Virus-Like Particles as a vaccinal strategy against tumors induced by papillomavirus infection

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Papillomavirus (PV) are small DNA viruses that infect mucosal and cutaneous epithelium and cause benign hyperproliferative lesions in human and animals. Occasionally these lesions can progress to cancer under appropriated environmental conditions. The human papillomavirus (HPV) is associated with many different types of cancer with approximately 450,000 newly diagnosed cases each year and a 50% mortality rate. The bovine papillomavirus (BPV) is associated with papillomatosis and cancer. Cattle cutaneous papillomatosis is not only a health problem but also has economic consequences. Occasionally, entire herds have to be culled if the papillomatosis does not regress. Infections with papillomatosis induce type-specific immune responses, mainly directed against the major capsid protein, L1. Based on the propensity of the L1 protein to self-assemble into virus-like particles (VLPs), our group has lead to research into the development of BPV and HPV vaccine strategies. This work focuses on the expression of L1 HPV gene to use in the *Pichia pastoris* expression system as a base to VLP production.

Keywords: VLP, *Pichia pastoris*, heterologous expression system, papillomavirus, vaccine

1. Introduction

Papillomaviruses are small double-stranded DNA viruses found in a wide variety of proliferative lesions of epithelial origin which are associated with different carcinogenesis process in human and other animals [1].

Human papillomaviruses (HPV) has been firmly identified as the major etiologic agent of neoplasia of cutaneous and mucosal epithelia [2]. Of the 200 or more types of HPV that have been discovered, about 35 are associated with cancer. Cervical cancer is the second most common cause of cancer-related deaths in women worldwide. More than 450,000 cases are diagnosed each year, resulting in nearly 250,000 deaths. HPV-16 is the most prevalent, accounting for more than half of cervical cancer cases [3].

In cattle, bovine papillomaviruses (BPV) are the etiologic agent of neoplasia of upper gastrointestinal tract and urinary bladder and cutaneous papillomatosis. Infection by papillomavirus requires epidermal or mucosal epithelial cells. Initial infection occurs in basal cells and the access to these cells occurs through micro-abrasions caused by various forms of physical trauma [4]. The correlation of BPV and cancer has been evaluated not only because of the economic repercussions of virus infection, but also because the system is an attractive experimental model to study HPV infection and carcinogenesis [5, 6]. Papillomavirus can not be grown in the laboratory as a source of antigen for serological tests and conventional killed virus vaccine development, so vaccine development is difficult [7]. Therefore, papillomaviruses vaccines currently under development employ genetic engineering technology. The main requirement for prophylactic papillomavirus vaccine is to induce neutralizing antibodies against the

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natural structural viral capsid proteins to prevent virus entry into the host cell [8]. The main protein components of the virion are proteins encoded by two open reading frames (ORF). The major capsid protein has a molecular weight of 55-60 kDa and is encoded by L1 ORF and the minor capsid protein, produced in a substantially less amount, is encoded by the L2 ORF and has an apparent molecular weight of 75-100 kDa [9]. The discovery that papillomavirus capsid proteins L1 and L2 (or L1 alone) self-assemble into virus-like particles (VLPs) when overexpressed in a heterologous expression system was decisive to papillomavirus vaccine research [10]. VLPs are not only immunogenic and safe, but also are able to induce strong cell-mediated and humoral immune responses [11, 12].

Expression of papillomavirus proteins has been achieved in several different model systems as bacteria, insect cells, mammalian cells, and yeast. Yeast as an expression host offers distinct advantages over the other expression systems both in terms of scale ability and productivity as well as the proven safety of yeast derived viral proteins for pharmaceutical applications, e.g., the hepatitis B vaccine [13].

We have selected the methylotrophic yeast *Pichia pastoris* to express the structural protein L1 of papillomavirus since several reports have shown that this yeast, unlike *Saccharomyces cerevisiae*, is capable of expressing glycoproteins that are both non-hyperglycosylated and lack a terminal 1,3-linked mannose addition, which are undesirable features that limit the pharmaceutical potential applications of recombinant expressed proteins [14]. *P. pastoris* is transformed by chromosome integration of the expression cassette into a specific locus to generate genetically stable transformants. This cassette can use the *AOX*1 strong promoter induced by methanol to drive the expression of recombinant proteins to high levels even with a single integrated copy of the expression cassette [14].

In this study, we focus on the strategy for production of a recombinant *Pichia pastoris* expressing HPV-16 L1 gene as a base system to VLP production.

2. Material and Methods

2.1 Bacteria and Yeast cells

Escherichia coli TOP 10 and *Pichia pastoris* GS115 were purchased from Invitrogen (Carlsbad, CA). The *E. coli* strain was grown in L-broth at 37ºC and *P. pastoris* was grown in YPD media at 30ºC.

2.2 Plasmids

The pBR322H16 plasmid containing the whole genome of HPV-16 was used to obtained L1 HPV-16 by PCR. The pGEM-T (Promega, Madison) was used as appropriated cloning vector for PCR products. Expression vector pPICZA, purchased from Invitrogen, contains the 5'AOX1 promoter region induced by methanol and the 3'AOX1 transcription terminators, as well as the *Sh ble* gene that gives Zeocin (Invitrogen) resistance.

2.3 Cloning strategy

DNA fragment encoding the full length HPV-16 L1 gene was generated by polymerase chain reaction (PCR) amplification using TripleMaster-Taq DNA polymerase HiFi (Eppendorf) and specific primers. The primers were constructed based on the sequences deposited in the Gene Bank. The forward primer for HPV-16 L1 gene was constructed to contain the yeast consensus Kozak sequence and a restriction site for *KpnI* in the 5' terminal. The reverse primer contains a terminal *SalI* restriction site terminal and the downstream stop codon was removed in order to have an attached 6 His-tag. The L1 fragment amplified was initially cloned into the pGEM-T easy vector, originating the pGEML1H16 construct, submitted to sequencing. After digestion of the pGEML1H16 with *KpnI* and *SalI*, the restriction fragment was purified using the Promega purification kit. The purified L1 fragment was subcloned into

the *P. pastoris* expression vector pPICZA, opened with *KpnI* and *SalI* enzymes, to originate the pPICZL1H16 construct.

2.4 DNA manipulation

All routine subcloning, ligation, transformation, restriction enzyme digestion, and plasmid DNA preparation were performed as described in standard texts [15].

2.5 PCR directed cloning

Two primers, FwL1H16 (5'- GGTACC *AWA**ATG**T*CTCTTTGGCTGCC -3', with *KpnI* site underlined and the Kozak sequence in italics) and RevL1H16 (5'- GTCGAC CAG CTT ACGTTTTTTGCG-3', with *SalI* site underlined), were used to generate a 1.5 Kb PCR fragment containing flanking *KpnI* and *SalI* restriction sites corresponding to L1 gene, using pBR322H16 as a template. This PCR fragment was ligated to the pGEM-T cloning vector generating the pGEML1H16 and sequenced by the dideoxi sequencing method. After digestion of the pGEML1H16 with *KpnI* and *SalI* the digestion fragment was purified and ligated into the corresponding *KpnI* and *SalI* sites of *P. pastoris* expression vector generating the pPICZL1H16 construct, with a 6 histidines tail in the C-terminus.

2.6 Pichia pastoris transformations

The recombinant plasmid pPICZL1H16 was linearized by *SacI* digestion and used to transform the *P. pastoris* strain GS115 by lithium chloride protocol [21]. A 50ml culture of *P. pastoris* was grown in YPD at 30ºC with shaking to OD_{600} of 0.8 to 1.0. Cells were harvested and washed with 25ml of sterile water and centrifuged at 1500xg for 10 minutes at room temperature. The water was discarded and cells resuspended in 1ml of 100mM LiCl. The cell suspension was transferred to a 1.5ml microcentrifuge tube and centrifuged at maximum speed for 15 seconds and LiCl was removed with a pipet. The cells were resuspended against in 400µL of 100mM LiCl. For each transformation 50µL of the cell suspension was dispensed into 1.5ml microcentrifuge tube and used immediately. The transformation was carried out by adding a reaction mixture containing 50% PEG, 1M LiCl, 2mg/ml single-stranded DNA and the linearized plasmid DNA. The reaction tube was submitted to vortex until the cell pellet was completely mixed and incubated at 30ºC for 30 minutes without shaking. After the reaction the tube was submitted to heat shock at 42ºC for 20 minutes. The tube was centrifuged at 7000 rpm and the transformation solution was removed with pipet. The pellet was resuspended in 1ml of YPD and incubated at 30ºC with shaking. After 1 hour and 4 hours, 25 to 100µl was plated on YPD containing 100µg/ml Zeocin. The plates were incubated for 2-3 days at 30ºC after which the transformants were analysed.

2.7 Analysis of the MUT^+ and MUT^S phenotypes of the GS115/pPICZL1H16 recombinants

After isolating the genome DNA from the GS115 transformed with pPICZL1H16, the following procedure was used to identify integrants and its Mut phenotype. Amplification of the interest gene was carried out with 5'AOX1 and 3'AOX1 primers. About 10µL of the *P. pastoris* culture was placed into a 1.5ml microcentrifuge tube and 5µL of a 5U/µL solution of lyticase was added and incubated at 30ºC for 10 minutes. The samples were frozen at -80ºC for 10 minutes and 5µL of this lysate was used to PCR. The product of amplification was analysed by 1.2% agarose gel electrophoresis.

2.8 Analysis of total mRNA extracted from *P. pastoris* recombinants

Recombinant colonies GS115/pPICZL1H16 isolated from Zeocin selective media plates were inoculated into 5ml of the minimal glycerol medium (MGY) and allowed to grow for 24h at 30ºC with vigorous shaking. To induce L1 gene expression, methanol was added to culture to a final concentration of 1%.

After incubation for 48h, methanol was added again and after a further 24h growth the cells were harvested. The total RNA was extracted from methanol induced culture samples using TRIzol reagent (Invitrogen). The L1 mRNA was detected by RT-PCR amplification using the kit SuperScript II (Invitrogen). The amplification was performed with primers Fw 5'-GGTCCATTAGGTGTGGG-3' and Rv 5'-AGCTGTCGCCATATGGTTCTG-3' and the L1 amplification product of 500pb confirmed by 1,2% agarose gel electrophoresis.

2.9 Analysis of L1 protein production

Cell extracts from GS115/pPICZL1H16 were analyzed by sodium dodecyl sulfate poliacrylamide gel electrophoresis (SDS-PAGE) separation to detect the L1 heterologous protein as follows: cells corresponding to an optical density of OD_{600}=1.0 were harvested, washed and resuspended in 500µL of PBS+1mM PMSF+1% Triton X-100, an equal volume of glass beads (0,5mm) was added and cells were disrupted by vortexing 10 times for 1min. After centrifugation for 10 min at 14000g, 10µL of the supernatant was loaded onto a 12% SDS-PAGE and the gel was stained with silver nitrate. Dot Blot analysis of same cell extracts was performed with anti-penta-His antibody (Qiagen, Germany). Membranes were blocked in 2% (w/v) bovine serum albumin in PBST (10mM Na2PO4, 150mM NaCl, and 1% Tween 20). Incubation was performed for 1-2 h in primary antibody diluted 1:2000 in blocking buffer. After washing three times with PBST, the membranes were incubated with secondary horseradish antibody proxidase (HRP) conjugated anti-mouse antibody at 1:4000 in blocking buffer for His-tag detection. Three washing steps were subsequently performed with PBST. Detection was done using DAB.

3. Results

3.1 Construction of the *P. pastoris* expression vector: pPICZL1H16

The results showed in the Fig. 1 indicate that the full length HPV-16 L1 gene was successfully cloned into pPICZA expression vector. The construct was designated pPICZL1H16.

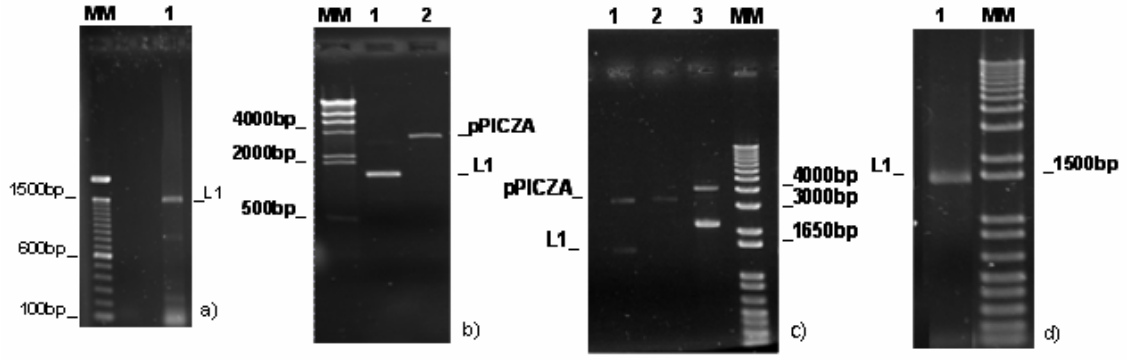

Fig. 1 In a) the PCR of HPV-16 L1gene with specific primers generated a 1.5 kb product confirmed by electrophoresis in 1.2% agarose gel: lane MM, molecular marker 100bp ladder (Promega); lane 1, 1.5Kb L1 amplicom. This product was inserted into pGEM-T easy vector and the pGEML1H16 was confirmed by *EcoRI* digestion (data not shown). In b) the product pGEML1H16 was cleaved by *KpnI* and *Sal1*, separated in 1.2% agarose electrophoresis and the 1.5kb fragment was purified and ligated to *P. pastoris* expression vector pPICZA previously digested also with *KpnI* and *SalI*; lane MM, lambda HindIII molecular marker; lane 1, HPV-16 L1 purified; lane 2, pPICZA purified. In c) analysis of the L1 HPV-16 clones: lane MM, molecular marker 1Kb plus DNA ladder (Promega); 1, clone DNA digested with *KpnI*/*SalI*; lane 2, pPICZA plasmid digested with *KpnI*, lane 3, pPICZA not digested. In d), is shown that L1 HPV-16 gene was successfully cloned into pPICZA vector by PCR

giving origin to pPICZL1H16 construct: lane MM, molecular marker 1Kb plus DNA ladder; lane 1, HPV-16 L1 amplicom.

3.2 Screening of GS115 transformants (GS115/pPICZL1H16)

With the use of the AOX1 primers or HPV-16 L1 primer associated with AOX1 primer was possible to verify the integration of HPV-16 L1 gene into *P. pastoris* cell genome. The results showed in the Fig. 2, indicate that pPICZL1H16 was successfully inserted into *P. pastoris* GS115 strain. The recombinant was designated GS115/pPICZL1H16.

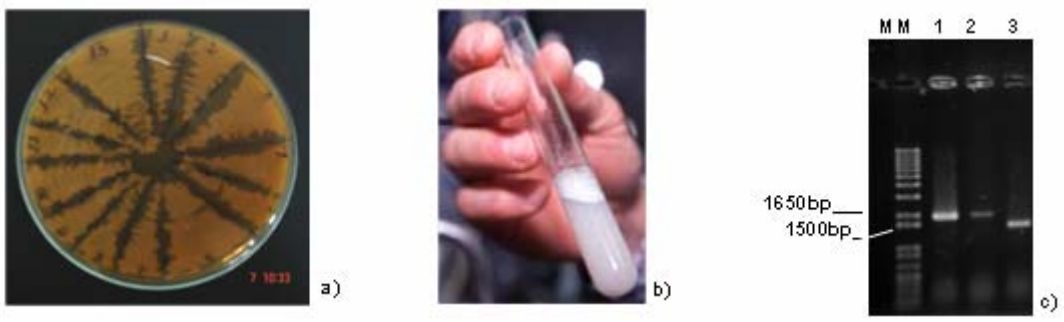

Fig. 2 In a) are shown GS115/pPICZL1H16 clones grown in plate containing YPD+Zeocin (see Material and Methods). In b), is shown a GS115/PPICZL1H16 clone grown in YPD+Zeocin liquid mediun (see Material and Methods). In c), the integration of pPICZL1H16 construct into GS115 was confirmed by PCR of the GS115 recombinant clones: lane MM, molecular marker 1Kb plus DNA ladder; lanes 1 and 2, PCR using AOX primers; lane 3, PCR using Fw L1 HPV-16/AOX1 primers. Both PCR amplifications have confirmed the presence of HPV-16 L1 gene.

3.3 Analysis of GS115/pPICZL1H16 phenotype

The results showed in Fig. 3, indicate that all GS115/pPICZL1H16 obtained clones are MUT^S phenotype.

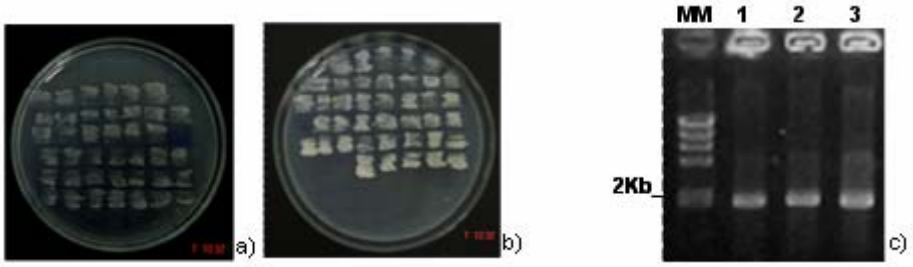

Fig. 3 In a) and b) are shown the GS115/pPICZL1H16 clones analysis for MUT phenotype by growth in plates with MMH (Minimal Metanol + Histidine) or MDH (Minimal Dextrose Medium + Histidine) medium, respectively. In c) is shown a PCR using AOX1 primers (according with *Pichia pastoris* Expression System manufacturer manual): only one band with 1500pb + 325pb indicate the MUT^S phenotype. Lanes 1, 2 and 3 identify the CL1, CL2, and CL3 clones respectively. The best growth of the clones in MDH medium associated with only one band amplification product in PCR indicate that these clones are MUT^S phenotype.

3.4 Analysis of HPV-16 L1 expression

Total RNA extracted from recombinant *P. pastoris* cells was analyzed for expression of the integrated L1 gene by RT-PCR. A 500bp integrated L1 gene DNA fragment was observed as an amplification product (Fig. 5), suggesting active transcription of the HPV-16 L1 gene. The protein

extract of induced and non induced clones were prepared and the proteins resolved in SDS-PAGE followed by staining with silver nitrate. The result shown in Fig. 5a indicates that L1 protein, represented by an approximately 55kDa band, can be visualized in all induced clones. A DotBlot using anti-Histidine antibody was performed and the result confirms the SDS-PAGE data, indicating the L1 protein expression (Figure 5b).

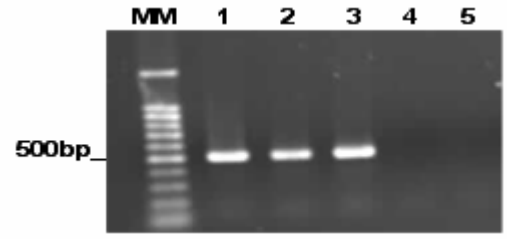

Fig. 4 RT-PCR of the GS115/pPICZL1H16 clones. MM, Ladder 100bp (Promega); 1, CL1; 2, CL2; 3, CL3. 4, reaction negative control. 5, GS115 wild type. The length of the product of RT-PCR for L1 gene is 500bp.

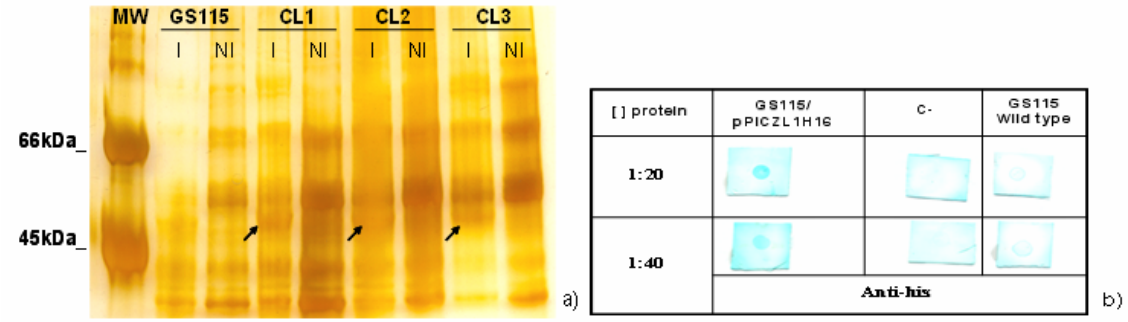

Fig. 5 a) silver nitrate stained SDS-PAGE of GS115/pPICZL1H16 clones and GS115 wild type *P. pastoris*. CL1-3, GS115/pPICZL1H16 *P. pastoris* clones; I, induced with methanol; NI, non induced with methanol. Arrows indicate the band (~55 kDa) correspondent to L1 protein. Note that in the GS115 wild type (I and NI) this band is not seen. b) Dot Blot using anti-His tag was performed to verify the expression of L1 protein expressed linked to a 6 histidine tag. The total protein was extracted from the CL3 clone and GS115 wild type and appropriatedly diluted before applied on the membrane. The Dot Blot was revealed by peroxidase reaction. C-, GS115/pPICZL1H16 protein extract hybridized only with secondary antibody.

4. Discussion

Traditionally most prophylactic vaccines for human viruses have consisted of live attenuated or inactivated virus. In the case of papillomavirus, due to the lack of a virus production system or proteins from natural sources to vaccine development the molecular cloning of the papillomavirus genes have been employed as a suitable source. Papillomavirus like-particles (VLPs) made from the major capsid protein L1 alone have been proven to induce protective immunity in animal model and L1-only VLPs are currently in clinical use [16]. A variety of expression systems have been investigated for expression of papillomavirus capside protein as potential systems for producing vaccines. Typically, overexpression of proteins in bacterial systems has been involved. However, expression of several different proteins in bacteria may yield inappropriately folded and/or glycosylated proteins [13]. To overcome these problems a variety of expression systems have been devised [13]. All these recombinant cell expression systems require stringent purification and sterile protocols to obtain sufficient papillomavirus L1 antigen [14]. Therefore, these processes are very expensive for developing countries that urgently require these vaccines [17].

P. pastoris is a methylotrophic yeast capable of metabolizing methanol as its sole carbon source. The first step in the metabolism of methanol is the oxidation of methanol to formaldehyde by alcohol oxidase enzyme using oxygen. Two genes code for alcohol oxidase, *AOX*1 and *AOX*2. The majority of alcohol

oxidase activity in the cell is attributable to the product of the *AOX*1 gene, which expression is controlled at the level of transcription. In methanol-grown cells approximately 5% the polyA+ RNA is from the *AOX1* gene and growth on glucose represses transcription, even in the presence of the inducer methanol. The loss of the *AOX*1 gene, and thus loss of most of the alcohol oxidase activity in the cell, results in a strain that is phenotypically Mut^S (Methanol utilization slow) [14, 18]. As an eukaryote, *P. pastoris* have many advantages of eukaryotic expression systems, such as protein processing, protein folding, and posttranslational modification, while being as easy to manipulate as *E. coli* or *S. cerevisae*. As an yeast, it shares the advantages of molecular and genetic manipulations and presents advantages over *S. cerevisae* in respect to recombinant expression protein levels and therapeutic use of the produced proteins. *P. pastoris* can produce 10-100-fold more heterologous protein and do not adds core terminal oligosaccharides α1,3 glycan linkages, which are believed to be primarily responsible for the hyper-antigenic nature of the recombinant proteins, making them particularly unsuitable for therapeutic use [14, 19].

In this paper we described the use of a recent expression system based on *P. pastoris* yeast cells to express the L1 papillomavirus protein as a first step to a vaccinal strategy based on VLPs against papillomavirus infection in human and animals. Initially we have constructed the expression vector pPICZL1H16 containing the L1 gene of HPV-16 by amplification with specific primers where the L1 gene have the initial ATG codon as part of a yeast Kozak consensus sequence [20] in order to improve the translation of L1 protein. The L1 gene produced by PCR was cloned into the pGEM-T easy vector and subcloned in frame with a his tag into the pPICZA expression vector for *P. pastoris* cells trasformation. Although spheroplasting have been used for yeast transformation, it is not recommended for transformation of *Pichia* with plasmids with the Zeocin resistance gene, because this method does not allow direct selection on Zeocin containing plates [14]. Therefore, we have used the LiCl transformation technique that showed good results, producing 40 recombinant clones selected by Zeocin resistance. All the clones obtained has shown the Mut^S phenotype, since they grow slowly in the presence of methanol and have presented a unique band after colony PCR with AOX primers. However, this is not an impairing characteristic, since the final yield of heterologous protein production can be even higher in Mut^S strains of *P. pastoris*.

After induction by methanol we have checked the transcription of the L1 gene of recombinant clones by RT-PCR, since in *Saccharomyces* was already verified the premature termination of transcription in TA rich sequences such as TTTTTATA, that resembles a sequence in HIV-1 gp 120, which resulted in premature termination of the mRNA when expressed in *Pichia* [14]. The RT-PCR analysis has confirmed that all 40 recombinant clones were positive for L1 gene mRNA transcription. The first clones (CL1-3) were grown on glicerol and after induction by methanol the L1 protein was detected in the appropriated aparent size (~55 kDa) in the electrophoresis gel. The 6-his tag fused to the L1 protein was used to confirm the identity of the produced protein by Dot blot analysis using an anti-his antibody.

The data presented in this work confirm the viability of the *P. pastoris* cells system to papillomavirus heterologous protein production, specially the capsid proteins that are natural candidates for the development of a vaccinal strategy against papillomavirus infection based on VLPs. The next steps will be to select the best producing clones, to standardize and scale up the production of L1 protein in fermenters, and after purification to analyze the ability of this heterologous protein to assemble in VLPs and, finally, to perform immunogenic analysis.

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