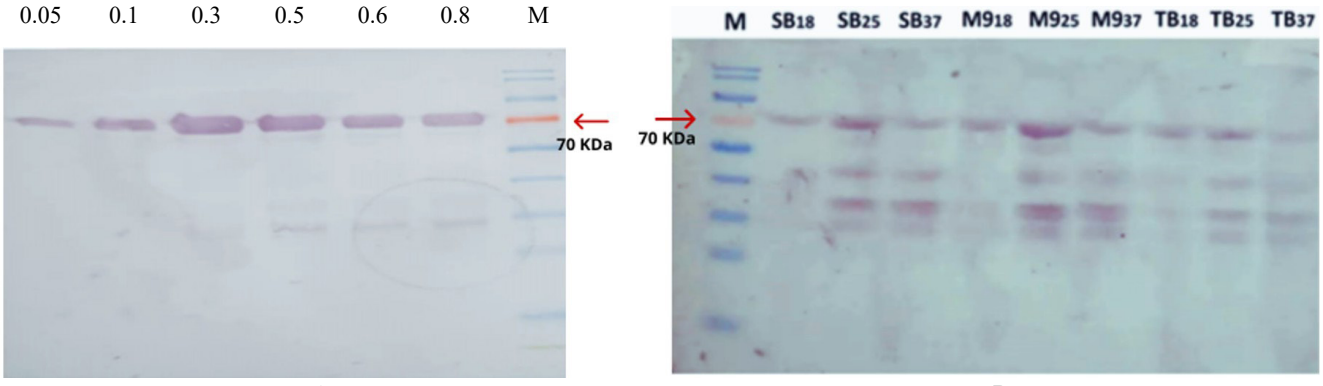


Figure 1. Western blot analysis of protein expression under different conditions: (A) various IPTG concentrations (0.05-0.8 mM) and M: marker, (B) different growth media and incubation temperatures. (SB, M9 and TB: growth medium, 18; 25; and 37: temperature in celcius; and M: marker)

between the His-tag on the recombinant protein and divalent metal ions (Ni^{2+} or Co^{2+}) immobilized on the resin. The protein sample was applied to the HisTrap column, which was pre-equilibrated with binding buffer, allowing the His-tagged protein to bind selectively. After washing to remove contaminants, the target protein was eluted using an imidazole gradient. The purification profile indicated a prominent peak with an absorbance value of 289.513, suggesting a high concentration of the target protein (Figure 2). Following elution, the protein concentration was quantified using an ELISA, confirming the presence of purified protein suitable for downstream applications (Table 3). A 30 mL protein sample with an initial concentration of 0.132 µg/50 µL was concentrated using an Amicon Ultra Centrifugal Filter, which retains macromolecules above a specific molecular weight cutoff. The final concentrations of 2 µg/mL and 4 µg/mL were achieved to meet experimental requirements. SDS-PAGE and Western blot analysis revealed the presence of the target protein at approximately 68 kDa, consistent with the expected molecular weight of the recombinant L1L2 chimeric protein (Figure 3). Transmission electron microscopy (TEM) was used to assess the formation of virus-like particles (VLPs). TEM imaging showed that the VLPs were not fully formed, indicating incomplete assembly of the chimeric protein into structured particles (Figure 4).

3.3. Immunogenicity Assay in Mice

The immunogenicity of the HPV type 52 L1L2 vaccine candidate was evaluated *in vivo* in mice using ELISA to quantify the production of specific antibodies. Serum samples were collected on days 28 and 42 post-vaccination, and the resulting antibody titers are summarized in Table 4. The results demonstrated that the L1L2 chimeric protein at a dose of 4 µg induced

Table 3. Quantity of purified protein using AKTA-purifier systems

Purified fraction(s)	Absorbance	Concentration (µg/50 µL)
AKTA 21.11.23 E 2B5	3.6755	0.13±0.0269
AKTA 21.11.23 E 3A1	3.6455	0.13±0.0212
AKTA 21.11.23 E 3A2	3.6620	0.13±0.0162
AKTA 21.11.23 E 3B1	3.6515	0.13±0.0042
AKTA 21.11.23 E 3B2	3.6410	0.13±0.0346
AKTA 21.11.23 E 3B5	3.6195	0.13±0.0268

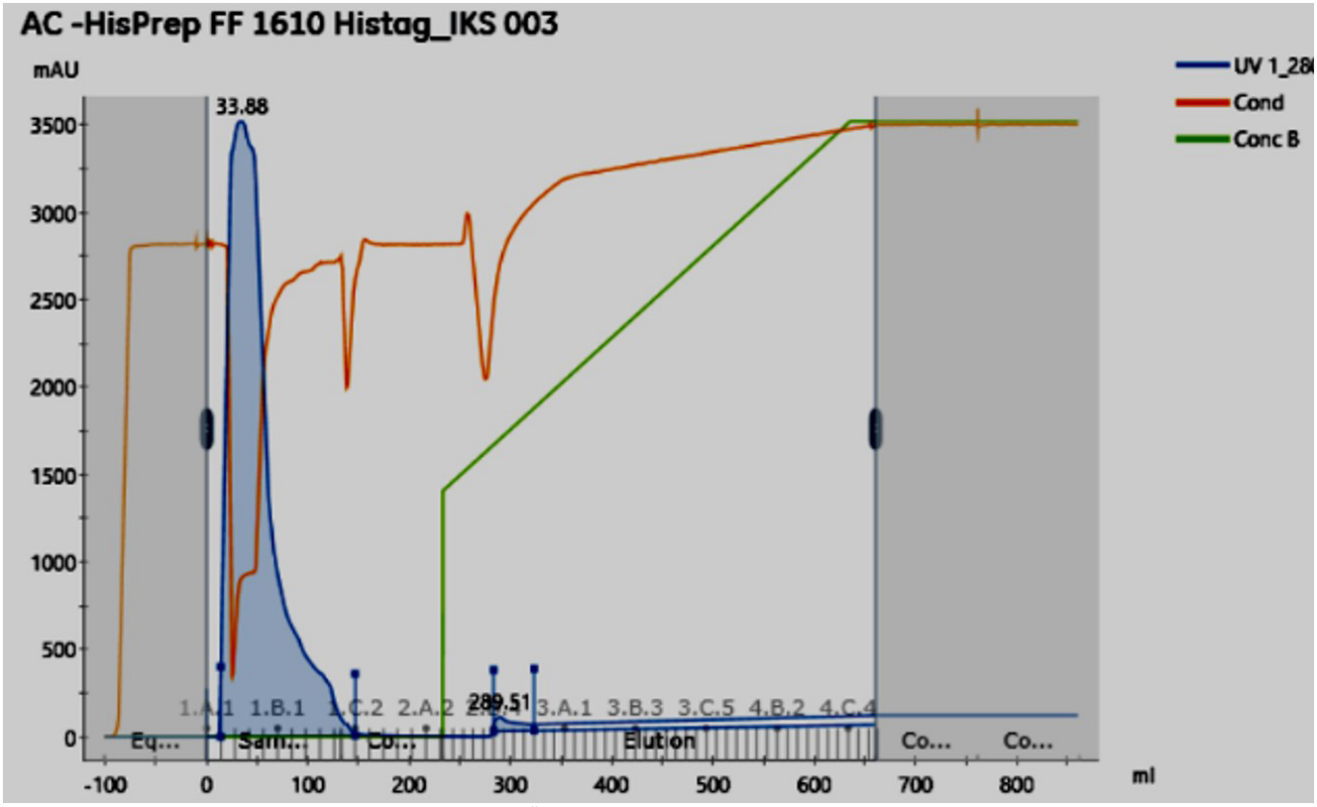
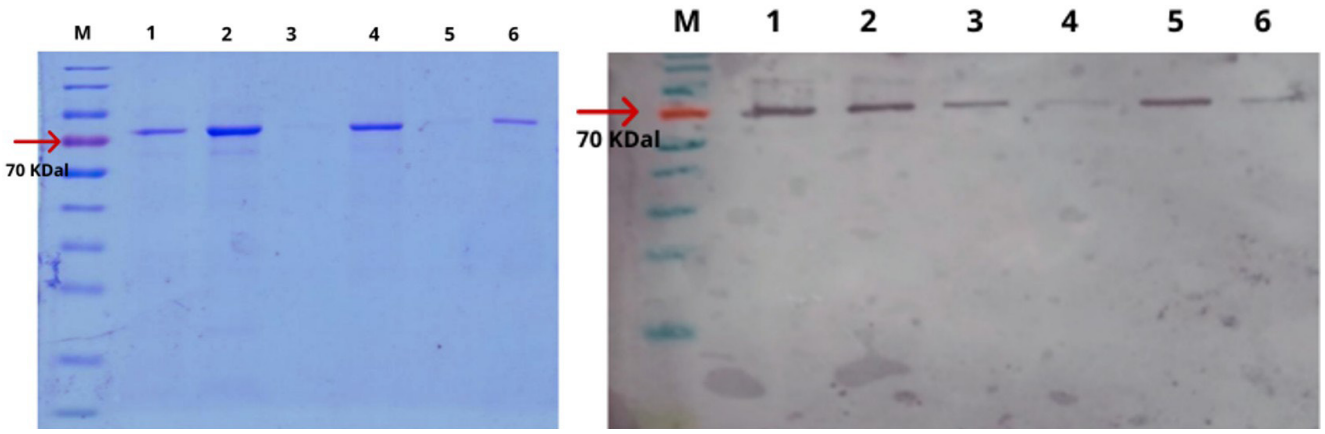


Figure 2. Protein purification profile obtained using the ÄKTA chromatography system. Blue line showed the UV detector; red line showed conductivity of the sample, equilibration buffer, elution buffer; green line showed concentration of buffer B during elution process which set to gradient between buffer A and B



A B

Figure 3. Analysis of purified protein fractions: (A) SDS-PAGE, (B) Western blot. L1L2 chimeric protein produced in batches 1–6 exhibits a molecular weight of approximately 68 kDa. M: protein marker; 1-6: number of fractions after protein purification using FPLC ÄKTA

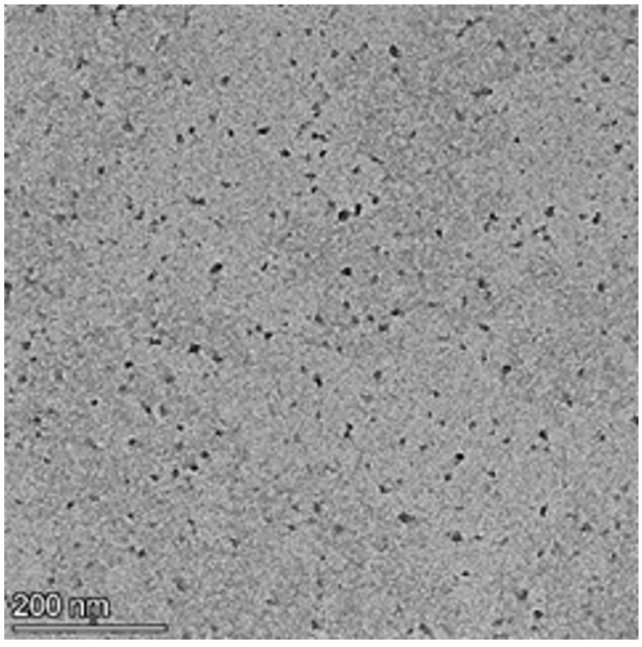


Figure 4.The TEM (transmission electron microscopy) analysis of the L1L2 chimeric vaccine candidate showed that the expected virus-like particles (VLPs) had not yet formed

Table 4. Antibody titer profiles of experimented mice using ELISA from different observation periods

Treatment(s)	Day-28 Concentration (ng/µL)	Day-42 concentration (ng/µL)
Positive control HPV type 52 L1 protein (Creative Diagnostic, 2 µg)	200.53	85.53
Positive control (Gardasil, 2 µg)	17.82	21.04
Negative control ($AlPO_4$ 20 µg/200 µL)	0.61	0.51
HPV type 52 L1L2 chimeric protein, dose 1 (2 µg)	-0.13	0.46
HPV type 52 L1L2 chimeric protein, dose 2 (4 µg)	1.82	1.94

detectable antibody responses on both day 28 and day 42. However, these responses were lower than those observed in the positive control group vaccinated with either the HPV type 52 L1 commercial protein or the commercial Gardasil vaccine.

For the neutralizing antibody assay, HPV type 52 pseudoviruses (PsV) were produced in HEK293FT cells, allowing for efficient PsV production. The neutralization assays demonstrated that serum from mice immunized with the 4 µg dose of the L1L2 chimeric protein exhibited significant neutralizing activity, as indicated by the reduction in fluorescent intensity at days 28 and 42 (Figure 5).

Histopathological analyses of the liver and kidneys were conducted to assess potential vaccine-induced tissue toxicity. Microscopic examination revealed treatment-dependent differences (Figure 6). In the negative control group, mild inflammatory cell infiltration was observed, accompanied by minimal vascular congestion, indicating a lower degree of immune activation. In the positive control group which was treated with HPV type 52 L1 commercial protein (Creative Diagnostic), hepatocellular necrosis and vascular congestion were observed, likely reflecting tissue damage associated with

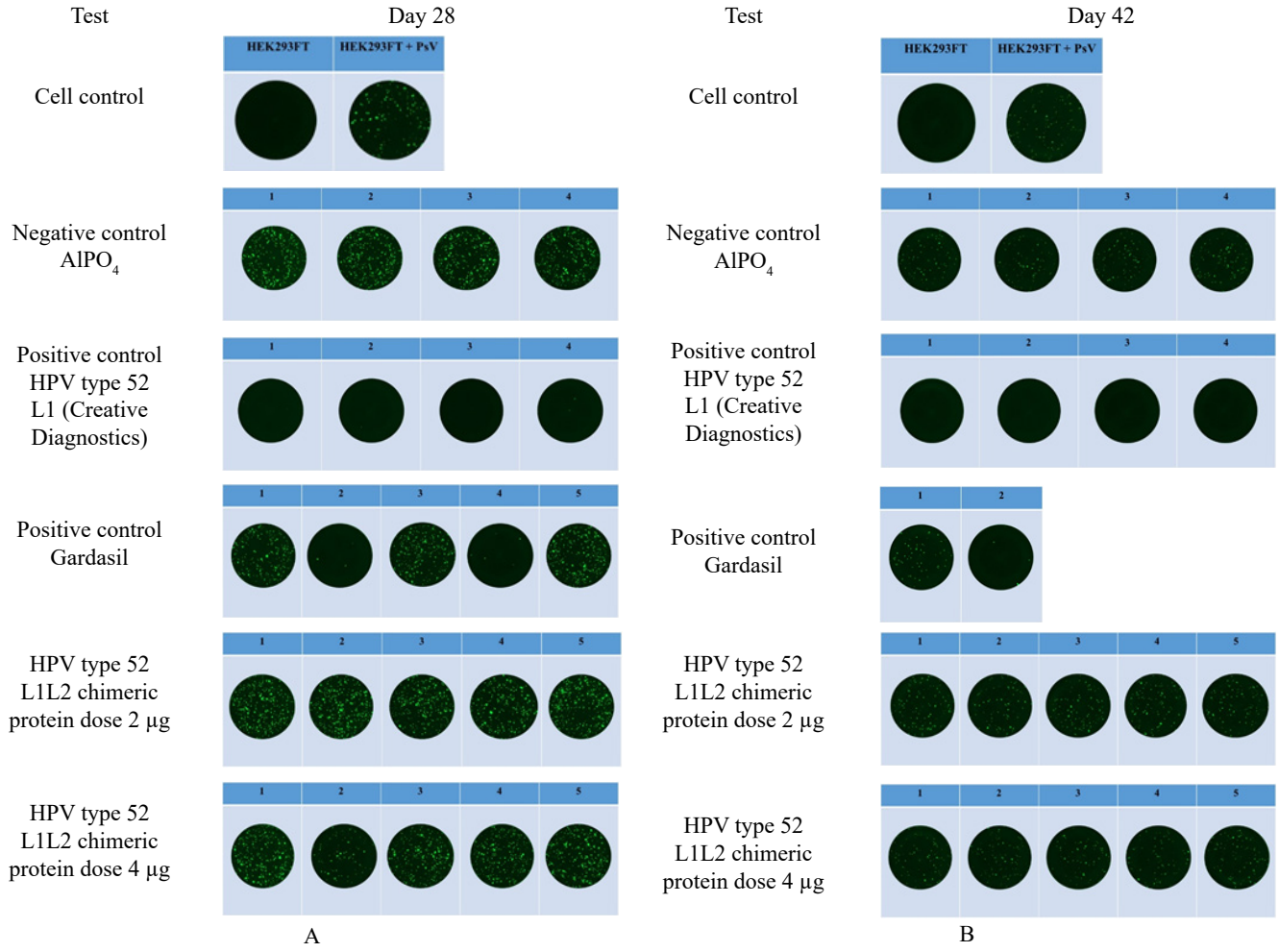


Figure 5. Neutralizing antibody assay using sera collected from mice at (A) day 28 and; (B) day 42 post-immunization. The cell controls included un-infected HEK293 FT cells and infected HEK293 FT cells. The negative controls were treated with $AlPO_4$ adjuvant. The positive controls included HPV type 52 L1 commercial protein (Creative Diagnostic) and Gardasil nonavalent commercial vaccine. The L1L2 chimeric protein was tested at doses of 2 µg and 4 µg, with mice 1–5 representing individual samples. The highest neutralizing activity was observed in the HPV type 52 L1 commercial protein, as indicated by the absence of green fluorescence. The L1L2 chimeric protein at 4 µg dose showed a slightly reduction in green fluorescence at day 42 post-immunization compared to the cell control infected with pseudovirus, indicating the formation of neutralizing antibodies in the mouse sera

immune activation or antigen-induced inflammatory responses (Figure 6A). On the other hand, the group which was given L1L2 chimeric protein did not show any congestion in the liver (Figure 6C) compared to the positive control group. The Mann Whitney test followed by Kruskal Wallis test showed $p<0.05$, which indicated that there was significant difference of liver congestion between groups. Furthermore, in both positive control and the L1L2 chimeric protein treated groups showed no congestion in the kidney as shown in Figure 6B and 6D, respectively. The Mann Whitney test followed by Kruskal Wallis test showed $p = 0.427$, which indicated that there was no significant difference of kidney congestion between groups.

4. Discussion

The L1L2 chimeric protein of HPV 52 was previously constructed (Ikramullah *et al.* 2024), and the expression system employed for recombinant protein production was *Escherichia coli* BL21 (DE3).

In this study, we optimized the heterologous expression of HPV type 52 L1L2 chimeric protein in *E. coli* BL21 to support further in vivo immunogenicity study in mice. The strain, *E. coli* is advantageous as a protein expression host due to its low-cost growth media, rapid growth rate, high protein expression levels, capacity for high cell density culture, relatively low production costs, and straightforward techniques

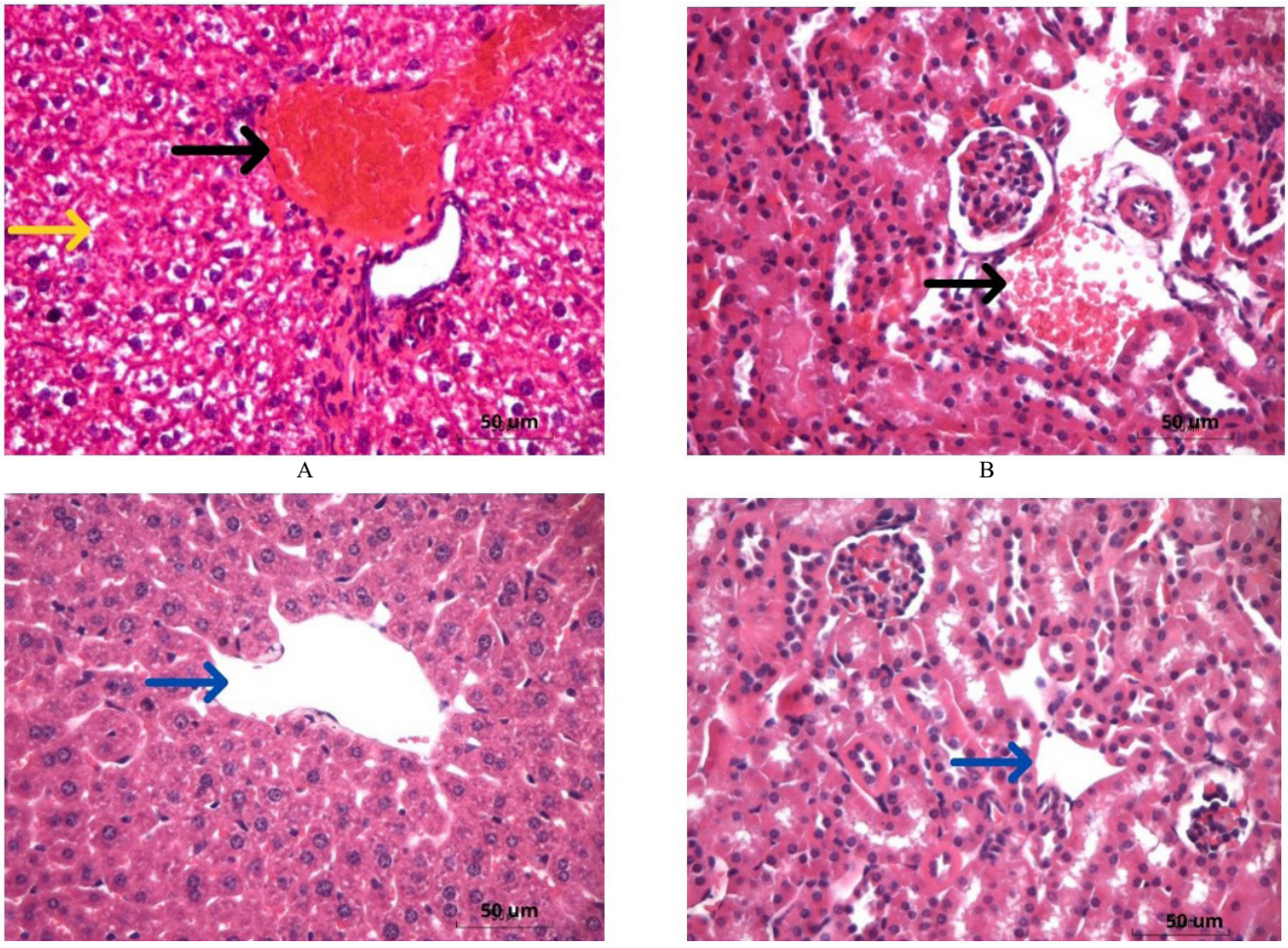


Figure 6. Histopathological evaluation of mouse organs: (A) liver of positive control (Creative Diagnostics) showing necrosis (yellow arrow) and vascular congestion (black arrow); (B) kidney of positive control group (Creative Diagnostics) showing vascular congestion; (C) liver of L1L2 chimeric vaccine group without vascular congestion (blue arrow); (D) kidney of L1L2 chimeric vaccine group showing absence of vascular congestion (blue arrow). Picture was magnified 400x, scale showed 50 µm

for recombinant protein production (Roos *et al.* 2020).

The selection of growth media and temperature proved critical for maximizing protein yield. In particular, M9 and Super Broth at 25°C supported higher levels of L1L2 chimeric protein, indicating that nutrient composition and growth conditions directly influence both the stability and quantity of recombinant proteins (Yu *et al.* 2022; Bobyr *et al.* 2024). The recombinant L1L2 chimeric protein can be expressed and purified effectively in *E. coli* using a His-tag fusion, which enhances solubility, stabilizes the protein against degradation, and facilitates affinity purification. To enhance the solubility of the recombinant protein, a His-tag fusion, consisting of six histidine residues (~0.84 kDa), was incorporated. The His-tag not only facilitated protein purification but also provided protection against degradation, increased recovery efficiency, and improved solubility without compromising the structural integrity or functional properties of the fusion protein (Mustopa *et al.* 2022; Ikramullah *et al.* 2024; Al-Adawiah *et al.* 2024).

Despite these advantages, the current results indicate that further optimization is required to achieve the formation of fully functional VLPs. Although the Western blot analysis confirmed that L1L2 chimeric protein were consistently detected all together with 6x His-tag, the TEM analysis revealed that VLP formation was suboptimal as it is differed from other reports, as shown in Figure 4 (Hendrix 2008; Panwar *et al.* 2022). This observation supports the notion that the recombinant protein may not have fully matured or assembled into VLPs under the current experimental conditions. Moreover, the incomplete assembly suggests that further optimization of expression conditions, folding, or post-translational processing may be required to achieve fully functional VLPs.

In vivo experiments using treated and untreated mice display different responses. While the L1L2 chimeric protein can stimulate an immune response, in the mice, its immunogenicity still requires further enhancement to achieve antibody levels comparable to those induced by established commercial vaccines. To assess the functional activity of these antibodies, neutralization assays are required. The World Health Organization (WHO) recommends neutralizing antibody assays as the gold standard for evaluating the protective potential of antibodies induced by HPV vaccines (WHO 2009). These assays measure the capacity of antibodies to inhibit viral entry into host cells, thereby preventing infection. Although technically demanding, they provide a comprehensive assessment of all potentially protective antibodies, regardless of immunoglobulin subclass (Schiller and Lowy 2018). Such assays evaluate whether the antibodies can effectively prevent pseudovirus entry into target cells, providing a direct measure of the candidate vaccine's protective potential against HPV infection (Chairunnisa *et al.* 2025).

The *in vitro* results showed that while the positive control group (Gardasil) showed variable antibody responses, the L1L2 chimeric vaccine group elicited consistent neutralization, suggesting stable induction of functional antibodies. These findings suggest that the L1L2 chimeric vaccine is capable of eliciting a protective immune response, supporting its further development as a potential HPV vaccine candidate.

For the last parameter, mice immunized with the L1L2 chimeric protein showed no signs of vascular congestion in both the liver and kidney, indicating the absence of significant organ toxicity (Pan *et al.* 2021). Moreover, statistical analysis using the Mann-Whitney and Kruskal-Wallis tests confirmed that the L1L2 chimeric vaccine group displayed lower histopathological severity scores compared with the positive control, supporting its safety and immunogenic potential.

To summarize our findings, the HPV 52 L1L2 chimeric protein, expressed in *E. coli* BL21, was successfully purified and obtained at sufficient concentrations. Transmission electron microscopy indicated that the produced L1L2 chimeric protein had not yet assembled into VLPs. Nevertheless, SDS-PAGE and Western blot analysis confirmed the purity of the protein, with a distinct band corresponding to a molecular weight of 68 kDa. *In vivo* evaluation in Balb/c mice demonstrated that the vaccine elicited HPV-specific antibody responses, although the immunogenicity observed was lower than that of commercially available HPV vaccines. Moreover, the *in vitro* neutralization assay showed detectable neutralizing activity by day 42. Further optimization of the protein formulation and delivery is expected to enhance its immunogenicity. Toxicological evaluation confirmed that the vaccine did not induce observable damage to the liver or kidneys, supporting its safety profile in mice.

Conflict of Interest

The authors declare that they have no conflict of interest.

Acknowledgements

The authors gratefully acknowledge the support of the National Research and Innovation Agency (BRIN) in collaboration with the Indonesia Endowment Fund for Education (LPDP) of the Ministry of Finance of the Republic of Indonesia for providing research funding through the "Riset dan Inovasi untuk Indonesia Maju" (RIIM) Program, Competition Batch 3 with the contract number: 4/IV/KS/05/2023 and 13955/IT3/PT.01.03/P/B/2023. The authors also thank the Primate Research Center IPB and BRIN, particularly the Research Center for Genetic Engineering, for their assistance.

References

Al Adawiah, R., Mustopa, A.Z., Budiarti, S., Umami, R.N., Hertati, A., Irawan, H., Ikramullah, M.C., Arwansyah, A., Mamangkey, J., Kartikasari, I., Darusman, H.S., 2024. Molecular dynamics simulation and purification of chimeric L1/L2 protein from human papillomavirus type 52 expressed in *Escherichia coli* BL21 (DE3). *J. Immunoass. Immunochemis.* 45, 395–414. https://doi.org/10.1080/15321819.2024.2376034

Bobyr, I.M., Bondarenko, V.L., Iungin, O.S., 2024. Optimization of culture media for industrial cultivation of the recombinant strain *Escherichia coli* BL21. *Український журнал природничих наук.* 2024, 17–24.

Buck, C.B., Pastrana, D.V., Lowy, D.R., Schiller, J.T., 2005. Generation of HPV pseudovirions using transfection and their use in neutralization assays. *Methods Mol. Med.* 119, 445–462. https://doi.org/10.1385/1-59259-982-6:445

Chabeda, A., van Zyl, A.R., Rybicki, E.P., Hitzeroth, I.I., 2019. Substitution of human papillomavirus type 16 L2 neutralizing epitopes into L1 surface loops: The effect on virus-like particle assembly and immunogenicity. *Front. Plant. Sci.* 10, 779. https://doi.org/10.3389/fpls.2019.00779

Chairunnisa, S., Mustopa, A.Z., Bela, B., Firdaus, M.E.R., Irawan, S., Arifah, R.K., Irawan, H., Nurfatwa, M., Umami, R.N., Ekawati, N., Hertati, A., Hasan, N., 2024. Expression and scale-up production of recombinant human papillomavirus type 52 L1 protein in methylotrophic yeast *Hansenula polymorpha*. *J. Genet. Eng. Biotechnol.* 22, 100342. https://doi.org/10.1016/j.jgeb.2023.100342

Chairunnisa, S., Mustopa, A.Z., Bela, B., Arifah, R.K., Umami, R.N., Firdaus, M.E.R., Ekawati, N., Irawan, H., Irawan, S., Nurfatwa, M., Hertati, A., Swasthikawati, S., Novianti, E., Kusumawati, A., Darusman, H.S., 2025. Expression, purification, and immunogenicity study of human papillomavirus type 52 virus-like particles produced in *Hansenula polymorpha. Biologicals.* 90, 101831. https://doi.org/10.1016/j.biologicals.2025.101831

Hendrix, S.L., 2008. Assessing human papillomavirus vaccine efficacy and safety. *J Am Osteopath Assoc.* 108, 8–12.

Huang, H., Hu, X.F., Zhao, F.H., Garland, S.M., Bhatla, N., Qiao, Y.L., 2015. Estimation of cancer burden attributable to infection in Asia. *J. Epidemiol.* 25, 626–638. https://doi.org/10.2188/jea.JE20140215

Ikramullah, M.C., Mustopa, A.Z., Wibawa, T., Hertati, A., Umami, R.N., Ratna, L.T., Irawan, S., Firdaus, M.E.R., Darusman, H.S., 2024. Cloning, expression, and bioinformatics modeling of human papillomavirus type 52 L1L2 Chimeric protein in *Escherichia coli* BL21 (DE3). *HAYATI J. Biosci.* 31, 891–902. https://doi.org/10.4308/hjb.31.5.891-902

Liu, C., Yao, Y., Yang, X., Bai, H., Huang, W., Xia, Y., Ma, Y., 2015. Production of recombinant human papillomavirus type 52 L1 protein in *Hansenula polymorpha* formed virus-like particles. *J. Microbiol. Biotechnol.* 25, 936–940. https://doi.org/ 10.4014/jmb.1412.12027

Longet, S., Schiller, J.T., Bobst, M., Jichlinski, P., Nardelli-Haefliger, D., 2011. A murine genital-challenge model is a sensitive measure of protective antibodies against human papillomavirus infection. *J. Virol.* 85, 13253–13259. https://doi.org/10.1128/jvi.06093-11

Lou, P.J., Phongsamart, W., Sukarom, I., Wu, Y.H., Zaidi, O., Du, F., Simon, A., Bernauer, M., 2024. Systematic literature review on the clinical and economic burden of human papillomavirus–related diseases in select areas in the Asia Pacific region. *Hum. Vaccin. Immunother.* 20, 2425535. https://doi.org/10.1080/21645515.2024.2425535

Mustopa, A.Z., Meilina, L., Irawan, S., Ekawati, N., Fathurahman, A.T., Triratna, L., Kusumawati, A., Prastyowati, A., Nurfatwa, M., Hertati, A., 2022. Construction, expression, and *in vitro* assembly of virus-like particles of L1 protein of human papillomavirus type 52 in *Escherichia coli* BL21 DE3. *J. Genet. Eng. Biotechnol.* 20, 19. https://doi.org/10.1186/s43141-021-00281-5

Pan, Q., Zhang, Y., Liu, A., Cui, H., Gao, Y., Qi, X., Liu, C., Zhang, Y., Li, K., Gao, L., Wang, X., 2021. Development of a novel avian vaccine vector derived from the emerging fowl adenovirus 4. *Front. Microbiol.* 12, 780978. https://doi.org/10.3389/fmicb.2021.780978

Pang, J., Thomas, N., Tsuchiya, D., Parmely, T., Yan, D., Xie, T., Wang, Y., 2021. Step-by-step preparation of mouse eye sections for routine histology, immunofluorescence, and RNA in situ hybridization multiplexing. *STAR protocols*. 2, 100879. https://doi.org/10.1016/j.xpro.2021.100879

Panwar, K., Godi, A., Cocuzza, C.E., Andrews, N., Southern, J., Turner, P., Miller, E., Beddows, S., 2022. Multiplex human papillomavirus L1L2 virus-like particle antibody binding assay. *MethodsX.* 9, 101776. https://doi.org/10.1016/j.mex.2022.101776

Parkin, D.M., Almonte, M., Bruni, L., Clifford, G., Curado, M.P., Pineros, M., 2008. Burden and trends of type-specific human papillomavirus infections and related diseases in the Latin America and Caribbean region. *Vaccine.* 26, 1–15. https://doi.org/10.1016/j.vaccine.2008.05.043

Roos, N., Breiner, B., Preuss, L., Lilie, H., Hipp, K., Herrmann, H., Horn, T., Biener, R., Iftner, T., Simon, C., 2020. Optimized production strategy of the major capsid protein HPV 16L1 non-assembly variant in *E. coli. Protein. Expr. Purif.* 175, 105690. https://doi.org/10.1016/j.pep.2020.105690

Ruan, Y., Li, H., Liu, M., Cao, G., Xu, X., Han, L., Li, F., 2023. A retrospective analysis of human papillomavirus (HPV) prevalence and genotype distribution among 25,238 women in Shanghai, China revealed the limitations of current HPV-based screening and HPV vaccine. *Cancer Epidemiol.* 84, 102372. https://doi.org/10.1016/j.canep.2023.102372

Schiller, J., Lowy, D., 2018. Explanations for the high potency of HPV prophylactic vaccines. *Vaccine.* 36, 4768–4773. https://doi.org/10.1016/j.vaccine.2017.12.079

Tripathi, N.K., Shrivastava, A., 2019. Recent developments in bioprocessing of recombinant proteins: expression hosts and process development. *Front. Bioeng. Biotechnol.* 7, 420. https://doi.org/10.3389/fbioe.2019.00420

Venkataraman, S., Hefferon, K., Makhzoum, A., Abouhaidar, M., 2021. Combating human viral diseases: will plant-based vaccines be the answer?. *Vaccines. 9*, 761. https://doi.org/10.3390/vaccines9070761

[WHO] World Health Organization, 2009. Human Papillomavirus Laboratory Manual. 1st ed. WHO; Geneva, Switzerland: 2009. Available at: https://www.who.int/publications/i/item/WHO-IVB-10.12. [Date accessed: 4 December 2025]

Yu, M., Chi, X., Huang, S., Wang, Z., Chen, J., Qian, C., Han, F., Cao, L., Li, J., Sun, H., Zhou, L., 2022. A bacterially expressed triple-type chimeric vaccine against human papillomavirus types 51, 69, and 26. *Vaccine*. 40, 6141–6152. https://doi.org/10.1016/j.vaccine.2022.09.010

Zhang, J., Ke, Y., Chen, C., Jiang, Z., Liu, H., Liu, Y., Cao, H., 2025. HPV cancer burden by anatomical site, country, and region in 2022. *Sci. Rep.* 15, 21048. https://doi.org/10.1038/s41598-025-06700-8

Zhao, X., Zhang, Y., Trejo-Cerro, O., Kaplan, E., Li, Z., Albertsboer, F., El Hammiri, N., Mariz, F.C., Banks, L., Ottonello, S., Müller, M., 2024. A safe and potentiated multi-type HPV L2-E7 nanoparticle vaccine with combined prophylactic and therapeutic activity. *NPJ Vaccines.* 9, 119. https://doi.org/10.1038/s41541-024-00914-z