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Expression and characterization of HPV-16 L1 capsid protein in *Pichia pastoris*

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Abstract

Human papillomaviruses (HPVs) are responsible for the most common human sexually transmitted viral infections. Infection with high-risk HPVs, particularly HPV16, is associated with the development of cervical cancer. The papillomavirus L1 major capsid protein, the basis of the currently marketed vaccines, self-assembles into virus-like particles (VLPs). Here, we describe the expression, purification and characterization of recombinant HPV16 L1 produced by a methylotrophic yeast. A codon-optimized HPV16 L1 gene was cloned into a non-integrative expression vector under the regulation of a methanol-inducible promoter and used to transform competent *Pichia pastoris* cells. Purification of L1 protein from yeast extracts was performed using heparin-sepharose chromatography, followed by a disassembly/reassembly step. VLPs could be assembled from the purified L1 protein, as demonstrated by electron microscopy. The display of conformational epitopes on the VLPs surface was confirmed by hemagglutination and hemagglutination inhibition assays and by immuno-electron microscopy. This study has implications for the development of an alternative platform for the production of a papillomavirus vaccine that could be provided by public health programs, especially in resource-poor areas, where there is a great demand for low-cost vaccines.

Introduction

Human papillomaviruses (HPVs) are epitheliotropic pathogens, etiologically associated with benign warts and malignant tumors. According to data from the World Health Organization (WHO), there are 630 million cases of sexually transmitted diseases (STD) associated to this virus worldwide. The annual incidence of sexually transmitted HPV infections is close to 5.5 million in the United States alone [1]. About 75% of sexually active people are exposed to HPV sometime in their lives [2]. Of the approximately 120 HPV types identified so far [3], more than 40 infect the epithelial lining of the anogenital tract and other mucosal areas of the body [4]. These types can be classified as lower high-oncogenic risk, according to their ability to promote malignant transformation. The high-risk HPVs are encountered in more than 99% of cervical tumors [5], and HPV16 is found in approximately 50% of the cases [6]. Cervical cancer is still the second most common cancer in women worldwide [7], although it is a disease that could theoretically be prevented.

The HPV capsid is composed of two structural proteins, L1 and L2. The papillomavirus major capsid protein L1 is intrinsically able to self-assemble into virus-like particles [8–12]. These particles are morphologically indistinguishable from native virions and present the conformational epitopes necessary for the induction of high titers of neutralizing antibodies [8].

Several approaches for expressing recombinant L1 from HPV16 have been tested using bacteria, e.g., *Salmonella typhimurium* [13], *Escherichia coli* [14, 15], *Shigella flexneri* [16], *Lactobacillus casei* [17], *Lactococcus lactis* [18], yeast, e.g., *Saccharomyces cerevisiae* [19–21], *Schizosaccharomyces pombe* [22], baculovirus-infected insect cells [23], transgenic plants, e.g., tobacco and potato [24], and mammalian cells [25]. Bacterial expression systems have proven to be quite limited in producing economically significant quantities of recombinant HPV-16 L1 VLPs [26]. Furthermore, protein preparations from bacteria carry the risk of contamination with endotoxins, a disadvantage compared with protein preparations from yeast cells. Other eukaryotic systems, such as insect and mammalian cells, have the disadvantage of low expression levels combined with complex growth requirements and slow growth rate, leading to high production costs, which may prevent the widespread application of a L1 vaccine in less developed countries. For this reason, expression systems using yeasts seem to be very attractive. We chose the *Pichia pastoris* system for heterologous protein expression because of the powerful genetic techniques available, high expression levels, rapid growth rate on relatively simple media and well-established fermentation technology, coupled with its economy of use. The efficient and tightly regulated promoter from the alcohol oxidase I gene (*AOX1*) was used to drive the expression of the HPV-16 L1 gene. The safety and convenience of yeast expression systems provide competitive processes for the production of a broad spectrum of biopharmaceuticals [27].

To date, two prophylactic HPV vaccines are on the market, Gardasil™ (Merck) and Cervarix™ (Glaxo-SmithKline). Studies have shown that these vaccines are safe, well tolerated and highly immunogenic. Gardasil™, produced in *Saccharomyces cerevisiae*, has been shown to be effective for 5 years for prevention of infection by HPV types 6, 11, 16 and 18 [28, 29]. Cervarix™, produced in baculovirus-infected insect cells, has also shown sustained efficacy for up to 4.5 years [29, 30]. However, these vaccines are still inaccessible to the majority of the population in economically disadvantaged regions. The commercial HPV vaccines cost at least US\$360 for one person (three doses), an amount that is much higher than the annual per capita health expenditure of less developed countries. Therefore, there is a great need for other strategies for the production of cheaper HPV vaccines that could be provided by public health programs, allowing a greater penetration into these

communities. Here we describe the production, assembly and characterization of HPV-16 L1 structural protein in *P. pastoris*, focusing on the development of a prophylactic vaccine for the public health system in Brazil.

Materials and methods

Strains and media

Escherichia coli DH5 [*F* 80*lacZ* M15 (*lacZYA*-argF)U169 *recA1 endA1 hsdR*17(*r_k⁻*, *m_k⁺*) *phoA supE44 thi-1 gyrA96 relA1* ⁻] (Invitrogen) was used as the host strain for amplification and propagation of recombinant DNA. *E. coli* was cultured at 37°C in LB medium (0.5% yeast extract, 1% NaCl, 1% tryptone) supplemented with 25 µg/ml zeocin (Invitrogen) when necessary. *P. pastoris* GS115 (*his4*) and KM71 (*his4, aox1*: ARG4, *arg4*) strains were purchased from Invitrogen. Yeasts were grown at 30°C on rich YPD medium (1% yeast extract, 2% Bactopeptone, 2% glucose) and BMMY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate buffer [pH 6.0], 1.34% yeast nitrogen base, 4 × 10⁻⁵% biotin, 4 × 10⁻³% histidine, and 0.5% methanol) supplemented with 100 µg/ml zeocin when necessary.

Construction of expression plasmids

An HPV16 L1 gene specifically codon-optimized for *P. pastoris* was amplified by polymerase chain reaction (PCR) from the plasmid vector pPICZB/L1 using Pfu DNA polymerase. PCR was carried out using the following oligonucleotide primers: L1 cod_opt *Forward* (5' ACC ATG TCT TTG TGG TTG CCA 3') and L1 cod_opt *Reverse* (5' GCG CGC TCT AGA CTA CTA TTA 3'). The resulting fragment was incubated with Taq DNA polymerase (Invitrogen) in the presence of 0.2 mM dATP and then ligated into pGEM-T Easy Vector (Promega). The L1 fragment was released from pGEM-T Easy after digestion with *SpeI*, filling in the single-stranded termini using Klenow polymerase (Invitrogen) and dNTPs, and finally, digestion with *EcoRI*. The L1 gene was ligated into the pPICHOLI expression vector (Mobitech) after digestion with *SaII*, filling in the single-stranded termini using Klenow polymerase (Invitrogen) and dNTPs, and digestion by *EcoRI*. The resulting expression vector was named pPICHOLI/L1. DNA sequence analysis of all constructs was performed on an automated DNA sequencer (ABI 3100) using the dideoxy termination method [31]. The wild-type HPV16 L1 gene was amplified by PCR from plasmid pEVmod/L1 [8], using specific oligonucleotide primers with unique restriction sites. The PCR product was inserted at the 5' *EcoRV* to 3' *SaII* fragment into the multiple cloning site of pPICHOLI expression vector. Transformation and induction from both yeast strains were performed as described below.

Yeast transformation

Five micrograms of pPICHOLI/L1 was added to 40 µl of competent cells and transferred into a 2-mm-gap electroporation cuvette that was precooled on ice. *P. pastoris* strains were transformed by electroporation at 1.5 kV, 200 µs, and 25 µF with a Gene Pulser II system (Bio-Rad). Immediately after the pulse, 1 ml cold 1 M sorbitol was added, and the suspension was transferred into a sterile 2-ml Eppendorf tube. Cells were grown for 2 h at 30°C with shaking. Aliquots of 150 µl were spread onto agar plates containing YPD supplemented with 100 µg/ml zeocin and incubated for 3 days at 30°C.

Analysis of transformants and protein expression

Yeast colony PCR was performed as described [32]. Briefly, yeast cells were transferred with a pipette tip to 1.5-ml microcentrifuge tubes containing 20 µL of 0.25% SDS. Tubes were vortexed for 10 s, heated to 90°C for 3 min and centrifuged at 10,000×*g* for 30 s.

About 1 μ L of the supernatant was added to the PCR mixture, which contained Triton X-100 at a final concentration of 1%. Yeast colonies that were positive for L1 DNA were inoculated in 5 ml of YPD medium supplemented with 100 μ g/ml zeocin and grown overnight with shaking (250 rpm) at 30°C. Fresh BMMY medium supplemented with 100 μ g/ml zeocin and 40 mg/ml histidine was inoculated 1:10 with the overnight culture (10% final concentration of cell suspension) and grown at 30°C with shaking to an OD₆₀₀ of 1.0. Methanol was added to final concentration of 0.5% (v/v) to induce protein expression, and cultures were grown at 30°C with shaking (250 rpm) for 2–3 days. To maintain the induction of the recombinant protein, 100% methanol was added every 24 h to the culture to a final concentration of 0.5%.

Cell lysis

Yeast cells were harvested by centrifugation at 2,100 $\times g$ for 5 min and resuspended in 0.3 ml of breaking buffer (50 mM sodium phosphate, pH 7.4, 1 mM PMSF, 1 mM EDTA, 5% glycerol). An equal volume of micro glass beads (0.5 mm) was added to the tubes, and a total of eight cycles of vortexing and incubation on ice (30 s each) were performed. Samples were centrifuged at 5,000 $\times g$ for 10 min, and the supernatant was transferred to a new microcentrifuge tube.

SDS-PAGE and Western blot analysis

SDS-PAGE was performed by the method of Laemmli [33]. Samples were diluted (10:1) in SDS-loading buffer (1 M Tris-HCl, pH 6.8, 10% SDS, 0.5% bromophenol blue, 50% glycerol, 1 M β -mercaptoethanol), boiled for 10 min and centrifuged at 10,000 $\times g$ for 5 min. Samples were then separated by gel electrophoresis using 10% polyacrylamide gels and either stained with Coomassie brilliant blue (Pierce) or electrotransferred to nitrocellulose membranes. The membranes were blocked overnight in 4°C in phosphate-buffered saline (PBS), pH 7.4, containing 0.05% Tween 20 (PBST) supplemented with 10% nonfat milk and then incubated for 2 h at room temperature with properly diluted CamVir anti-HPV16 L1 monoclonal antibody (Chemicon). The membranes were washed three times in PBS-T for 10 min and then incubated for 1 h at room temperature with peroxidase-conjugated goat anti-mouse immunoglobulin (IgG, Sigma-Aldrich) diluted 1:10,000 in PBST with 5% nonfat milk. This was followed by three 10-min washes in PBST. The L1 protein was detected using an enhanced chemiluminescence detection kit (ECL, GE Healthcare).

Heterologous protein quantitation

For the determination of expression levels of the recombinant L1 protein, two L1-expressing *P. pastoris* clones were subjected to analysis after 48 h of induction. Yeast cell extracts were properly diluted in SDS-loading buffer (1:60 and 1:150) and compared to different amounts of purified recombinant L1 VLPs expressed in insect cells [23]. Samples were applied to an SDS-10% polyacrylamide gel and blotted as described previously. L1 detection was performed as described above and the quantification of L1 protein in the cell lysates was obtained after comparison of the relative intensity of the bands with purified L1 of known concentration by densitometry [17].

Protein purification

After 48 h of induction, cultures were harvested by centrifugation at 4,600 $\times g$ for 10 min at 4°C in a Sorvall S1500 rotor and resuspended in 100 ml of cold lysis buffer (PBS, 1 mM EDTA, 1 mM PMSF). Cells were broken by eight 30-s cycles of vigorous vortexing in a Beadbeater (Biospec Products Inc.) containing 0.5-mm micro glass beads. Clarification of cell lysate was carried out by centrifugation for 30 min at 7,200 $\times g$ and 4°C, followed by filtration in an AP20 Millipore membrane. Affinity chromatography was performed using 5

ml of heparin–Sepharose CL-6B (GE Healthcare) in a 1.0-cm-diameter column preequilibrated with PBS containing 0.3 M NaCl. The clarified cell lysate was pumped onto the column at 0.5 ml/min and then washed with 20 bed volumes of equilibration buffer containing 0.4 M NaCl. Elution was performed at 1.0 ml/min with a 10-volume salt gradient from 0.5 to 2.0 M NaCl. Absorbance at $_{280\text{nm}}$ was monitored using a spectrophotometer (Biomate 3, Thermo Electron Corporation). Samples were analyzed by SDS-PAGE and Western blot procedures.

Disassembly and reassembly of virus-like particles

Disassembly of VLPs was carried out by dialysing the aliquots containing the VLPs against a solution of PBS, pH 8.2, containing 0.166 M NaCl, 2 mM DTT and 2 mM EDTA. For the reassembly procedure, samples were dialysed against 2 l of PBS, pH 7.0, containing 0.5 M NaCl at 4°C with four changes of buffer [34]. Both the disassembly and reassembly procedures were performed in the presence of 0.01% polysorbate 80 (Sigma) [35]. Aliquots were then loaded onto sucrose cushions (10–35%, w/v) in PBS containing 0.5 M NaCl and 0.01% polysorbate 80 and ultra-centrifuged for 16 h at $183,000\times g$ in a 70Ti rotor (Beckmann). Pellets were resuspended in 500 μl of PBS containing 0.5 M NaCl and 0.01% polysorbate 80.

Electron microscopy

Samples were adsorbed to carbon-coated grids and negatively stained with 2% uranyl acetate. Grids were allowed to air-dry prior to examination with a Zeiss EM 109 transmission electron microscope operated at 80 kV. Micrographs were taken with various magnifications.

Ultrastructural immunocytochemistry

Ten-microliter drops containing the purified HPV-16 L1 VLPs were adhered to 2% parlodion and carbon-coated nickel grids for 10 min at room temperature. Thereafter, they were incubated for 1 h at room temperature in a humid chamber with a primary mouse monoclonal neutralizing antibody against L1 protein from HPV 16 (# C65315 M, Biodesign International, see <https://meridianlifescience.com/bioSpecs/C65315M.doc> for more information on this product) diluted 1:100 in PBS/1.5% BSA/0.01% Tween 20. After rinsing with PBS/1% BSA, samples were incubated for 30 min at room temperature in a humid chamber with a secondary antibody (goat anti-mouse IgG complexed with 10-nm gold particles (Sigma)) diluted 1:100 in PBS/0.5% BSA/0.05% Tween 20, rinsed with PBS and distilled water, contrasted with 2% uranyl acetate for 5 min, and examined with a Zeiss EM 109 transmission electron microscope operated at 80 kV.

Hemagglutination and hemagglutination inhibition assays

Fresh mouse blood (1 ml) was collected in a tube containing 1,000 U of heparin. The sample was suspended in 9 ml of PBS containing 1 mg/ml BSA and centrifuged for 5 min at $1,000\times g$. Erythrocytes were resuspended in 10 ml of PBS containing 1 mg/ml BSA and centrifuged for 5 min at $1,000\times g$. After repeating this procedure three times, the erythrocyte pellet was finally diluted to 1% (v/v) in PBS containing 1 mg/ml BSA. An aliquot of 100 μl of the erythrocyte suspension was mixed with the same volume of VLPs and added to a 96-well V-shaped plate, incubated for 3 h at 4°C, and photographed. For the hemagglutination inhibition assay, VLPs were first incubated with 400 ng of anti-HPV16 L1 neutralizing monoclonal antibody (# C65315 M, Biodesign International, see <https://meridianlifescience.com/bioSpecs/C65315M.doc> for more information on this product) for 1 h at room temperature before being added to the erythrocyte suspension.

Results

Expression of HPV16 L1 protein in *Pichia pastoris*

A codon-optimized L1 gene was expressed in *P. pastoris* strains GS115 and KM71 under the control of the AOX promoter. A 56-kDa protein was detected in the lysates of both *P. pastoris* strains, recognized as HPV16 L1 by Western blotting using the CamVir anti-HPV16 L1 monoclonal antibody (Fig. 1, KM71 and GS115 transformants). Lower molecular-mass bands were also observed. These species are possibly degradation products of L1, since they were not seen in total extracts of *P. pastoris* that did not show expression of the recombinant protein. Other groups have also found degradation patterns after expression of the HPV16 L1 protein in insect cells [8], yeast [36] and bacteria [14, 15, 26, 37]. L1 was not detected in negative control extracts (*P. pastoris* transformed with empty pPICHOLI vector, Fig. 1, lanes EV) and in the non-induced clone (Fig. 1b, lane BI), showing that expression of the heterologous protein was specifically induced in the presence of methanol. In contrast, a *P. pastoris* KM71 strain transformed with the same plasmid containing the wild-type HPV16 L1 gene did not express the L1 protein (Fig. 1a, lane WT), indicating the need for codon optimization for HPV16 L1 expression in *P. pastoris*. Both *P. pastoris* strains, KM71 and GS115, showed similar levels of recombinant protein expression. The concentration of L1 protein in yeast cell extracts was estimated by densitometry based on a standard curve produced using VLPs of known concentration expressed in insect cells as described [17]. The results showed that the *P. pastoris* clones (two KM71 clones) had an average expression of 14.2 and 13.4 mg/l of L1 protein, respectively (data not shown).

L1 purification and characterization

Purification of L1 protein was achieved after heparin–Sephacrose chromatography as described (“Materials and methods”) [38]. The high-salt-eluted species was a single protein species of approximately 56 kDa (Fig. 2a) that was specifically recognized by the monoclonal antibody anti-L1 (HPV16) CamVir (Fig. 2).

The majority of the L1 protein was eluted in fractions from 0.5 M NaCl to 0.7 M NaCl. A significant amount of the L1 protein did not bind to the resin or was eluted with 0.4 M NaCl. This observation may be attributed to L1 monomers, since it has been demonstrated that the interaction between L1 and heparin requires intact capsomeres or VLPs [39]. Purified L1 protein was then analyzed by electron microscopy. Although some VLPs were seen, samples consisted predominantly of pentamers, as well as amorphous material (Fig. 3a). A disassembly and reassembly protocol was then used, as described previously [40]. After reassembly, samples were concentrated by centrifugation and visualized by electron microscopy (Fig. 3b). Intact particles were observed in these preparations, and the VLPs appeared more regular and defined (45–50 nm). In addition, an antibody recognizing conformational epitopes of the HPV16 L1 protein bound to the VLP surface, showing the correct assembly of the particles, as demonstrated by ultrastructural immunocytochemistry (Fig. 3c).

L1 VLPs hemagglutinate mouse cells, and hemagglutination can be blocked by a neutralizing antibody

L1 VLPs purified after expression in *P. pastoris* hemagglutinated mouse erythrocytes (Fig. 4, line A). Hemagglutination was not observed in the negative controls (Fig. 4, lines C and D). Inhibition of L1-VLP-induced hemagglutination was observed using a specific antibody that recognizes only conformational epitopes of the HPV16 L1 protein, confirming the proper assembly of VLPs expressed in *P. pastoris* and the presence of neutralizing conformational epitopes in these particles (Fig. 4, line B).

Discussion

Human papillomavirus infections are the most common sexually transmitted diseases in the world [41], and the association between high-risk HPV infections and cervical cancer development is corroborated by both epidemiological and biochemical evidence. The development of an efficient prophylactic vaccine against these high-risk HPV types, especially against HPV16, could greatly reduce the incidence rates of the disease associated with this virus. Traditionally, the majority of prophylactic viral vaccines are composed of live, attenuated or inactivated viruses. However, the difficulties of obtaining sufficient quantities of papillomavirus in culture, as well as concerns about the potential risks involving the administration of a vaccine containing viral oncogenes, have mobilized great effort toward the development of a subunit vaccine.

Clinical studies have demonstrated that either quadrivalent or bivalent vaccines composed of L1 VLPs have high efficacy against HPV infections for the high-risk HPV types 16 and 18 [28, 30, 42]. However, a significant impact on morbidity and mortality rates associated with HPV infections requires that these vaccines be generally available. For this reason, alternatives for producing an effective and low-cost vaccine must be sought.

The *P. pastoris* expression system, based on strong promoters, allows the expression of large quantities of heterologous proteins and has been successfully used in large-scale processes [43–45]. Under our conditions, the expression of L1 protein of HPV16 was only achieved with a L1 gene that was codon-optimized for *Pichia*. Many groups have demonstrated the necessity of codon optimization to improve the expression levels of recombinant proteins in *P. pastoris*, for example, equistatin [46], anti-human anti-T cell immunotoxin [47] and the F2 domain of erythrocyte-binding antigen from *Plasmodium falciparum* [48]. The plasmid expression system used was non-integrative. Compared to integrative systems, the episomally replicating plasmids have greater flexibility, have less variable expression levels (recombination of genomically integrating vectors can occur in many different ways that affect expression [49]) and require neither the integration procedure nor the multicopy selection step. Integrative vectors need to be linearized before yeast transformation, and incomplete cassette integration or relatively low rates of integration into the genome have been observed [50]. Moreover, the episomal expression vector can be readily transferred to other host strains such as protease-deficient yeast, because they can be conveniently recovered. The drawback of episomally replicating *P. pastoris* plasmids is that continuous antibiotic selection is necessary as plasmids containing a *Pichia*-specific autonomous replication sequence (PARS) lack long-term replication stability [51,52]. This fact contrasts to integrative vectors, which are generally highly stable and, for this reason, more suitable for production purposes. Regarding expression levels, a study has demonstrated that the expression level obtained using a vector containing PARS1 is at least equal to or better than that observed with an integrative expression system [52]. Since both systems have advantages and disadvantages, the choice of a proper expression system depends on the intended purpose. Since our purpose was to evaluate the feasibility of *P. pastoris* as a L1 expression host, the use of an episomally replicating plasmid represents an advantage because of its simplicity and faster results.

The amount of L1 in the yeast lysates was relatively high. In comparison to another yeast system, in which *S. cerevisiae* was used to express the L1 protein from HPV-11 [36], the *P. pastoris* expression system showed a higher expression level (13.8 µg/ml vs. 2.7 µg/ml) in cell lysates, although the copy number of episomal plasmids containing a 2-µm replication origin in *S. cerevisiae* cells under selective conditions seems to be higher (approximately 100 copies per cell [53]) than the copy number of a PARS1-containing plasmid in *P. pastoris* (about 13 copies per yeast cell [51]). However, the purification of HPV16 L1 protein from

the yeast cell lysate was difficult. Several attempts to purify the recombinant protein were made, and the process employing heparin–Sepharose affinity chromatography was the most successful. Joyce et al. [38] investigated the ability of HPV11 L1 VLPs to bind to glycosaminoglycans such as heparin. VLPs were able to bind to heparin–Sepharose resin at salt concentrations as high as 0.5 M NaCl and could be eluted using a linear NaCl gradient. A conserved region in the final 15 amino acid residues of the L1 protein of the general type XB BBBXB (B = Arg, Lys or His), similar to the consensus sequences of known heparin-binding proteins, has been suggested to mediate the interaction between L1 and heparin. However, others have shown that deletion of this region does not affect the interaction between L1 and heparin [54]. It has also been suggested that the interaction between heparin and the HPV capsid requires an intact outer surface conformation, which provides a conformational cluster of basic amino acids instead of a linear stretch of positively charged amino acids [39].

The HPV16 L1 protein purified after expression in *P. pastoris* self-assembled into VLPs, as visualized by electron microscopy. However, the L1 protein appeared unstable, and the low yield may be attributed to protein degradation, aggregation, or a tendency to absorb nonspecifically to surfaces. Polysorbate (Tween) 80 was previously shown to minimize aggregation [35], and thus it was added in all procedures following the final step of the purification process, substantially reducing protein loss. A significant amount of the initial preparation was comprised of VLPs inadequately assembled or appearing amorphous. It has been shown that the incubation of VLPs at low ionic strength and basic pH in the presence of low concentrations of reducing agent, followed by incubation at neutral pH and high ionic strength led to the generation of homogeneous VLPs with enhanced structural stability and higher immunogenicity [40]. Using this protocol, we were able to produce properly folded VLPs (Fig. 3b). Hemagglutination and hemagglutination inhibition assays, as well as immunocytochemical detection, supported the functionality of the produced VLPs and the presence of conformational neutralizing epitopes, since the HPV neutralizing antibody that we used was conformation-dependent.

Immunization with L1 capsomeres induces virus-neutralizing antibodies [55]. L1 capsomeres are regarded as potential cost-effective vaccine candidates, not only because they can be produced in bacterial expression systems, but also due to their higher stability compared to VLPs. Comparative immune studies of L1 capsomeres and VLPs have shown that VLPs induce higher titers of neutralizing antibodies, independent of the route of immunization [56]. However, these studies were done at limiting doses of antigen without adjuvant. With adjuvant, capsomeres are likely comparable to VLPs, and thus our system may not require preparation of VLPs, but rather purification of the dissociated capsomeres for use as the vaccine immunogen. Further studies will be required to verify this possibility. Importantly, this study supports the feasibility of producing HPV16 L1 VLPs in *P. pastoris*, which has direct relevance for the manufacture of a cost-effective prophylactic vaccine for Brazil's public health system. Although the proof of concept has been demonstrated (expression of functional HPV16 L1 VLPs in *P. pastoris*), the method must be improved before moving to biomanufacture. Considering that the described expression system is non-integrative, it is necessary to use the antibiotic zeocin to maintain the plasmid, which is problematic from an industrial point of view. We are now investigating the use of a different genetic system to integrate the L1 expression cassette into the *P. pastoris* genome as a possible solution to this problem, since the resulting recombinant clone would be stable and not require the use of antibiotics and would therefore be more compatible with biotechnological protein production.

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Fig. 1.

Expression of HPV16 L1 in *Pichia pastoris* KM71 (a) and GS115 (b) strains after 48 h of induction. Total cellular extracts were analyzed using an anti-L1 (HPV16) monoclonal antibody (Camvir), as described in “Materials and methods”. Recombinant *Lactobacillus casei* expressing L1 was used as positive control (*Lc*) [17]. The 56-kDa protein band identified as L1 is indicated by an *arrow*. Total extracts from different recombinant *P. pastoris* KM71 and GS115 clones after transformation with the pPICHOLI plasmid carrying the codon-optimized L1 gene and after induction with methanol are indicated as KM71 or GS115 transformants. As a control, *P. pastoris* carrying the empty vector (EV) or the wild-type HPV16 L1 gene (WT) after methanol induction are shown, as well as a GS115 transformant (GS115 clone 1) before induction (BI) with methanol

Fig. 2. HPV16 L1 protein purification by heparin-sepharose affinity chromatography. **a** Nitrocellulose membrane containing purified L1 fractions stained with Ponceau S and **b** Western blot analysis. *M* molecular mass standard, *N* not adsorbed on heparin-sepharose, *W* wash, *E* elutions, *A* column entry sample, *C* positive control (*P. pastoris* KM71/L1). L1 is indicated by arrows. Fractions eluted from heparin-Sepharose with 0.5, 0.6, 0.7 and 1.0 M NaCl are indicated

Fig. 3.

Transmission electron microscopy of yeast-derived VLPs after purification by affinity chromatography. **a** VLPs purified from heparin–sepharose without disassembly and reassembly. Samples were negatively stained with 2% uranyl acetate (magnification of 20,000 $\times$; *bar* 100 nm). **b** Electron microscopy of purified L1 protein after disassembling and reassembling procedures (magnification of 50,000 $\times$; *bar* 100 nm). Samples were negatively stained with 2% uranyl acetate. **c** Immunocytochemical labeling of VLPs samples. Goat anti-mouse IgG coupled with 10-nm gold particles was used in this experiment as secondary antibody (magnification of 20,000 $\times$; *bar* 100 nm)

Fig. 4.

Pichia pastoris-produced L1 VLPs agglutinate mouse erythrocytes. Samples were incubated for 3 h at 4°C and photographed. *A1* 25 ng VLPs; *A2* 50 ng VLPs; *A3* 100 ng VLPs; *A4* 200 ng VLPs. *B1–B4* the same VLP concentrations previously incubated with neutralizing monoclonal antibody. *C1–C4* negative control, erythrocytes incubated with PBS; *D1* and *D2* negative control, erythrocytes incubated with the neutralizing monoclonal antibody