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Ovine papillomavirus type 3 virus-like particle-based tools for diagnosis and detection of infection

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ABSTRACT

The design of prophylactic and diagnostic tools specific to animal papillomaviruses is hampered by the difficulties of viral in vitro manipulation and by the scarce availability of dedicated biotechnological tools. This paper reports the production of Ovine Papillomavirus 3 (OaPV3)-based virus-like particles (OaPV3-VLPs) in the baculovirus system and their use to investigate host humoral immune response through the establishment of an indirect ELISA test., Polyclonal sera and monoclonal antibodies were generated against OaPV3-VLPs, and their isotype and reactivity were determined. Additionally, antibodies allowed OaPV3 detection in ovine squamous cell carcinoma (SCC) samples by immunohistochemistry. Results encourage the standardization of OaPV3-specific prophylactic and serological diagnostic tools, and open new perspectives for the study of host-viral interaction and SCC development.

1. Introduction

The family *Papillomaviridae* comprises a heterogenous group of small, non-enveloped viruses with circular dsDNA genome of about 8 kb in size. Papillomaviruses (PVs) have been found in almost all vertebrates and have been associated to either benign or malignant lesions in cutaneous and mucosal tissues [35,46,49]. To date, 498 reference genomes have been filed into the PApillomaVirus Episteme database (PaVE: https://pave.niaid.nih.gov/). Among them, 222 are human PVs (HPVs) while the remaining 276 genotypes have been isolated from 110 different animal species. Despite their species-specificity, interspecies transmission has been reported, such as in the case of bovine PVs (BPVs) able to infect several animal species, and ovine PVs (OaPVs) detected in bovines and in pig [28,26,29,30,23,11,39,13,12].

To date, 4 PV species have been identified in sheep, namely OaPV1, OaPV2, OaPV3, and OaPV4 [1,47,33]. According to PaVE classification, OaPV1, OaPV2 and OaPV4 belong to the genus *Deltapapillomavirus*, whereas OaPV3 belongs to *Dyokappapapillomavirus*. The 3 ovine delta viruses mainly associate to healthy skin and result in benign proliferative lesions (fibropapillomas), whereas OaPV3 was detected at 65 % prevalence in cases of cutaneous squamous cell carcinoma (cSCC) [1,47,51,27]. Furthermore, OaPV3 was also found in non-SCC samples, albeit with a lower prevalence (30 %), supporting the hypothesis of a critical role for OaPV3 as a co-inducer in the development of malignant cancer in association with other risk factors, such as UV exposure [51]. The ability of PV to induce cancer has been partly related to the presence of a conserved retinoblastoma tumour suppressor binding sequence motif LXCXE [55]. A LXCXE motif is absent in the E7 of ovine deltapapillomaviruses whereas it is conserved in the dyokappa OaPV3 and this relates to the different transforming and immortalizing properties of ovine papillomaviruses [1,47,48,33].

Only a few diagnostics OaPV3 tools are available and are limited to several in house molecular direct tests developed by different labs and to a single indirect tool based on a serum raised against the E6 protein expressed into the *E. coli* system [7]. Also, no information is reported in literature about host-virus interaction, particularly the specific immune responses raised during infection. The production of specific serological tools for OaPV3 diagnosis and for investigating viral-host interaction is

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hampered by the unavailability of an efficient in vitro cultivation system.

Many strategies have been developed to overcome these issues, and the production of PV Virus-Like Particles (VLPs) [21] have proven particularly effective for the great antigenic resemblance with native viruses. PV VLPs have been produced in several expression systems, such as *Escherichia coli*, yeast cells, *Leishmania*, mammalian cells, plants, and insect cells, exploiting the L1 ability to self-assemble into particles resembling the original virus capsids [21,41,25,3,18,31,14]. VLPs do not contain the viral genome and have been used to develop non-infectious vaccines such as Gardasil and Cervarix [15,10].

Beside their use for the development of monoclonal antibodies and polyclonal sera for diagnosis, VLPs can be used to investigate viral infection, viral cycle, and specific host immune responses. VLPs also have a potential role for the delivery of drugs or other molecules, including vaccines [32,56,24,5,17,4].

While HPV-based VLPs were extensively produced and studied, development of VLPs based on animal PVs is still limited [44,22,43,19,20,42,53,54,50].

In this paper we report the production of OaPV3-based VLPs in insect cells, by using a recombinant baculovirus system. VLPs were used to immunize several host species (rats, mice, and sheep) and specific polyclonal sera and monoclonal antibodies were developed and tested for direct diagnosis of OaPV3 by IHC. OaPV3 VLPs were also used to develop a OaPV3 specific ELISA test. OaPV3-ELISA was primarily used to demonstrate the presence of specific antibodies in naturally infected sheep. The developed OaPV3-based tools will allow the improvement of our knowledge on the key features of OaPV3 interaction with its natural host and promote the standardization of OaPV3-specific prophylactic and serological diagnostic tools.

2. Materials and methods

2.1. Generation of OaPV3 VLPs

OaPV3 VLPs were produced in insect cells by using the MultiBac system [38]. Briefly, wild type (WT) and humanised versions of the OaPV3 L1 gene (GenBank accession number NC_038516) were cloned into the shuttle vector pFBDM, a bicistronic plasmid containing 2 multiple cloning sites (MCS1 and MCS2) allowing double transcription of the gene of interest. Recombinant pFBDM vectors carrying alternatively the WT or humanised OaPV3 L1 gene versions were used as donors for producing recombinant bacmids in the *E. coli* system. Bacmids were used to produce OaPV3 VLPs in insect cells.

2.1.1. OaPV3 L1 codon optimization

The OaPV3 L1 sequence was optimised for human codon usage by using the "Optimizer" tool (https://genomes.urv.es/OPTIMIZER/). Two distinct humanised sequences (namely H1 and H2) were generated by applying 2 different algorithms: 1) One amino acid-one codon (optimizes all codons of the query sequence, i.e. all codons that codify the same amino acid are substituted by the synonymous codon most frequently used in the reference set); 2) Guided random (Monte Carlo algorithm that randomly selects codons based on the frequencies of use of each codon in the reference set) [34]. The Kozak sequence was added upstream each codifying sequence. To clone the humanised sequences into the bicistronic pFBDM vector, specific restriction sites were inserted upstream and downstream the L1 ORF (EcoRI and SphI at 5' before the Kozak' sequence and BstBI and XhoI at 3' downstream the L1 ORF). An internal XhoI restriction site generated by the guided random approach was eliminated by introducing a silent mutation (position 195, TCC to TCA = S). The two resulting sequences, OaPV3 L1H1 and OaPV3 L1H2, were synthetised and cloned into plasmids pBMH/OaPV3L1H1 and pBMHL1H2 by Biomatik (Ontario, Canada) and their sequences are reported in the Fig.S1 and S2.

2.1.2. Generation of OaPV3 L1 shuttle vectors and bacmids

In order to clone the WT OaPV3 L1 into the MCS1 and MCS2 of pFBDM vector, the WT OaPV3 L1 gene was amplified from pUC19/OaPV3, a cloning vector containing the entire OaPV3 genome [1] by using 2 different couples of primers (L1/Kozak/BamHI_F 5′-AAGGATCCGCCACCATGGCCGTGTGGGTGCCCAATG-3′ and L1/STOP/NotI_R 5′-AGGCGGCCGCTTATTTATTGTTTAATTTTCGCCTACG-3′; L1/Kozak/XhoI_F 5′-AACTCGAGGCCACCATGGCCGTGTGGGTGCCCAATG-3′ and L1/STOP/NheI_R 5′-CGCGCTAGCTTATTTATTGTTTAATTTTCGCCTACG-3′). Each primers couple contained the appropriate restriction sites for cloning WT OaPV2 L1 into pFBDM (BamHI and NotI for MCS1, and XhoI and NheI for MCS2). A Kozak sequence was inserted into each of the 2 forward primers.

PCRs were conducted with the Platinum Pfx™ DNA Polymerase (Invitrogen) according to manufacturer's instructions, checked by agarose electrophoresis, and purified with the QIAquick PCR Purification Kit (Qiagen).

Three different pFBDM recombinant plasmids (pFBDM/OaPV3L1WT/MCS1and2, pFBDM/OaPV3L1H1/MCS1and2, and pFBDM/OaPV3L1H2/MCS1and2) were generated, each of them alternatively carrying 2 copies of the same version of the OaPV3 L1 gene (WT, H1, or H2). Briefly, purified WT OaPV3 L1 PCR product and pBMH/OaPV3L1H1 and pBMH/OaPV3L1H2 plasmids were digested with the appropriate restriction enzymes and cloned into pFBDM vector (digested with the same enzymes) by using the Rapid DNA Dephos&Ligation Kit (Roche), following manufacturer's instructions. One Shot™ TOP10 Chemically Competent *E. coli* (Invitrogen) were transformed with the ligation products and positive colonies were selected for ampicillin resistance. Plasmid were extracted with the PureLink™ Quick Plasmid Miniprep Kit (Invitrogen). Inserts sequences were confirmed by Sanger sequencing. DH10multiBac electrocompetent *E. coli* were alternatively transformed with 50 ng of pFBDM/OaPV3L1WT/MCS1and2, pFBDM/OaPV3L1H1/MCS1and2, or pFBDM/OaPV3L1H2/MCS1and2 plasmids by electroporation. Bacteria were selected for antibiotic resistance (50 μg/mL kanamycin, 7 μg/mL gentamicyn, 100 μg/mL ampicillin, and 10 μg/mL tetracyclin) and blue/white screening allowed identifying clones containing recombinant bacmids. Bacmids were generated via T7-mediated transposition of the WT, H1, or H2 versions of the OaPV3 L1 from the donor vectors (the pFBDM shuttle plasmids) to the acceptor vector (DH10MultiBac). Recombinant bacmids were then extracted with the PureLink™ Quick Plasmid Miniprep Kit (Invitrogen) and sequenced to confirm the correct cloning of the original sequences.

2.1.3. Transfection of insect cells with recombinant bacmids

Sf9 cells were cultivated in monolayer in TNM-FH medium (supplemented with 10 % FBS, 1 % glutamine and 1 % peni-strepto) at 27 °C in non-humidified, non-CO_2 equilibrated incubator, and maintained at a density of 1×10^6 cells/mL.

Sf9 cells were transfected with 2 clones of each recombinant bacmid. Twenty-four hours before transfection Sf9 cells were seeded in 24 cm^2 flasks. Prior to transfection, Sf9 cells were washed with 1 mL of complete Grace's medium (10 % FBS, 1 % peni-strepto, 0.1 % Pluronic F-68, pH 6.02). The transfection solution was prepared by mixing 1 μg of bacmid DNA with 1 mL of buffer B (25 mM HEPES, 125 mM $CaCl_2$, 140 mM NaCl, pH 7.1), added to the Sf9 cells drop by drop, and cultures were incubated for 5 h at 27 °C. Afterwards, cells were washed twice with TNM-FH complete medium and incubated for 6 days as described above. As a positive control, Sf9 cells were infected with HPV16 baculovirus.

Plasmid #226 pIE HR (Novagen), encoding ß-galactosidase under the control of a baculovirus immediate-early promoter, was used for evaluating efficiency of transfections.

2.1.4. Propagation and testing of recombinant baculovirus stocks

In order to generate and propagate recombinant baculovirus stocks, supernatants collected from transfected Sf9 cultures were used to infect

new Sf9 cells. Briefly, 5 mL of infected cell supernatant were added to new Sf9 cells (2×10^6 cells in 25 cm^2 flasks) and flasks were gently agitated for 1 h to promote the infection. After 6 days of incubation, 5 mL of supernatant was recovered from each infection and used for a new round of infection. After 6 days of incubation, supernatants were collected to obtain the first virus stocks for each construct. This amplification process was repeated for two additional rounds increasing the flask size. The final virus stock (P6) for each construct was collected and stored until use.

Supernatants initially obtained from transfected Sf9 cells were also tested for OaPV3 L1 production by SDS-PAGE and western immunoblotting (see above). Briefly, Sf9 cells were seeded on 25 cm^2, infected with 1 mL of virus stock, and incubated for 1 h. After washing with fresh medium, cells were incubated with 10 mL of TNM-FH complete medium for 3 days at 27 °C. After medium removal, cells were washed with PBS, scraped from the flask surface, and collected by centrifugation. Cell pellets were resuspended in 300 μL of Laemmli buffer and subject to SDS-PAGE and western immunoblotting with the MD2H11 monoclonal antibody (see above).

2.1.5. Infection of High Five™ cells with OaPV3 L1 recombinant baculovirus

High Five™ cells were grown in suspension at the density of 2–3 × 10^6 cells/mL in EX-CELL® 405 Serum-Free Medium (supplemented with 1 % glutamine and 1 % penicillin–streptomycin) at 27 °C in non-humidified, non-CO_2 equilibrated incubator under vigorous shaking (120 rpm). Prior to infection, 250 mL of cell suspension were centrifuged at 680× g for 30 min at 4 °C and pellets were resuspended in 42 mL of EX-CELL® 405 Serum-Free Medium, alternatively inoculated with P6 recombinant baculoviruses (expressing OaPV3L1WT, OaPV3L1H1, or OaPV3L1H2) at a MOI of 5, and incubated at RT for 1 h under gentle shaking. Afterwards, 250 mL of EX-CELL® 405 Serum-Free Medium were added to the cell suspensions and cultures were incubated for 3 days at 27 °C under constant agitation (117 rpm). Subsequently, cells were harvested by centrifugation at 150× g for 15 min at 4 °C. Pellets were washed twice with ice-cold PBS and spun again. Cell pellets were used for OaPV3 VLPs purification.

2.1.6. Purification of OaPV3 VLPs

High Five™ cells pellets were resuspended in 20 mL of extraction buffer (5 mM MgCl2, 5 mM $CaCl_2$, 150 mM NaCl, 0.01 % Triton X-100, 20 mM HEPES pH 7.4) supplemented with 1 mM PMSF, and sonicated (2 × 1 min, power 3, 20 W). After, lysates were centrifuged at 10,000 rpm (Sorvall, SA-600 rotor) for 10 min at 4 °C and supernatants were collected. Residual pellets were resuspended in 6 mL of extraction buffer with 0.1 mM PMSF, sonicated again (45 sec, power 4.5) and spun as above. The 2 supernatants were combined and clarified by 2 consecutive centrifugation steps at 9000 rpm for 10 min at 4 °C (Sorvall, SA-600 rotor). Clarified supernatants were then subjected to ultracentrifugation in a two-step gradient. Briefly, 7 mL of a 40 % sucrose solution was layered over 8 mL of 3.5 M CsCl solution into a swinging bucket tube (Beckman ultracentrifuge, SW 32 rotor). The clarified supernatants were loaded over the two-step gradients and centrifuged at 27,000 rpm for 3 h at 10 °C (Ultracentrifuge, SW28 rotor). The interphases between sucrose and CsCl and the complete CsCl layers were collected, resuspended, transferred into Quickseal tubes (Beckman) and centrifuged at 50,000 rpm for 16 h at 20 °C (Ultracentrifuge, TFT65 rotor). The bottom of the tubes was punctured with a needle and 500 μL fractions were collected. All the fractions were dialyzed against water with 0.025 μm dialysis membranes (Millipore) and tested for the presence of VLPs by SDS-PAGE, western immunoblotting (against the MD2H11 antibody), and electron microscopy. After SDS-PAGE, western immunoblotting, and electron microscopy, dialyzed fractions containing high amounts of proteins were pooled and used for further analyses.

2.1.7. Electron microscopy

Transmission electron microscopy (TEM) was used to visualise OaPV3 VLPs. Five μL of dialyzed fractions were applied onto the carbon-coated copper grids and negative stained with 2 % uranyl acetate, air-dried, and examined with a transmission electron microscope CM200 FEG (FEI) operating at 200 kV. Pictures were taken at 27,500X magnification using a slow scan CCD (2 k × 2 k) camera.

2.2. SDS-PAGE and immunoblotting

SDS-PAGE and western immunoblotting were conducted as previously described [6]. Briefly, samples were resuspended in Laemmli buffer and incubated at 95 °C for 5 min and resolved into 10 % polyacrylamide gel after clarification. Gels were stained with SimplyBlue™ SafeStain (Invitrogen) and images were acquired with a VersaDoc MP 4000 Imaging System (Bio-Rad). Gel replicates were blotted into nitrocellulose membranes by either using a Mini Trans-Blot® cell (Bio-Rad) or a Trans-Blot® SD semi-dry transfer cell (Bio-Rad), following manufacturer's instructions. Membranes were blocked with 5 % skim milk in PBS containing 0.05 % Tween-20 (PBS-T) for 1 h and incubated with the appropriate primary antibody diluted in 2 % skim milk in PBS-T for 1 h at RT. Membranes were washed 3 times with PBS-T and incubated with the appropriate HRP-conjugated secondary antibody diluted in 2 % skim milk in PBS-T at RT for 1 h. After washes, membranes were developed with the Luminata Forte Western HRP substrate (Millipore) and chemiluminescence was detected by exposing membranes to X-ray films or with VersaDoc MP 4000 Imaging System (Bio-Rad).

2.3. Evaluation of humoral response in naturally infected sheep

2.3.1. Identification of OaPV3-infected flocks and samples collection

Two flocks persistently infected by OaPV3 were previously identified in Sardinia [1]. A total of 86 sheep were selected and sera and skin swabs were collected. Samples were stored at −80 °C until use. Sera were tested in VLPs-based ELISA (see below). Total DNA was extracted from the skin swabs by means of the QIAamp® DNA Mini Kit (Qiagen) and subjected to specific L1 PCR. Briefly, two specific primers were designed based on OaPV3 L1 sequence (NC_038516), namely OaPV3-L1 591F (5′-AGAAGACGGGAACATGATGG-3′) and OaPV3-L1 1158R (5′-CTCAGGGGTGAGGTCCACTA-3′) and used in PCR under standard conditions. Each DNA sample was tested in triplicate and appropriate positive and negative controls were included.

2.3.2. Sheep and rat immunisation

Two sheep were immunised subcutaneously 3 times with 100 μg of dialysed OaPV3 VLPs per each immunization. VLPs were diluted 1:1 with complete (1st immunization) or incomplete Freund's adjuvant (2nd and 3rd immunization and final boost) and administered every 20 days. Pre-Immune sera were collected before the first inoculum. Hyperimmune sera were collected 15 days after the last immunisation.

Two rats were immunised with 10 μg of dialysed OaPV3 VLPs at different inoculation sites (one subcutaneously and one intraperitoneal). Rats were immunised 3 times at 2 weeks intervals. OaPV3 VLPs were diluted in Freund's adjuvant as described above. Pre-immune sera were collected before the first inoculum and hyperimmune sera 15 days after the 3rd immunization. Wistar rats (280–300 g) were housed in the animal facility of the Department of Veterinary Medicine (University of Sassari, Italy) at constant temperature (24 ± 1 °C) and humidity (60 ± 5 %) with a 12 h light–dark cycle.

Dilutions of sheep and rat hyperimmune sera (1:100, 1:500, 1: 1000, and 1:2000) were tested by western immunoblotting against purified OaPV3 VLPs and using the appropriate pre-immune sera (1:100) as negative controls.

2.3.3. OaPV3 VLPs-based ELISA

ELISA conditions were initially optimized by coating plates with

serial two-fold dilutions of OaPV3 VLPS (1:150 to 1:96,000) and performing assays with 2 dilutions (1:200 and 1:800) of rat and sheep hyperimmune sera.

Upon ELISA tuning, Nunc®MaxiSorp™ 96 well flat bottom plates were coated with 200 ng/well VLPs suspended in 50 μL PBS and incubated o/n at 4 °C. The next day plates were washed with PBS-T and blocked for 1 h at 37 °C with 3 % BSA in PBS-T (300 μL/well). After washing, plates were incubated for 1 h at 37 °C with sheep sera (1:50) collected from OaPV3 naturally infected flocks. Plates were washed 3 times with PBS-T and incubated 1 h at 37 °C with 50 μL HRP-conjugated α-sheep secondary antibody diluted 1:10,000 in 3 % BSA in PBS-T. Plates were washed 3 times with PBS-T and 50 μL of TMB substrate (Sigma-Aldrich) were added to each well. After 5 min incubation signals were detected with a Multiplate Reader Multiskan EX or a Tecan Infinite F50 (Thermo Scientific) at 620 nm. Pre-immune and hyperimmune sheep sera were respectively used as negative and positive controls. The ELISA cut-off value was calculated as an OD value greater than the mean of the negative serum plus three standard deviations (OD > 0.416).

2.4. In situ detection of OaPV3

2.4.1. Production and characterization of monoclonal antibodies

Five pathogen-free BALB/c mice were immunised with OaPV3 VLPs. Mice were subjected to 3 biweekly subcutaneous injections in the neck area, and an additional final intraperitoneal boost injection was administered to mice three days before splenectomy. Lymphocytes extracted from the spleen were used for the hybridomas production and spleen cells were used as feeder cells. BALB/c mice were maintained under pathogen-free conditions at the animal facility of the German Cancer Research Center (DKFZ, Heidelberg, Germany).

For shot preparation, 40 μg of dialysed OaPV3 VLPs were diluted 1:1 with complete (1st immunization) or incomplete Freund's adjuvant (2nd and 3rd immunization and final boost). Sera samples were collected before the first immunization (pre-immune sera) and prior to sacrifice. Immunised mice were used for splenectomy and generation of monoclonal antibodies. Prior to the hybridomas cell fusion, the mouse humoral response was evaluated by means of titration of specific antibodies in OaPV3 VLPs-ELISA.

Hybridomas producing OaPV3 VLPs monoclonal antibodies were generated as previously described [37]. Briefly, after immunisation with OaPV3 VLPs, both feeder and immunised mice were euthanised with CO_2, disinfected with 70 % ethanol, and spleens collected aseptically. Spleens were homogenised by pressing them through a metal mesh (mesh size 0.5 mm) into a Petri dish using the plunger of 10 mL syringe. Splenocytes from immunised mice were washed twice with RPMI medium and collected by centrifugation at 2000 rpm for 5 min. Similarly, feeder cells were washed with HAT medium and collected by centrifugation. Cultured Sp2/0 cells were trypsinised, washed twice with RPMI medium, spun (2000 rpm, 5 min), and counted.

Splenocyte from immunised mice were mixed with 3×10^8 Sp2/0 cells and the in a 50 mL conical tube and centrifuged. After discarding the medium, tubes with the mix splenocyte- Sp2/0 cells were placed in a beaker of warm water and kept at 37 °C during all the fusion process.

For hybridoma generation, 1.5 mL of warm PEG was added to each tube drop by drop within 90 s and stirred for an additional minute. After PEG addition, RPMI medium was added as following: 1 mL at 1 min, 3 mL at 2 min, and 16 mL at 4 min. Cells were then centrifuged at 2500 rpm for 10 min and after 5 min rest supernatants were discarded. Pellets were resuspended in HAT medium, mixed, and added to bottles with feeder cells. Fusion cells were distributed on 20 96 well flat-bottom plates, wrapped in a plastic foil, and incubated at 37 °C, 5 % CO_2 for 7–10 days. To identify stable cell lines, clones derived from cell fusion were subcloned 3 times. Before every sub-cloning step, cell colonies were tested to produce specific antibodies by OaPV3 VLPs-based ELISA. Cells derived from positive clones were transferred into new 96-wells plates containing 150 μL/well complete RPMI (5 % FCS, 1 % penicillin–streptomycin, and 1 % glutamine) and feeder cells, plating 1:2 serial dilutions to reach the theoretically number of 1–2 cells/well. After the third subcloning, all plates were tested by ELISA and 4 positive clones with the highest performance were selected. Hybridoma cells were propagated in complete RPMI medium, and 4 monoclonal antibodies (mAb) were identified as #GH3E, #GH1G, #GH6F, and #GH2B. mAb isotype was determined by using the Mouse Monoclonal Antibody Isotyping Reagents (Sigma-Aldrich) in ELISA. Briefly, plates coated o/n at 4 °C with 50 μL of 1:1000 MD2H11 (a mAb raised against HPV16 L1, Santa Cruz), and blocked with 3 % BSA for 1 h at 37 °C. OaPV3 VLPs diluted 1:200 in 3 % BSA were added, and plates were incubated at 37 °C. After washing, 50 μL of each hybridoma supernatant were added to each well and plates were incubated 1 h at 37 °C. Then, plates were washed and HRP-conjugated typing antibodies (rabbit- α- IgG 1, IgG 2a, IgG 2b, IgG 3, IgA, and IgM) were added to the wells diluted 1:3000 in 3 % BSA. A goat α-mouse IgG-HRP secondary antibody (Dianova) was used as positive control. After 1 h room temperature (RT) incubation, plates were washed and TMB as added. Reaction was detected after 10 min incubation with a Multiskan EX ELISA reader at 405 nm. mAbs were also tested against OaPV3 VLPs by western immunoblotting and dot blot. Briefly, 1 μL OaPV3 VLPs aliquots were spotted on nitrocellulose membrane. Membranes were then saturated with 5 % skim milk, washed, and incubated with 4 different mAb dilutions (from 1:50 to 1:1000) for 1 h. After washing, membranes were incubated 1 h with 1:50,000 HRP-conjugated α-mouse secondary antibody (Southern Biotech). After washing, signals were developed as described for western immunoblotting. MD2H11 was used as a positive control.

2.4.2. Immunofluorescence

To evaluate utility of mABs for immunofluorescence, the antibodies were used to stain HeLa TK4 cells expressing OαPV3 L1 and L2. pGEM expression plasmids were constructed for OαPV3 L1 and L2 using the two previously described methods of gene humanization, H1 (a single codon for each amino acid) or H2 (guided random selection of codons based on known frequencies of codon usage) HeLa TK4 cells were transfected with pGEML1-IRES-L2 H1 or pGEML1-IRES-L2 H2 and Turbofect reagent (Thermo Scientific). 48 h post transfection cells were fixed, permeabilised, incubated with hybridoma supernatants, and subsequently with an anti-mouse IgG Alexa Fluor® 594 conjugate secondary antibody. HeLa TK4 cell nuclei were counterstained with DAPI and images were acquired with a Leica fluorescence microscope. As a positive control, HeLa TK4 cells were transfected with #988_HPV16 vector and challenged with the 1.3.5.15 HPV16 mAb.

2.4.3. Immunohistochemistry

For immunohistochemistry, 3 μm sections were mounted on SuperFrost™ slides (Thermo Scientific) and were dewaxed and rehydrated with Dewax and HIER (heat-induced epitope retrieval) Buffer H, pH 8.8 (ThermoScientific) at 98 °C for 20 min. Tissues were then blocked for endogenous peroxidase with 30 min incubation in Dako REAL Peroxidase-Blocking Solution (S2023, Dako, Glostrup, Denmark), and for non-specific binding with 2 % BSA in PBS for 1 h at RT followed by a second incubation with 2.5 % Normal Horse Serum (ImmPRESS reagent kit, Vector Labs, Burlingame, CA, USA). Slides were incubated with hybridoma supernatants diluted 1:50 and the MD2H11 mAb (1:500) while α-OaPV3 VLPs rat hyperimmune sera (1:100) was used as positive controls. All primary antibodies were diluted (Max Vision Biosciences) and incubated for 16 h at 4 °C.lides were incubated with an HRP-conjugated secondary antibody (Southern Biotech) for 30 min at RT. After staining with 3,3′-Diaminobenzidine (ImmPACT DAB, Vector Laboratories, Burlingame, CA, USA) tissues were counterstained with hematoxylin, cover-slipped with Eukitt Mounting MediumTM (BiOptica, Milan, Italy) and observed under light microscopy. Negative controls were carried out by replacing the primary antibody with antibody diluent. Images of all sample tissues were obtained with a Nikon Eclipse 80i microscope with a Nikon DS-Fi1 camera (Nikon Instruments Inc.,

Melville, NY).

2.5. Ethical statement

All animal procedures complied with standards stated in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Academy of Sciences, Bethesda, MD, USA). Animal procedures were also approved by the University of Sassari Animal and Use Committee (Organismo Preposto al Benessere e alla Sperimentazione Animale dell'Università degli Studi di Sassari, OPBSA) with protocol number 87039. Animal experiments involving the production of monoclonal antibodies were performed under the license A12/11, Regierungspräsidium Karlsruhe, Germany.

3. Results

3.1. Generation of OaPV3 VLPs

To maximise VLPs expression, 3 recombinant MultiBac bicistronic pFBDM shuttle vectors were successfully constructed each one containing 2 copies of one version of the OaPV3 L1 gene in a head-to-head arrangement: pFBDM/OaPV3L1WT/MCS1and2 containing WT L1 gene, pFBDM/OaPV3L1H1/MCS1and2 containing the H1 version of the L1 gene, and pFBDM/OaPV3L1H2/MCS1and2 containing the H2 version of the L1 gene. Also, the corresponding 3 bacmids were successfully generated in DH10multiBac *E. coli*. Correct gene cloning and sequence of shuttle vectors and bacmids in the shuttle vectors were confirmed by restriction enzyme digestion and Sanger sequencing.

Recombinant baculoviruses were produced and amplified in Sf9 insect cells. Western immunoblotting confirmed OaPV3 L1 expression in all the tested clone except in H1-clone11. Moreover, OaPV3 L1 was expressed at comparable level in all the 5 positive clones, independently from the gene version, either humanised or WT (Fig. 1).

High Five™ cells were successfully infected with recombinant baculoviruses carrying the 3 versions of the L1 gene. Gradient ultracentrifugation of the cell crude extracts generated several fractions containing the OaPV3 VLPs.

Initially, the presence of OaPV3 L1 in fractions obtained from crude extracts by density ultracentrifugation was verified by SDS-PAGE and western immunoblotting (Fig. 2). A distinct abundant 55 kDa band was detected in all the 3 crude extracts derived from High Five™ cultures alternatively infected with OaPV3 WT, H1, and H2 baculoviruses. SDS-PAGE and western immunoblotting also shown the presence of L1 in all the fractions, with significant enrichment in some fractions (WT 3, 6–8; H1 2–6; and H2 4–5, 7–8). TEM microscopy confirmed morphology and correct assembly of OaPV3 VLPs in all the fractions expressing L1, independently from the gene version, either WT or codon optimised (Fig. 2).

3.2. Evaluation of humoral response in naturally infected sheep

An indirect ELISA test was established based on OaPV3 VLPs (OaPV3 VLPs-ELISA). OaPV3 VLPs-ELISA allowed detecting specific antibodies in sheep naturally infected by OaPV3 (Fig. 3). About 63 % (54/86) of sera tested positive by OaPV3 VLPs-ELISA, with OD values ranging from 25 % to 30 % of the pooled hyperimmune sheep sera used as positive control. A single sample, collected from a sheep without apparent lesions, showed a high OD value (85 % of the pooled hyperimmune sheep sera). Differences in OD values between groups of sera were statistically significant ($p < 0.05$). Comparison of ELISA and OaPV3-L1 PCR are shown in Fig. S11. Briefly, among seropositive animals (54), 3 sheep tested also positive to OaPV3-L1 PCR while 51 seropositive sheep tested negative by PCR. Out of 83 PCR negative animals, 51 sheep were seropositive (61.4 %). Fig. S12 reports the correlation between seroreactivity and presence of SSC lesions. Among 54 seropositive animals, 2 presented cSCC while 52 did not show any proliferative malignancy.

3.3. α-OaPV3 VLPs sera production and titration

Hyperimmune polyclonal sera produced in rats and sheep immunised with OaPV3 VLPs were tested against purified OaPV3 VLPs by western immunoblotting. Rats hyperimmune sera efficiently recognised the target even at the highest dilution (1:2000) independently from the inoculum route, either subcutaneous or intraperitoneal (Fig. S3). Similarly, hyperimmune sheep sera efficiently recognised the OaPV3 L1, although the sheep 2 serum generated a weaker signal at the 1:1000 and 1:2000 dilutions (Fig. S4). Specific antibodies were titrated by OaPV3 VLPs ELISA (Figs. S5 and S6). Titration of the 2 hyperimmune rat sera produced curves showing comparable trends with high OD values at dilutions ranging from 1:150 to 1:2400 and (OD values decreased with further serial VLPs dilutions). Similar results were obtained with sheep hyperimmune sera. When titrated in OaPV3 VLPs ELISA, sheep hyperimmune sera displayed trends comparable to what observed with rat sera.

Polyclonal serum collected from the mouse before splenectomy (final bleeding) was titrated by OaPV3 VLPs-ELISA. Briefly, both 1:200 and 1:800 mouse polyclonal serum dilutions tested positive against OaPV3 VLPs quantities ranging from 700 ng to 11 ng (Fig. S7).

3.4. mAbs selection, titration, and isotyping

After hybridoma generation, 4 single clones where selected based on

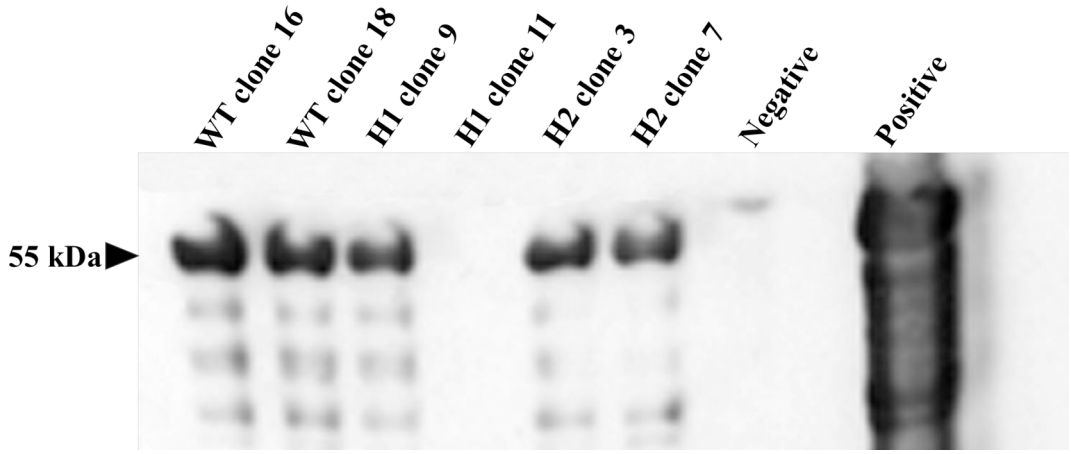

Fig. 1. Western immunoblotting representative of reactivity of baculovirus clones expressing OaPV3 L1 encoded by the 3 different L1 gene versions (WT, H1, and H2) against MD2H11 mAb. Untransfected Sf9 insect cells and Sf9 cells infected with HPV16 baculovirus were respectively used as negative and positive controls.

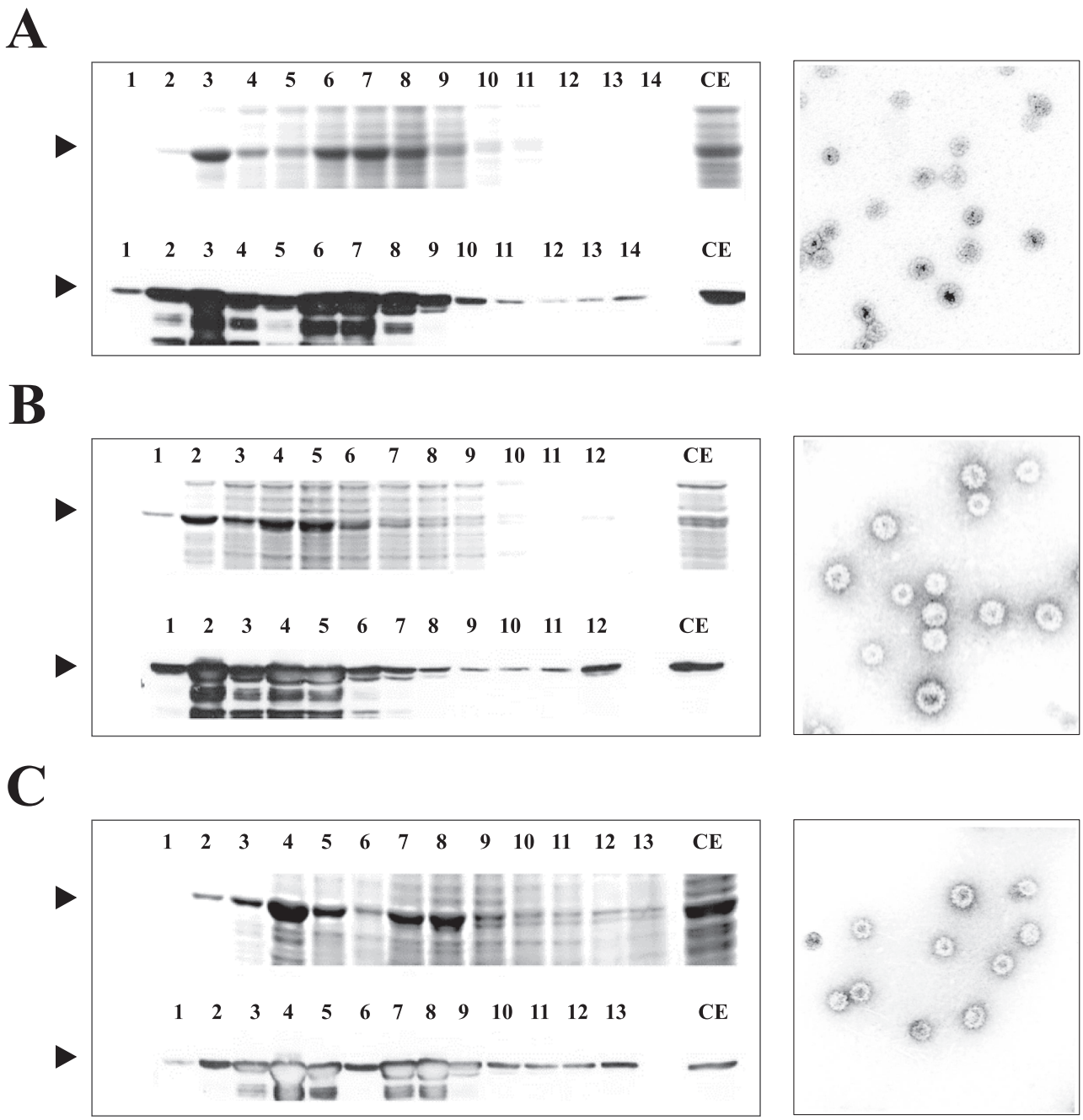

Fig. 2. SDS-PAGE (left upper lanes) and western immunoblotting (left lower lanes) of fractions and crude extracts (left panels) derived from High Five™ cultures alternatively infected with OaPV3 WT (A), H1 (B), and H2 (C) baculoviruses. A representative image of TEM morphology of the corresponding VLPs is shown (right panels). Fractions are progressively numbered. CE: crude extract. Arrows indicate 55 kDa.

high reactivity of culture supernatants against OaPV3 VLPs (data not shown). The corresponding mAbs were identified as #GH_6F, #GH_3E, #GH_2B, and #GH_1G. Titration and isotyping of α-OaPV3 VLPs mAbs are reported in Fig. S8 and S9. Briefly, all 4 mAbs specifically recognised OaPV3 VLPs, with decreasing performance from #GH_2B to #GH_3E, #GH_6F, and #GH_1G. Based on isotyping test, #GH_6F and #GH_1G mAbs were classified as IgG 1, whereas #GH_2B and #GH_3E were assigned to IgG 2b.

3.5. mAbs reactivity in western immunoblotting, dot blot, and immunofluorescence

All 4 mAbs failed to produce a detectable signal when challenged with OaPV3 VLPs by western immunoblotting, while MD2H11 used as a positive control and recognising an L1 external and exposed region showed an intense signal. On the contrary, all mAbs tested positive in dot blot, also at high dilutions, with #GH_2B and #GH_3E producing more intense signals (Fig. 4). MD2H11 produced only a faint signal in dot blot, showing an inability to bind assembled OaPV3 VLPs. Considered together, dot blot and western immunoblotting results indicate that all mAbs recognise conformational OaPV3 VLPs epitopes. All 4 mAbs also reacted with OaPV3 VLPs assembled in HeLa TK4 cells independently from the humanised version of the L1 and L2 genes used (Fig. 5).

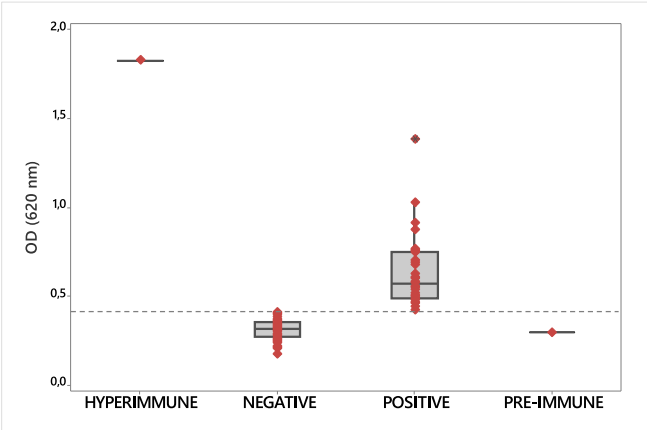

Fig. 3. Boxplot showing OD values of ELISA results for discrimination of OaPV3 seropositive and seronegative sheep. Positive: OaPV3 seropositive sheep; Negative: OaPV3 seronegative sheep; Pre-immune: sheep sera collected at T0; Hyperimmune: pooled hyperimmune sheep sera. Red diamonds shapes represent individual OD values. Dotted line indicates the cut-off value (0.416).

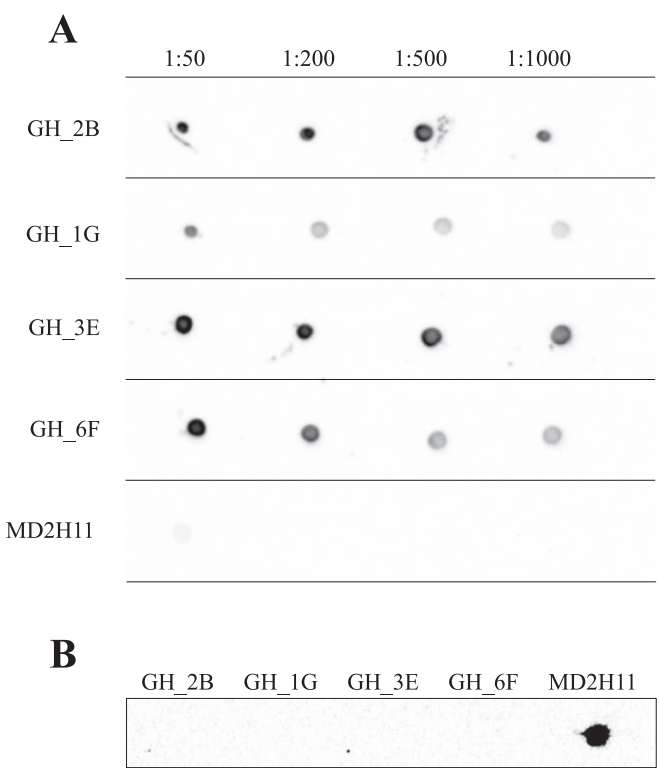

Fig. 4. Dot blot (A) and western immunoblotting (B) reactivity of mAbs against OaPV3 VLPs. Human MD2H11 mAb was used as a control.

3.6. *Immunohistochemistry detection of OaPV3*

Rat polyclonal serum raised against OaPV3 VLPs was tested in IHC on sheep SCC samples (Figs. 6 and 7). Rat α-OaPV3 VLPs serum efficiently recognised VLPs antigens in OaPV3 PCR-positive ovine SCC samples, showing strong immune-reactivity comparable to the MD2H11 positive control (Fig. 6). At higher magnification a strong cytoplasmic signal could be detected into keratin pearls (Fig. 6). Serial sections from the same tissues were tested with the hybridoma supernatants containing #GH_2B, #GH_3E, #GH_6F, and #GH_1G mAbs (Fig. 7). All α-OaPV3 VLPs mAbs displayed immunoreactivity in the cytoplasm of epithelial neoplastic cells in ovine SCCs, although with different intensity being strong for #GH_2B and #GH_3E and mild for #GH_6F and #GH_1G.

4. Discussion

Papillomaviruses (PV) are small dsDNA viruses with tropism for vertebrate mucosal and cutaneous epithelial cells. Despite their seeming simplicity, PVs replicate through a complex cell cycle, in which each step is achieved in a different cell stage during keratinocytes differentiation.

This peculiar feature hampers the application of classical virology techniques such as in vitro isolation and cultivation on eukaryotic cell lines, and consequently the development of dedicated diagnostic and prophylactic tools. Organotypic raft cultures based on keratinocytes harbouring HPVs allowed new opportunities to explore PVs biology, but this technique remains technically demanding and time-consuming. The possibility of producing viral particles structurally and immunologically mirroring native PV virions, such as DNA-free Virus Like Particles (VLPs) and plasmids-harbouring Pseudoviruses (PsVs) allowed to overcome these limits.

The production of VLPs and PsVs have mainly focused on HPVs but the application of this technologies to animal PVs remains underexplored and is complicated by limited access to dedicated immunological tools. The development of specific antibodies, VLPs, and PsVs, represents an essential step to study animal PVs oncogenic properties and their interaction with the host.

This work reports the production of VLPs based on the SCC-associated OaPV3 ovine PV in the baculovirus system. VLPs were successfully used to develop a dedicated indirect ELISA which allowed the measurement of humoral immune response in naturally infected sheep.

Upon comparing ELISA results with PCR data and lesion presence it was observed that diseased sheep identified as ELISA-positive tested negative in PCR. Also, several healthy sheep tested positive both in PCR and ELISA. However, it was demonstrated that during genital HPV natural infection the production of α-L1 antibodies starts very slowly without reaching high titres; furthermore, titre rapidly falls after clearance of viral infection [8,52]. In the case of OaPV3, it can be speculated that in sheep testing negative to L1 PCR and positive to ELISA, PV infection has been successfully cleared by the host immune system. On the other hand, it cannot be excluded that sheep have a persistent infection with viral loads maintained under the detection limit due to the host immune response. Further studies are necessary to clarify the dynamics of the OaPV3 infection and of the related host immune response.

The production of VLPs also allowed the development of specific polyclonal sera and monoclonal antibodies (mAbs), which proved useful to identify OaPV3 in sheep cutaneous SCCs by immunohistochemistry. Previous attempts to produce OaPV3 VLPs in *E. coli* failed due to the inability of obtaining soluble L1-based VLPs, which constantly formed inclusion bodies, probably related to L1 incorrect folding and assembly [7].

The production of VLPs in the baculovirus system was not influenced by the gene version used (WT, H1, and H2), as demonstrated by western immunoblotting and TEM. Indeed, TEM analysis confirmed the assembling of proper capsid structures, opening the possibility of using OaPV3 VLPs as surrogates for the development of specific prophylactic and diagnostic tools. VLPs represent excellent platforms for the development of safe and effective vaccines and diagnostic antigens since they are non-infectious and non-replicating due to the absence of viral DNA [45]. VLPs have been successfully used as prophylactic vaccines against high-risk HPVs, as the already developed and licensed HPV vaccines such as Gardasil® (4vHPV, Merck), Gardasil9® (9vHPV, Merck), and Cervarix®, (2vHPV, Glaxo Smith Kline), able to induce the production of neutralizing antibodies [15,16,10].

In this work, we demonstrated that the administration of OaPV3 VLPs to sheep induces a strong humoral response, due to their densely

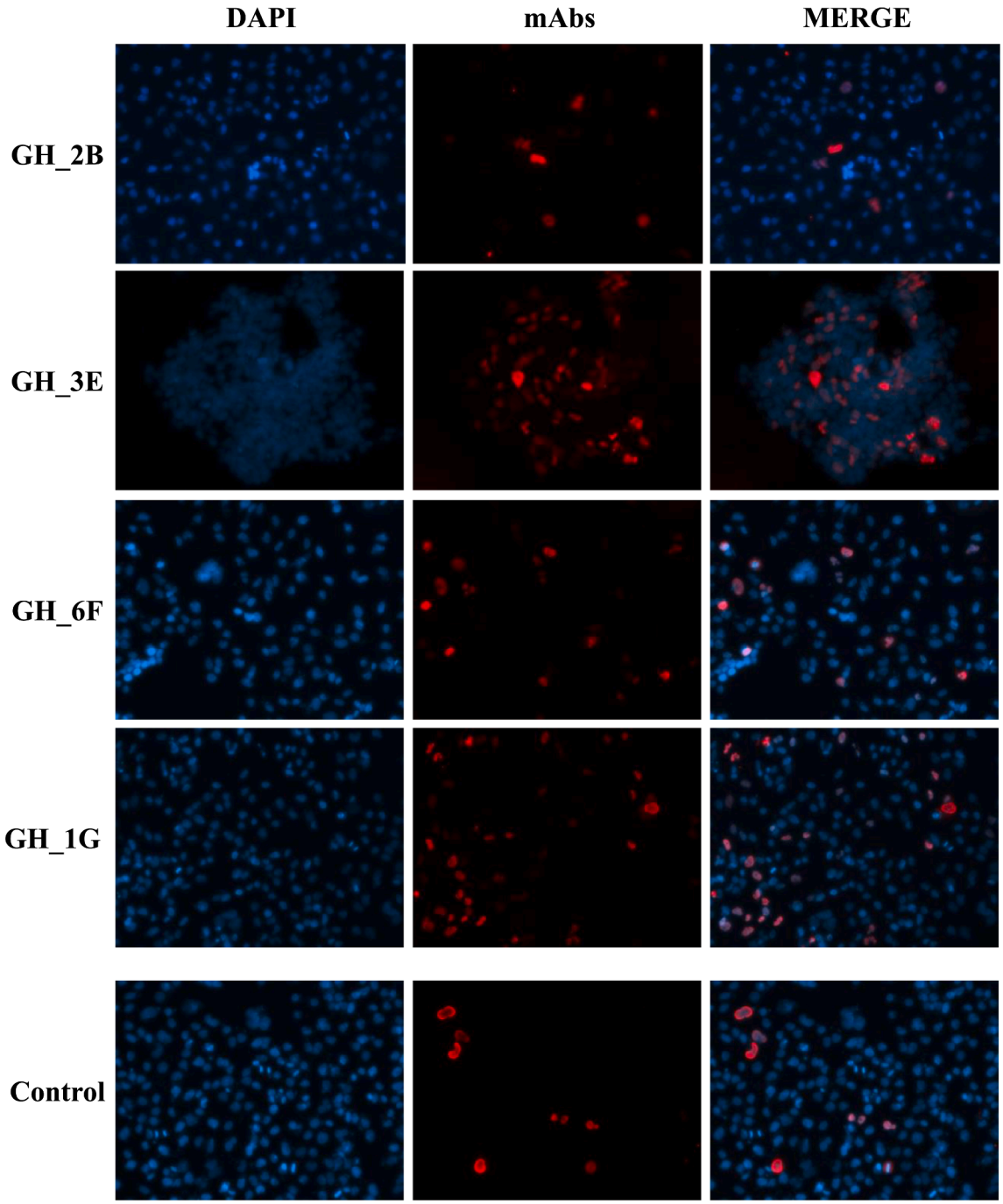

Fig. 5. mAbs immunofluorescence reactivity with HeLaT K4 cells expressing OaPV3 L1 and L2. Control line show reactivity of HeLa TK4 cells transfected with #988_HPV16 vector and challenged with the 1.3.5.15 HPV16 mAb.

repeated display of viral L1 antigens in a conformation close to the authentic virion. Based on these preliminary data, OaPV3 VLPs may be considered suitable vaccine candidates and their effectiveness in the prevention of squamous cell carcinoma in sheep must be assessed, as well as their use to develop therapeutic vaccines in association with surgical resection of the lesions. Indeed, the administration of VLPs-based vaccines in surgically treated human patients has been proven to reduce lesion recurrence [36,40]. PVs recurrence is still under-investigated in animals, but some information is available for BPV in equine sarcoid, in which correlate to the dissemination through peripheral blood [2]. Notably, the recrudescence of ovine PV lesions must be still investigated.

To date, efficient tools for OaPV3 serological diagnosis are still lacking. As a first attempt, polyclonal rat sera were raised against the OaPV3 E6 and L1 recombinant proteins produced in *E. coli*. When tested in immunohistochemistry (IHC) on PCR-positive samples, the α-E6 serum produced a strong positive staining, while the α-L1 signal was weakly detected mainly in well-differentiated keratinocytes in the stratum corneum [7]. In this work, VLPs were used to generate 4 specific monoclonal antibodies (mAbs). All α-OaPV3 VLPs mAbs were highly reactive to the native VLPs antigens and recognise only conformational epitopes, as demonstrated by reactivity in ELISA and dot blot, but not western immunoblotting. When probed in IHC, the 4 mAbs recognised OaPV3 antigens in positive tissues, but not in the negative controls. In particular, #GH_2B and #GH_3E showed a stronger staining compared to #GH_6F and #GH_1G. The degree of responsiveness mirrors what

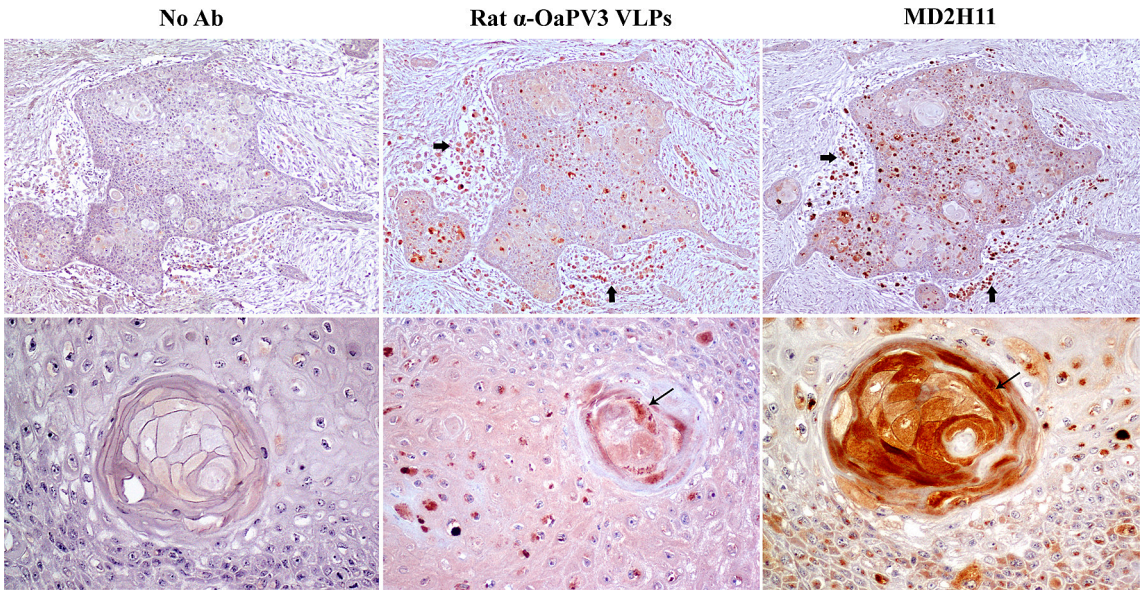

Fig. 6. IHC Immunoreactivity of rat α-OaPV3 VLPS on OaPV3-positive SCC samples. A strong cytoplasmic OaPV3 immunostaining can be observed in the neoplastic epithelial cells of SCCs (top middle picture) and in keratin pearls (bottom middle picture) (thin arrows). A strong signal is also detected in the inflammatory infiltrate near the island of the neoplastic epithelial cells (thick arrows). MD2H11 (positive control): mAb raised against HPV16. No Ab (negative controls): OaPV3-positive SCC samples with the omission of the primary Ab.

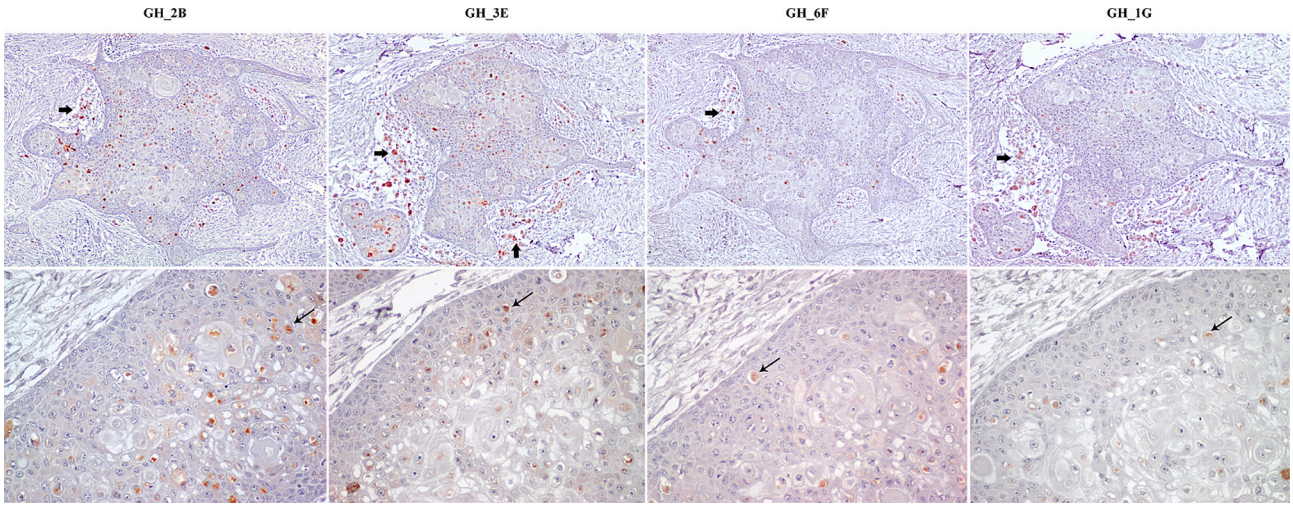

Fig. 7. Representative immunoreactivity of α-OaPV3 VLPs mAbs as tested by IHC on OaPV3-positive SCC samples. A strong (#GH_2B and #GH_3E) and mild (#GH_6F and #GH_1G) cytoplasmic OaPV3 immunoreactivity can be observed in the neoplastic epithelial cells of SCCs (thin arrows). The signal is present in the inflammatory infiltrate near the island of the neoplastic epithelial cells (thick arrows). Upper and lower lines show different magnifications of the same tissue samples.

seen in ELISA in which #GH_2B and #GH_3E produced a stronger signal compared to the others mAbs. Taken together, results suggest that hybridoma supernatants contains different concentrations of reactive mAbs. To standardise this diagnostic method, hybridoma supernatants containing the α-OaPV3 VLPs mAbs should be purified and quantified. Based on literature, we can speculate that mAbs can possibly neutralize OaPV3 infection; in fact, it is well known that antibodies raised against conformational epitopes exposed on PV virions usually have neutralizing properties [9]. Further studies are needed to explore the neutralizing properties of α-OaPV3 VLPs mAbs. In conclusion, our results encourage the standardization of OaPV3-specific VLPs-based prophylactic and serological diagnostic tools, and open new perspectives for the study of host-viral interaction and SCC development.

CRediT authorship contribution statement

Carla Cacciotto: Writing – original draft, Investigation, Formal analysis, Data curation. **Gian Mario Dore:** Validation, Investigation, Data curation, Conceptualization. **Tiziana Cubeddu:** Investigation, Formal analysis, Data curation. **Giovanni Pietro Burrai:** Investigation, Formal analysis, Data curation. **Antonio Giovanni Anfossi:** Investigation. **Elisabetta Antuofermo:** Investigation, Formal analysis, Data curation. **Maria Vittoria Varoni:** Investigation, Data curation. **Maria Piera Demontis:** Investigation, Data curation. **Rosanna Zobba:** Investigation, Formal analysis, Data curation. **Marco Pittau:** Investigation, Conceptualization. **Martin Müller:** Writing – original draft, Methodology, Conceptualization. **Alberto Alberti:** Writing – original draft, Supervision, Resources, Project administration, Methodology, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2024.06.001.

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