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Novel biotechnological platform based on baculovirus occlusion bodies carrying *Babesia bovis* small antigenic peptides for the design of a diagnostic enzyme-linked immunosorbent assay (ELISA)

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Abstract

Baculoviruses are large DNA virus of insects principally employed in recombinant protein expression. Its ability to form occlusion bodies (OBs), which are composed mainly of polyhedrin protein (POLH), makes them biotechnologically attractive, as these crystals (polyhedra) can incorporate foreign peptides and can be easily isolated. On the other hand, peptide microarrays allow rapid and inexpensive high-throughput serological screening of new candidates to be incorporated to OBs. To integrate these 2 biotechnological approaches, we worked on *Babesia bovis*, one of the causative agents of bovine babesiosis. Current molecular diagnosis of infection with *B. bovis* includes enzyme-linked immunosorbent assay (ELISA) techniques, which use merozoite lysate obtained from infected bovine erythrocytes. However, it is important to produce recombinant antigens that replace the use of crude antigens. Here, we describe a new biotechnological platform for the design of indirect ELISAs based on 5 antigenic peptides of 15 amino acid residues of *B. bovis* (ApBb), selected from a peptide microarray and expressed as a fusion to POLH. An Sf9POLH$_{E44G}$ packaging cell line infected with recombinant baculoviruses carrying POLH-ApBb fusions yielded higher levels of chimeric polyhedra, highlighting the advantage of a trans-contribution of a mutant copy of polyhedrin. Finally, the use of dissolved recombinant polyhedra as antigens was successful in an ELISA assay, as *B. bovis*-positive sera recognized the fusion POLH-ApBb. Thus, the use of this platform resulted in a promising alternative for molecular diagnosis of relevant infectious diseases.

Keywords Baculovirus · Occlusion bodies · Antigenic peptides · ELISA · *Babesia bovis*

Introduction

The baculovirus expression vector system (BEVS) is one of the most widely used eukaryotic systems to produce recombinant proteins. Baculoviruses are viruses of insects, which have a biphasic life cycle consisting of 2 different viral forms: occlusion derived virus (ODV) and budded virus (BV) (Blissard and Rohrmann 1990). ODVs are embedded in a protein matrix comprised mostly of the viral polyhedrin protein that assembles into occlusion bodies (OBs) or polyhedra. OBs are structures of resistance that protect ODVs of adverse environmental conditions and are responsible for initiating primary infection when they are ingested by larvae.

Autographa californica nucleopolyhedrovirus (AcMNPV) polyhedra are particles of about 1 μm in diameter that can be easily purified by simple centrifugation steps and this makes them excellent candidates for expressing recombinant proteins that can be incorporated into the OBs (Je et al. 2003). Fusions

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between polyhedrin and proteins of interest allow the morphogenesis of chimeric polyhedra capable of being simply isolated with high yield and purity (Chang et al. 2003; Kim et al. 2007). The expression of recombinant antigens in OBs minimizes cross-reaction, as baculoviruses do not infect vertebrates (O'Reilly et al. 1994). Therefore, the potential of recombinant proteins incorporated to polyhedra is vast.

For many diseases, the development of new diagnostic tests based on indirect enzyme-linked immunosorbent assays (ELISA) is partly limited by the availability of well-characterized antigens containing immunodominant B cell epitopes and the production of purified forms of these proteins. Traditionally, recombinant antigens are obtained from microbial sources (either prokaryotic or eukaryotic) that allow high production yields. However, recombinant proteins expressed in these systems may be co-purified with microbial proteins. Bovine serum, particularly, reacts strongly to bacterial co-purifying contaminants, producing a high background signal that affects the interpretation and standardization of ELISA results. Thus, bacterial lysates are added as blocking agents to overcome these limitations (Nguyen et al. 2014).

On the other hand, peptide microarrays have emerged as one of the most prominent and revolutionary technologies currently available for the multiplexed serological screening (Pellois et al. 2002). This technology, along with the availability of complete genomes, allowed to develop computational methods to assess defined molecular properties for each potential diagnostic target in a genome of reference and developed a scoring function used to rank all peptides from a large eukaryotic pathogen proteome to be printed into a microarray (Carmona et al. 2012). This approach allowed the prediction and/or prioritization of a subset of diagnostically relevant peptides instead of screening the entire "peptidome" of the pathogen.

With this background, we propose to combine the microarray screening of bioinformatically predicted antigenic peptides with the use of OBs as carrier particles in a promising technology to highly produce and simply purify antigens for diagnosis. To assess this, we used the parasite *Babesia bovis*, one of the causative agents of bovine babesiosis, as a prototype.

Bovine babesiosis is an acute or chronic tick borne-transmitted disease of cattle that is caused by protozoan hemoparasites of the genus Babesia, mainly *Babesia bovis* and *B. bigemina* (Bock et al. 2004). These apicomplexan parasites are transmitted by ticks and cause fever, hemolytic anemia, and anorexia. Mortality and anemia caused by bovine babesiosis impact directly in economic losses.

Serological tests are used to determine the presence of anti-Babesia spp. antibodies in herds (Mahoney and Ross 1972). Among these tests are the indirect fluorescent antibody test (IFAT) and ELISA. ELISA, which uses whole merozoite crude antigens prepared from highly enriched *B. bovis* infected erythrocytes, has undergone extensive evaluation and is considered a standard assay by OIE (De Echaide et al. 1995; Molloy et al. 1998; Waltisbuhl et al. 1987; OIE 2009). These ELISA tests show optimum values of specificity and sensitivity (above 95%) (De Echaide et al. 1995). However, these tests are performed with crude antigens because the production of native parasite antigens is difficult, time consuming, and involves maintenance of *B. bovis* merozoites in donor bovine red blood cells. To date, there are no commercial ELISA tests based on recombinant antigens for *B. bovis*. For this reason, the identification of new diagnostic candidates that would replace the use of crude parasite lysates is crucial.

Taking these difficulties into consideration, in this work, we applied the microarray technology to identify candidates from the in silico predicted *B. bovis* genome and the bioinformatic peptide prediction pipeline for B-cell epitopes. From this search, we selected 5 chemically synthesized antigenic peptides (ApBb; P1-P5) of 15 amino acids each and evaluated the performances of each ApBb alone or the 5 peptides in tandem, as a fusion to polyhedrin and incorporated into polyhedra. Previously, Je et al. (2003) reported that the contribution of wild type (wt) polyhedrin is essential to increase the probability of formation of chimeric polyhedra. For that reason, all the obtained recombinant baculoviruses presented an extra copy of the polyhedrin gene (cis-containing). Protein yields were then compared between infections of the traditional Sf9 cell line and packaging cell lines expressing polyhedrin in trans. In this work, we could select 5 B. bovis antigenic peptides not assayed until now. We also designed and obtained 6 recombinant baculoviruses that were able to include these peptides into their OBs. Furthermore, we highly produced the chimeric polyhedra and successfully employed them as antigens in an indirect ELISA.

Materials and methods

Sequence selection and peptide design

B. bovis annotated genes and translated protein sequences (assembly ASM16539v1) were downloaded from GenBank (Brayton et al. 2007). The *B. bovis* proteome was analyzed using a bioformatic antigen prediction pipeline to detect the most promising diagnostic peptidic antigens, as previously described (Carmona et al. 2012). Briefly, each protein was scored based on predicted sub-cellular localization, presence of internal protein sequence repeats, trans-membrane domains and codon usage bias. Next, all 15-mer peptides derived from each *B. bovis* protein, were ranked based on predicted antigenicity, intrinsic disorder, glycosylation state and sequence similarity against bovine (host species) and *Theileria parva* (a potentially cross-reactive apicomplexan pathogen of bovines)

proteomes. Synonymous codon usage bias was used as a proxy for translational efficiency, which is correlated with protein abundance in protozoan parasites, such as *T. cruzi*. We verified that *B. bovis* highly expressed proteins, such as ribosomal proteins, histones, tubulin, Heat Shock Proteins, and VESAs (variant erythrocyte surface antigen-1) showed a biased codon usage. All proteins were scored according to their codon usage similarity to that of the highly expressed genes.

Microarray

Serological screening

Peptide microarrays (JPT Peptide Technologies, Berlin, Germany) were employed in the serological screening. Five array slides were assayed with the following samples: 2 slides with pools (3 subjects per pool) of sera from bovines infected with *B. bovis*; 1 slide with a pool (3 subjects) of sera from *Babesia*-negative bovines; 2 slides with a pool (3 subjects) infected both with *B. bovis* and *B. bigemina*. Serological status of all sera was confirmed by the standardized ELISA assay for both Babesia parasites (De Echaide et al. 1995; Mastropaolo et al. 2009).

Serum samples were diluted 1:10 in TBS buffer (50 mM Tris-Cl, pH 7.6; 150 mM NaCl) and incubated overnight at 4 °C in a humidity chamber. After washing 3 times with TBS, secondary antitotal bovine immunoglobulin G conjugated to Cy5 (Jackson Immuno Research) diluted (1 mg/ml) 1:400 in TBS was added. The slides were incubated for 60 min at 30 °C in the humidity chamber and afterwards they were washed several times with TBS, including a step with deionized water. The slides were dried carefully under a N_2 flow before reading in the scanner.

Quantitation and analysis of peptide reactivities

The slides were scanned using the fluorescence reader ArrayWorx BioChip Reader (Applied Precision, LLC) at maximal resolution (pixel size 10 μm). The laser excitation wavelength was 633 nm (red) and the emission filter employed was 670 nm (band pass 30), according to the secondary antibody fluorophore (Cy5).

Digital array images were analyzed and peptide spots intensities were quantified with the software GE ImageQuant with no background subtraction. On the other hand, 1% of the spots signals were discarded from the analysis as they presented bad quality signals due to fluorescence smearing, spikes or other forms of non-homogeneous spot intensities. The average intensity for each peptide was calculated from subarray triplicates. All subarrays carry spots of bovine IgG attached to the glass slides as positive controls. These spots were positive in all slides assayed.

Also, all slides contain some spots with bovine, human and mouse proteins (alpha casein, bovine IgM, mouse and human IgG, etc.) attached to the slides by the manufacturer of the array to test them as candidate negative controls.

Cell lines and virus

Spodoptera frugiperda Sf9 cells obtained from American Type Culture Collection (ATCC) were cultured at 27 °C as a monolayer in TNMF-H insect medium (Sigma) supplemented with 10% fetal bovine serum (FBS, Internegocios, Argentina) and 1% antibiotic–antimycotic solution (GIBCO). Stably transformed packaging cell lines Sf9POLH (López et al. 2010) and Sf9POLH$_{E44G}$ (López et al. 2011) previously obtained in our laboratory were maintained with TNMF-H medium supplemented with 10% FBS and 10 μg/ml of blasticidin. Sf9POLH$_{E44G}$ is similar to Sf9POLH but instead of expressing wt POLH, it expresses POLH mutated in aminoacid 44 and originates bigger polyhedra with viral occlusion deficiencies when infected with a baculovirus. The *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) wt stock used in this study was originally obtained from Pharmingen. Wt and recombinant baculoviruses were propagated in Sf9 cells. Cell cultures were visualized at an AE200 Motic inverted microscope.

Obtainment of the recombinant baculoviruses

Recombinant baculoviruses were generated using the Bac-to-Bac methodology according to the supplier's suggestions (Invitrogen). Transfer vectors were obtained using vector pFBDPOLH/POLH derived from pFastBac-Dual (Invitrogen) as a backbone, which was previously constructed in our laboratory.

Oligonucleotides

The oligonucleotides used in this work were purchased to Genbiotech S.R.L. (sequences in Table 1).

Transfer vector construction

The final sequences of DNA of each peptide were optimized using the database CUTG (Codon Usage Tabulated from GenBank) (http://www.kazusa.or.jp/codon/), which considers the codon usage of *Spodoptera frugiperda*. This optimization was performed to discard possible false sequences from Sf9 insect cells, which come from *Spodoptera frugiperda*. The sequence of the 5 individual ApBb (P1, P2, P3, P4 and P5) was designed as a continuous construction to be cloned in a pUC57 bone plasmid (Fig. 1a). This vector was specially designed and synthesized to carry an ORF comprising the five ApBb in tandem (TANDEM) separated from each other

Table 1 Oligonucleotides used in this work

Oligonucleotide	Sequence (5′ to 3′)	Function
P1 for	aaactagtaggccgcttggaggtggcggc	Amplification of P1-P1 sequence
P1 rev	aaagcggccgccgcctaagctttcacat	Amplification of P1-P1 sequence
P2 for	aaactagtacatgtgaaagcttaggcggt	Amplification of P2-P2 sequence
P2 rev	gggcggccgcgtaccttagtttcctccgc	Amplification of P2-P2 sequence
P3 for	aaactagtaaaactaaggtaccaggcggt	Amplification of P3-P3 sequence
P3 rev	gggcggccgcattcctagtttattttgcg	Amplification of P3-P3 sequence
pPOLseq for	ggagataattaaaatgataacc	Recombinant bacmid analysis
pFastBacDual rev	ggctgattatgatcctctag	Vector sequencing

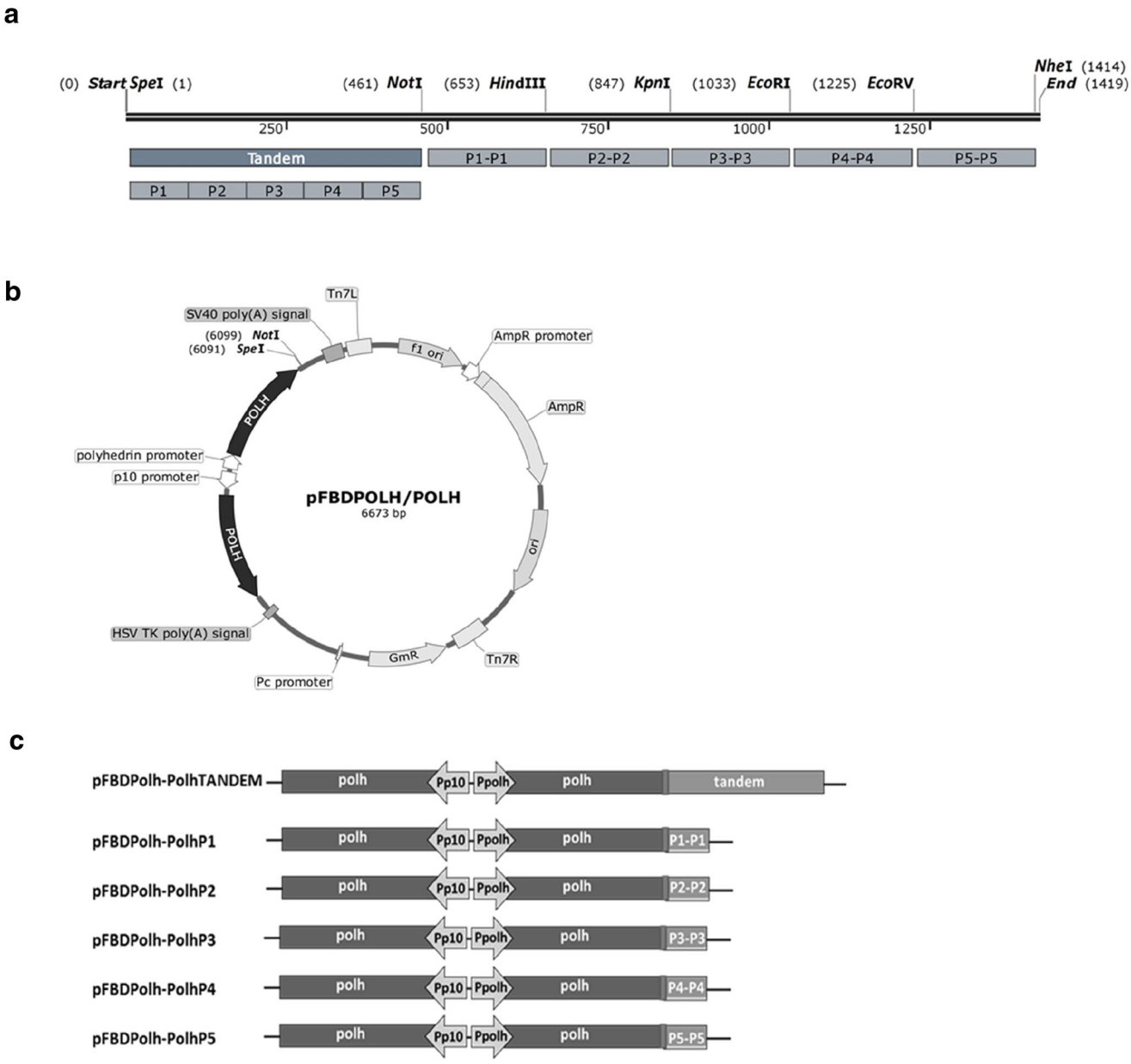

Fig. 1 Designed constructs carrying the sequence of the 5 selected *Babesia bovis* antigenic peptides (ApBb) used in this work. **a** Chemically synthesized ORF comprising the 5 ApBb (P1–P5) in tandem and each in duplicates (GeneBank accession number, MF806608), extracted from plasmid pUC57-AgBb. **b** Map of receptor transfer plasmid pFBDPOLH/POL, carrying 2 copies of polyhedrin sequence, one under P10 promoter and the other under the polyhedrin promoter. **c** Schematic illustrations of the DNA regions to be transposed to AcMNPV genomes for the obtainment of the recombinant baculoviruses

by a flexible linker region of 3 replications glycine-glycine-glycine-serine (GGGS) to allow a correct exposure of the peptides to the antibodies (Chen et al. 2013), and five ORFs corresponding to each ApBb repeated twice, separated by different restriction enzyme sites and connected by the same linker regions (P1- P1, P2-P2, P3-P3, P4-P4 and P5-P5) (GeneBank accession number: MF806608). The final vector, called pUC57-AgBb, was synthesized by GenScript™.

The plasmid diagrams were obtained using SnapGene® software (from GSL Biotech; available at snapgene.com).

The construction of baculoviruses AcPOLH/POLH-P1, AcPOLH/POLH-P2, AcPOLH/POLH-P3, AcPOLH/POLH-P4, AcPOLH/POLH-P5, and AcPOLH/POLH-TANDEM were performed using 2 different strategies for the insertion the genes of interest into pFBDPOLH/POLH vector (Fig. 1b). This vector has 2 copies of native polyhedrin (wt), one under the P10 promoter and the other under the polyhedrin promoter without its stop codon. All sequences were planned so that they can be cloned following the reading frame of the polyhedrin under its own promoter (schematized in Fig. 1c).

For the construction of vector pFBDPOLH/POLH-TANDEM, we digested the plasmid pUC57-AgBb with the restriction enzymes *Not*I and *Spe*I, to release the TANDEM fragment of approximately 450 bp. Then, this fragment was introduced into vector pFBDPOLH/POLH after digestion with the same enzymes. For the construction of vectors pFBDPOLH/POLH-P4 and pFBDPOLH/POLH-P5, we used plasmid pUC57-AgBb. This plasmid was digested with *Eco*RI and *Eco*RV as well as *Eco*RV and *Nhe*I to release the P4 and P5 fragments (both of approximately 190 bp), respectively. These fragments were introduced into vector PFBDPOLH/POLH digested with *Not*I restriction enzyme and previously treated with Klenow enzyme to blunt cohesive ends.

Finally, the vectors pFBDPOLH/POLH-P1, pFBDPOLH/POLH-P2, and pFBDPOLH/POLH-P3 were constructed. For this purpose and due to complications in blunt cloning, P1, P2, and P3 fragments were amplified by PCR from the pUC57-AgBb vector by using specific oligonucleotides for each fragment that allowed cloning into *Not*I and *Spe*I restriction sites into pFBDPOLH/POLH. In all cases, the presence of each insert in several clones was analyzed by digestion with the restriction enzyme *Hpa*I followed by sequencing using the pFastBacDual rev oligonucleotide.

Obtainment of recombinant BV

To generate the series of recombinant bacmids, the donor plasmids mentioned above were transformed into DH10Bac competent cells containing the pMON7124 helper plasmid and the AcBacmid using the Bac-to-Bac system (Invitrogen). To verify if the transposition into the bacmid was successful, the recombinant bacmid DNAs were analyzed by 2 different PCRs, one with the universal primer pairs M13 for and M13 rev and the other using the pair of oligonucleotides pPOLseq for (Table 1) and M13 for.

To generate the first passage of the recombinant baculovirus (P1), the recombinant AcBacmids mentioned above were used to transfect Sf9 cells using Cellfectin II reagent (Invitrogen) and standard procedures. At 5 days post transfection (dpt), the P1 BVs recovered were named AcPOLH/POLH-TANDEM, AcPOLH/POLH-P1, AcPOLH/POLH-P2, AcPOLH/POLH-P3, AcPOLH/POLH-P4, and AcPOLH/POLH-P5.

Samples of polyhedra

A total of 3.5×10^7 Sf9, Sf9POLH and Sf9POLH$_{E44G}$ cells in T175 flasks were infected at a multiplicity of infection (moi) of 10 with the recombinant baculoviruses previously obtained. At 5 days post infection (dpi), polyhedra were purified according to O'Reilly et al. (1994). Briefly, the cells were collected, washed with 1 × PBS pH 6.2 and the cellular pellet was re-suspended in equal volumes of 0.5% sodium dodecyl sulfate (SDS) in glass hemolysis tubes, followed by centrifugation in a low speed centrifuge (Eppendorf) at 3000*g* for 20 min. The pellets were re-suspended in equal volumes of 0.5 M NaCl, followed by centrifugation at 3000*g* for 20 min. The pellets containing insoluble material (polyhedra) were finally re-suspended in equal volumes of ultrapure water.

Scanning electron microscopy (SEM) of polyhedra samples.

Polyhedra samples isolated from different infection conditions (as described above) were washed in phosphate buffered saline (PBS), attached to poly-L-lysine-coated glass coverslips, and immersed in fixative (2% glutaraldehyde, 2% paraformaldehyde buffered in 0.1 M sodium cacodylate, pH 7.3). Fixation was carried out at room temperature for 1 h. Primary fixation was followed by rinsing in the same buffer and postfixation for 30 min in 1% osmium tetroxide. After fixation, the cells were dehydrated through an ascending acetone series, critical-point-dried in CO_2, covered with a layer of gold, and observed in a SEM JEOL 1200EX-II microscope in the Laboratory of Microscopy and Related Skills of the National Center of Agronomic Investigations (CNIA), National Institute of Agricultural Technology (INTA), Buenos Aires, Argentina.

Protein quantification and Western blot assays

For its characterization, chimeric polyhedra were sedimented and dissolved using 0.1 M Na_2CO_3. The proteins were separated by SDS-PAGE using a 12% acrylamide gel.

For quantification assays, gels were Coomassie blue stained and ImageJ software (NIH) was used to the measure the intensities of the bands corresponding to wt POLH or POLH-ApBb. For that purpose, the image was first converted into 8-bit and the measured areas were standardized to obtain a pixel curve. The pixel values obtained were extrapolated from a curve of increasing concentrations of BSA, to quantify the amount of each protein in milligrams (mg). Grouped data were represented as the mean ± SEM. Analyses were performed using Prism version 5.0 (GraphPad Software, San Diego, CA).

For Western blot assays, gels were transferred to a nitrocellulose membrane at 200 mA for 1.5 h. The membrane was incubated with an anti-polyhedrin chicken-made polyclonal antibody (GenTex®) diluted 1:3000 in TBS-T and 2% skim milk or bovine sera diluted 1:50 in TBS-T and 2% skim milk. The membrane was then incubated for 30 min with anti-chicken IgG alkaline phosphatase-conjugated antibody (SIGMA) diluted 1:1000 or with an anti-bovine IgG alkaline phosphatase-conjugated antibody (SIGMA) diluted 1:30,000. Antibody binding was visualized using the reactive NBT/BCIP (Thermo Fisher Scientific).

Bovine serum samples

A total of 48 post-vaccination bovine sera positive for *B. bovis* by current indirect iELISA (De Echaide et al. 1995) (INTA Rafaela, Santa Fe, Argentina) and 52 sera from negative animals (born and raised in Tandil, Buenos Aires, Argentina which is a *R. microplus* tick-free region) were used. To test specificity, we used a set of *B. bigemina* ($n = 16$) and *Anaplasma marginale* ($n = 4$) serologically positive sera. These sera were tested negative for *B. bovis* antibodies by the current MZ–iELISA (De Echaide et al. 1995).

All the serum samples used in this work were obtained by cattle manually restrained (< 5 min) and blood samples were aseptically collected by jugular venipuncture (Vacutainer ™, Becton Dickinson, < 0.0005% blood volume; one sampling per animal).

ELISA

Polyhedra samples were dissolved in 1 M Na_2CO_3 buffer for 30 min at 37 °C and F96 Maxisorp Nunc-Immuno plates (Themo Fisher Scientific, MA, USA) were coated with 2 μg of the dissolved polyhedra in buffer 0.03 M carbonate/0.07 M bicarbonate, pH: 9.6 at 4 °C overnight. After blocking with PBS containing 5% skim milk, 50-fold diluted bovine serum from animals infected with *B. bovis*, or not infected (control), was added to each well and incubated at room temperature (r.t.) for 1 h. After washing the wells 3 times with PBS containing 0.05% Tween 20 (Sigma-Aldrich), 1000-fold diluted HRP-conjugated anti-bovine (KPL) was added and incubated at r.t. for 1 h at 37 °C. After washing 4 times, the reaction was developed by the addition of ABTS substrate (Sigma Aldrich) in 0.1 M Citrate Buffer pH 4 with 0.05% H_2O_2. The absorbance was measured at 405 nm using a Multiskan spectrophotometer (Labsystems).

Three strong positive samples, previously tested by the reference indirect ELISA, were included in each plate as reference controls and used as duplicates. Additionally, 3 negative sera reference from a free-tick region were also included as duplicates in each plate.

In all cases, the average of values of each group was used. The absorbance of each serum was expressed as positivity percentage (%P) regarding the value of the positive controls according to the following value = [(A_{450nm} of serum sample × 100)/Average of A_{450nm} of positive control sera].

Taking into consideration the number of known negative serum samples analyzed, the cutoff value was defined as the mean of the negative samples + 2 standard deviations.

Results

Peptide selection

We first analyzed the *B. bovis* proteome to detect the most promising diagnostic peptide antigens. For this purpose, we used a bioinformatics antigen prediction pipeline, as previously described by Carmona and co-workers (2012). In this analysis, we assessed properties such as sub-cellular localization or expression level for the whole protein. In addition, at a higher resolution, we evaluated a set of local properties as repetitive motifs, disorder (structured vs. natively unstructured regions), trans-membrane spans, genetic polymorphisms (conserved vs. divergent regions), predicted B cell epitopes, and sequence similarity against bovine proteins and other potential cross-reacting species.

The pipeline defined a linear function generating a ranking of overlapping peptides of 15 amino-acid residues from the "in silico" proteome of the parasite. A list of 89 candidate peptides resulted from the bioinformatic analysis and after a manual curation from the peptides of highest scores (Table S1). These peptides, which were derived from 71 different protein products, were present in the microarray in 3 internal replicas. The list includes peptides from already reported and/or characterized *B. bovis* antigens such as the small heat shock protein, the rhoptry associated protein-1 (Rap-1), the merozoite surface protein (MSA), the variant erythrocyte surface antigen, the apical membrane antigen (AMA-1), the spherical body proteins 1 and 2 (SBP 1 and 2) and the 12D3 antigen.

The exploratory experimental validation herein performed contained these 15-mer peptide-chips and used 2 slides with pools of sera from bovines infected with *B. bovis* and 1 slide

with sera from Babesia-negative bovines. To discard possible *B. bigemina* cross-reacting antibodies, we also analyzed 2 slides with pools infected both with *B. bovis* and *B. bigemina*. The binding was quantified for each spot in the array and statistically significant signals were determined as described in Materials and methods. We selected 5 spots corresponding to 5 individual peptides that were specific for *B. bovis* positive sera (Table 2).

Recombinant baculovirus construction

The first step for obtaining the recombinant baculoviruses was the design of the transfer vectors which would carry the sequences of interest to be cloned as fusion to polyhedrin. All the plasmids were based on pFBDPOLH/POLH vector (Fig. 1b). They carried the fusion of polyhedrin to the 5 ApBb in tandem (TANDEM) separated from each other by a flexible linker region, or with each of the five ORFs corresponding to ApBb repeated twice and connected by the same linker peptide. The fusions were placed under the polh promoter sequence. In addition, every cassette carried also a copy of polyhedrin under P10 promoter (Fig. 1c). The final 6 recombinant baculoviruses obtained using Bac-to-bac methodology were AcPOLH/POLH-P1, AcPOLH/POLH-P2, AcPOLH/POLH-P3, AcPOLH/POLH-P4, AcPOLH/POLHP5, and AcPOLH/POLH-TANDEM.

Three dpi Sf9 cells showed cytopathic effects by optic microscopy. We confirmed the presence of OBs similar to those observed in an infection with wt AcMNPV in the case of infections to the lines Sf9 (Fig. 2aI) and Sf9POLH (not shown). In the case of infections to $Sf9POLH_{E44G}$ cell line, some cells contained bigger polyhedra corresponding to E44G mutant polyhedrin phenotype (Figure 2AII). Through scanning electron micrographs, we further analyzed polyhedra morphology of the isolated chimeric OBs. Although in Fig. 2b we only show a SEM photograph of POLH-TANDEM polyhedra, every chimeric OB sample presented similar morphology.

Chimeric polyhedra analysis

We then evaluated the efficiency of incorporation of the different ApBb by Western blot. With this aim, we performed infections of Sf9 cells with the 6 recombinant baculoviruses at a high moi and 5 days post infection, polyhedra were isolated as described in Materials and methods. Subsequently, each polyhedron sample was dissolved and electrophoresed by SDS-PAGE. Fusion proteins incorporated into OBs were analyzed with a polyclonal anti-polh and an infected bovine serum as primary antibody. The anti-polh antiserum recognized all the fusion proteins (Fig. 3a), whereas, conversely, a positive bovine serum only recognized some of them (Fig. 3b). Then, we performed the analysis with randomly selected positive sera. In this case, every ApBb was recognized at least once by some positive serum, whereas the fusion containing the tandem peptide was recognized by every infected bovine serum that previously recognized some of the peptides alone (data not shown). With this evidence, we selected POLH-TANDEM fusion for further production and antigenicity analysis.

To analyze the production of ApBb by the recombinant baculoviruses, we compared the expression of the fusion proteins with or without trans-contribution of polyhedrin by packaging cell lines. We employed 3 cell lines: Sf9, Sf9POLH and $Sf9POLH_{E44G}$ at the same moi, in triplicates. First, we measured the proportion of fusion protein incorporated to chimeric OBs, by performing infections with the baculovirus AcPOLH/POLH-TANDEM. The resulting chimeric polyhedra were purified as stated in M&M, dissolved, resolved by SDS-PAGE, and Coomassie blue stained. The average of the intensities of the bands corresponding to POLH (29 KDa) and POLH-TANDEM (43 kDa) in each sample was conversed to milligrams (mg) of protein obtained, by extrapolating the data with a standard curve of increasing quantities of BSA. The results are shown in a bar graph (Fig. 4a). Infection of the $Sf9POLH_{E44G}$ line displayed the greatest amount of total proteins per polyhedra sample, although ANOVA statistic test showed non-significant differences to other samples. Indeed, the proportion of recombinant protein included into polyhedra was independent of the cell line used, being approximately 30% of the total proteins in each sample.

Then, we compared ApBb yields in the different cell lines, by performing infections with the baculovirus AcPOLH/POLH-TANDEM and AcPOLH/POLH-P5. As explained above, we quantified the intensities of the bands of fusion

Table 2 Amino acid sequence of the 5 antigenic peptides of *Babesia bovis* (ApBb)

B. bovis gene symbol	Name	Derived peptide	Aminoacid sequence of peptide
BBOV_II000710	Hypothetical protein	P1	MPPMNEPMPPMNGPM
BBOV_IV010380	Membrane protein	P2	VKHGKRSHDKHRGGN
BBOV_II006080	Subtilisin-like protein	P3	DGNNKPRRSPKRKIN
BBOV_IV011230	Apical membrane antigen 1	P4	RGSDDTSESSDRYSG
BBOV_IV011230	Apical membrane antigen 1	P5	ASRGQLLNSRRGSDD

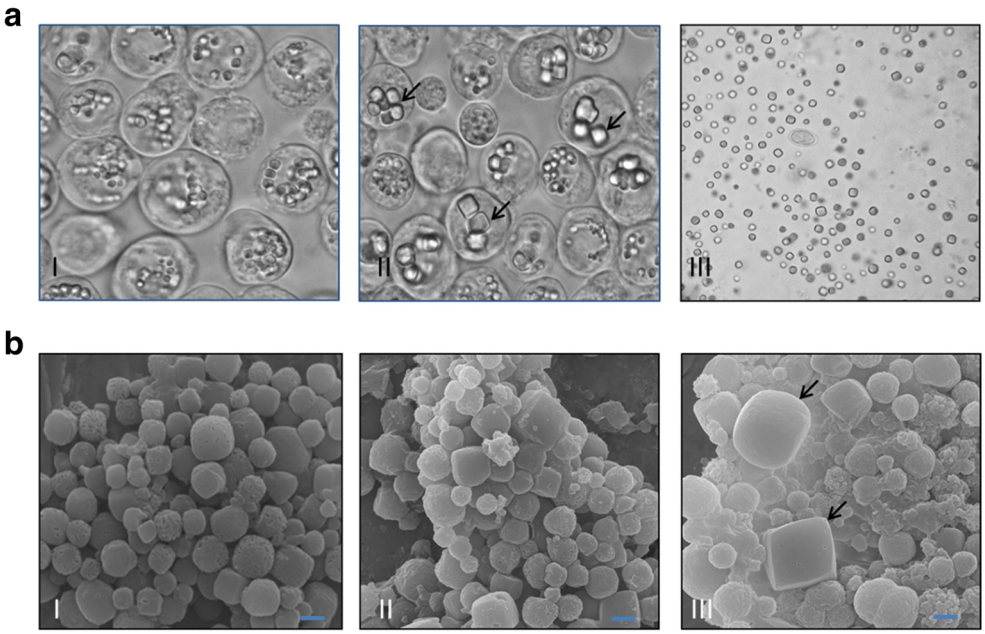

Fig. 2 Chimeric polyhedra samples. **a** I and II: Visualization by optical microscopy of Sf9 and Sf9POLH$_{E44G}$ cell lines, respectively, infected with the baculovirus AcPOLH/POLH-TANDEM. Amplification: 100 ×. III: Polyhedra sample after purification from infected cells. Amplification: 40 ×.Arrows point the formation of larger polyhedra due to the contribution of mutant polyhedrin by the line Sf9POLH$_{E44G}$ **b** SEM analysis of chimeric POLH-TANDEM polyhedra (as an example) purified from infections to I: Sf9 cell line II: Sf9POLH cell line and III: Sf9POLH$_{E44G}$ cell line. The arrow indicates the formation of larger polyhedra. Scale bar: 1 μm

proteins in Coomassie blue stained gels, and calculated the mg of recombinant proteins rendered by each polyhedra sample. The results showed that yields were significantly different for POLH-P5 (ANOVA, $F\ (1.8) = 16.8$, $p < 0.005$) in the 3 cell lines assayed, but not for POLH-TANDEM (Fig. 4b). Taking these results into account, we employed the Sf9POLH$_{E44G}$ cell line to obtain chimeric polyhedra in large quantities and reached levels of up to 0.3 mg of recombinant protein per bottle of 75 cm^2 of infected cells.

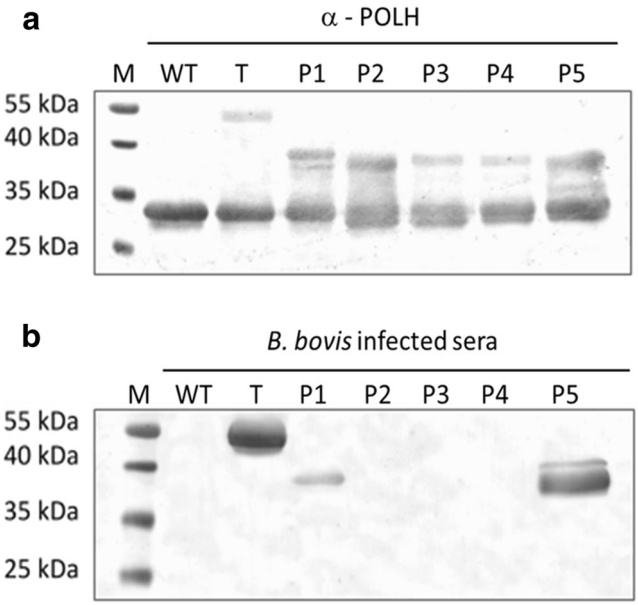

Fig. 3 Characterization of chimeric polyhedra by Western Blot. Samples of dissolved polyhedra isolated from infections with AcMNPV (wt), AcPOLH/POLH-TANDEM (T); AcPOLH/POLH-P1 (P1), AcPOLH/POLH-P2 (P2); AcPOLH/POLH-P3 (P3); AcPOLH/POLH-P4 (P4) and AcPOLH/POLH-P5 (P5). (M) Molecular weight marker. **a** Detection of POLH with a polyclonal chicken-made anti-polh antibody. POLH-TANDEM (43 kDa) and POLH-ApBb (38 kDa) fusions efficiently incorporated into the OBs are marked with arrows. **b** Antigenic evaluation of ApBb with a bovine serum positive for *B. Bovis*. An example of the differential reactivity of a bovine serum to TANDEM (T), peptide 1 (P1), and peptide 5 (P5) can be observed

Assessing peptide performance by ELISA

To evaluate the antigenic capacity of ApBbs incorporated into the AcMNPV OBs, we performed an ELISA test in which the dissolved chimeric POLH-TANDEM polyhedra were challenged with 48 bovine positive sera and 52 sera negative to *Babesia bovis*. Each serum was assayed in duplicate and the cutoff point was defined as the average plus 2 standard deviations of the values obtained with the negative samples. The positivity percentages obtained are shown in Fig. 5. In the conditions assessed, ApBb could recognize 60.4% of positive sera with variable values but above the cut-off point. On the other hand, 48 of 52 control sera resulted negative. Regarding species specificity, the POLH-TANDEM indirect ELISA (iELISA) was tested with samples originated from regions where the tick *R. microplus* is present and may transmit other hemoparasites (i.e., *B. bigemina* and *Anaplasma marginale*) to bovines. All except one of the *A. marginale* positive sera ($n = 4$) scored below the cutoff established value and were considered negative; meanwhile, all *B. bigemina* were negative to the ApBb iELISA.

Discussion

In this work, we propose an integrated approach for the discovery and production of novel B-cell epitopes that could be

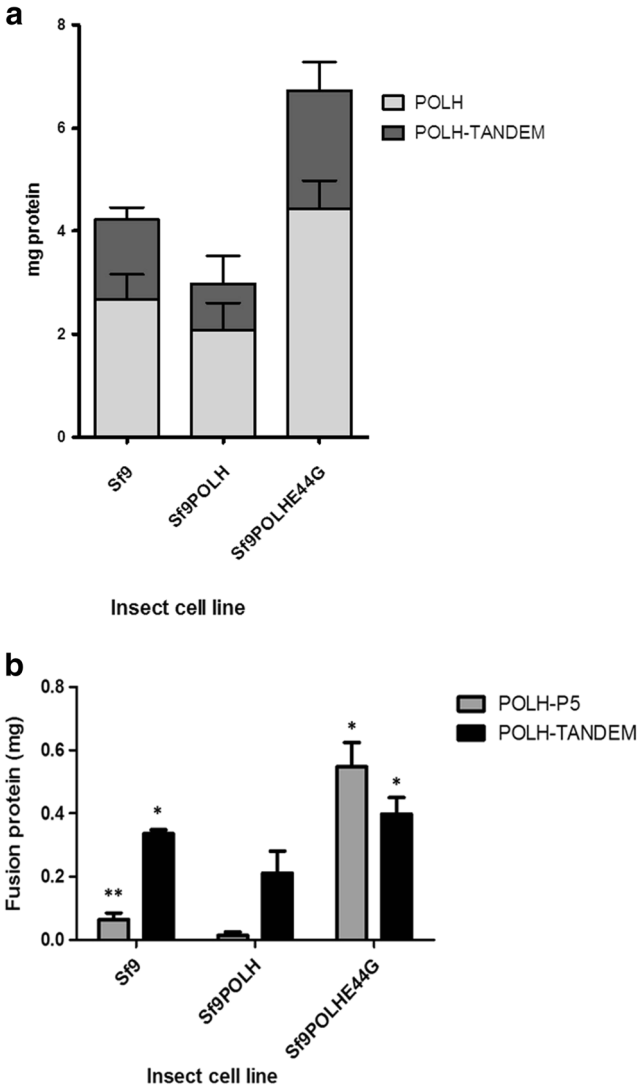

Fig. 4 Analysis of POLH-ApBb yields. **a** Composition of OBs obtained by infection of cell lines Sf9, Sf9POLH and Sf9POLH$_{E44G}$ with baculovirus AcPOLH/POLH-TANDEM. Bars represent the total proteins of each polyhedra sample. In dark gray is the proportion of POLH-TANDEM and in light gray the proportion of POLH in each sample. **b** Comparison of yields of POLH-TANDEM vs POLH-P5 in the 3 cell lines. The amount of POLH-P5 (gray bars) and POLH-TANDEM (black bars) fusion proteins obtained from recombinant polyhedra. Error bar: standard deviation. ** $F\ (1.8) = 50.82$ $p < 0.001$; * $F\ (1.8) = 16.8$, $p < 0.005$. The milligrams of fusion protein in **a** and fusion protein and polyhedrin in **b** was obtained from an average of intensities of bands in an SDS-PAGE stained with Coomassie blue, and extrapolated from a standard curve with increasing concentrations of BSA

useful for the diagnosis of bovine babesiosis. Our strategy involved the prediction of antigenic peptides of *Babesia bovis* by bioinformatics analysis followed by screening of short synthetic peptides in microarray platforms, expression by baculovirus-insect cell system and further validation with ELISA. Even though the biological model chosen here was the bovine babesiosis caused by *B. bovis*, our strategy can be completely applied to other diagnostic systems where B cell epitopes are still unknown and where expression of peptides results tedious.

Serological test based on proteins expressed in prokaryotic systems is often confronted with the problem of high reactivity of normal bovine sera with contaminants derived from bacteria. The solution involves the inclusion of complex and costly purification steps in the antigen-obtaining protocols. These drawbacks are often overcome with different mammalian expression systems for the production of proteins of interest. In these systems, the high costs discourage the production of antigens for diagnostic tests for veterinary use in farm animals. BEVS appears as an attractive alternative, as baculoviruses are not pathogens for vertebrates or plants (O'Reilly et al. 1994) and the risk of cross reacting antigens is almost null.

Particularly, baculovirus polyhedra offer many biotechnological advantages, since they are particles that can be easily purified by low centrifugation. This characteristic makes them excellent candidates to express recombinant proteins in concentrations higher than those obtained by a simple recombinant baculoviruses (Je et al. 2003). Moreover, the smaller the peptides are, the better they can be incorporated into polyhedra without altering its morphogenesis, while maintaining its simplicity of purification (unpublished results).

In this work, we analyzed the microarrays containing the selected 89 peptides and the 5 identified candidates that are significantly more reactive to sera from bovines infected with *B. bovis*. Only 2 of these peptides belong to an already characterized *B. bovis* antigen called apical_membrane_antigen_1 (Gaffar et al. 2004). The other 3 peptides are uncharacterized yet and belong to hypothetical membrane and subtilisin-like proteins respectively (Table 2). The potential of the respective proteins as diagnostic candidates still deserves further investigation. Initially, these peptides were not recognized by specific bovine sera when they were synthesized in a biotinylated form and used as antigens in an indirect ELISA test on plates with streptavidin. This may be because their small size prevented their correct presentation after the adsorption to the plates (data not shown). Thus, we propose that the fusion of the antigenic peptides to polyhedrin and their inclusion into baculovirus polyhedra could be indeed a practical integral solution. Indeed, polyhedrin could be not only a mean to improve and simplify its purification, but also a carrier that allow epitopes to be correctly exposed to the antibodies.

As inclusion of a foreign protein depends on an interaction between native polyhedrin and the polyhedrin fusion protein, a contribution of wt polyhedrin is necessary for the correct incorporation of recombinant peptides into polyhedra (Je et al. 2003; Sampieri et al. 2015). This extra copy of polyhedrin can be carried in cis by the recombinant baculovirus or can be supplied in trans by a co-infection with AcMNPV wt or by infecting packaging cell lines.

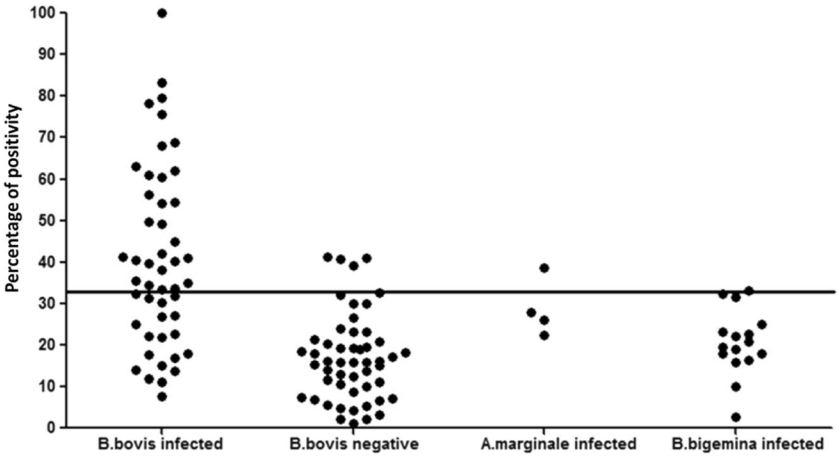

Fig. 5 ELISA assay to evaluate the antigenic capacity of the dissolved polyhedra carrying POLH-TANDEM by using sera from animals infected with *B. bovis* and from uninfected and sera from cattle exposed to *B. bigemina* and *A. marginale* as negative controls. Each spot corresponds to one serum sample expressed as percentage of positivity in relation to a positive control showing an arbitrary absorbance value of 1. The cutoff point (black horizontal line) was calculated as the mean of negative sera to *B. bovis* plus 2 standard deviations

Our results show that all ApBbs were efficiently expressed as fusion to polyhedrin and successfully incorporated into polyhedra due to the presence of wt polyhedrin in cis in Sf9 cells. We were able to isolate all of them, based on their insolubility at neutral pH with 2 simple centrifugation steps. The analysis by Western blot demonstrated the presence of the fusion proteins (Fig. 3). All 5 candidates reacted to serum of *B. bovis* infected animals in Western blot (data not shown). Indeed, peptide 5 and the TANDEM reacted with a stronger signal than the other peptides. Furthermore, the TANDEM was the peptide that reacted to most sera. For this reason, we selected this polypeptide for the ELISA test, by including it into occlusion bodies.

To improve renders of chimeric polyhedra in a co-infection-independent manner, we used packaging cell lines, which are Sf9 cells stably transformed with the polyhedrin gene expressing such protein when infected, ensuring the production of polyhedra even in virus of polh- genotype. We assayed 2 previously developed insect cell lines: Sf9POLH (López et al. 2010) and Sf9POLH$_{E44G}$. Sf9POLH carries a copy of the polyhedrin wt, whereas Sf9POLH$_{E44G}$ carries a copy of mutant polyhedrin at amino acid 44 and generates polyhedra of greater surface that have occlusion deficiencies (López et al. 2011). When we analyzed the composition of chimeric polyhedra obtained by infection of the 3 lines with baculovirus AcPOLH/POLH-TANDEM, the higher yields of total polyhedra protein were in infected Sf9POLH$_{E44G}$. Nevertheless, in all cases, the proportion of fusion protein POLH-TANDEM was around 30% of the total protein (Fig. 4a). This proportion reminds us of polyhedrin protein structure that allows its auto-assembly into trimers that subsequently stick together to form polyhedra tetramers (Coulibaly et al. 2009; Ji et al. 2010). With our results, we suggest that inclusion of fusion proteins into polyhedra may be by forming trimers composed by POLH-ApBb:POLH in a 1:2 ratio, respectively. This is in accordance with previous reports which described that the ratio of the recombinant fused polyhedrin fragments to the wt polyhedrin is very important for the formation and quantity of foreign protein that could be incorporated into crystal (Sampieri et al. 2015).

In terms of fusion proteins yields, when comparing POLH-TANDEM with POLH-P5 in the 3 lines, infected Sf9POLH$_{E44G}$ rendered equal or higher levels of recombinant protein than Sf9 cell line, independent of the recombinant baculovirus used; but only POLH-P5 production showed significant differences (Fig. 4b). This result suggests that renders may vary depending on the size of the fusion protein and the cellular system employed. Nevertheless, production of mutant polyhedra contributes advantageously to the production of fusion proteins With this evidence, we used the Sf9POLH$_{E44G}$ cell line for producing POLH-TANDEM polyhedra in ELISA because, in addition to the rise of total proteins, the mutant polyhedra present a reduction of baculovirus total proteins that also may contaminate samples. In our hands, the production of 220 ng of chimeric protein POLH-TANDEM enough to coat an ELISA plate has an approximate cost of U$S 0.46, 15 times less expensive than equivalent quantities of immobilized synthetic peptides.

Finally, the ApBb present in polyhedra further dissolved at alkaline pH were efficiently recognized in ELISA assays, thus demonstrating that polyhedrin fusion did not affect their immunological recognition and probably improved the exposure of B-cell epitopes to antibodies. The TANDEM construct efficiently reacted to 60.4% of *B. bovis*-positive sera with no cross reaction with sera from *B. bigemina*-infected bovines. This high species-specificity confirms that the previous selection of *B. bovis* positive spots in the microarray analysis was accurate and helped to discard potential cross reactive *B. bigemina* peptides. The large variation of positivity percentages observed in the ELISA test reflects what is usual in

serum samples of bovines that received the *B. bovis* live attenuated vaccine and then were naturally challenged in the field by the bite of infected ticks. On the other hand, almost 40% of positive *B. bovis* sera failed to show positive signals in the POLH-TANDEM ELISA. Therefore, further improvements need to be made to the POLH-TANDEM construct including other peptides that also showed high antigenic scores. Similar results were obtained by List et al. (2010) in a bioinformatics selection of peptides complemented with screening on a high-throughput microarray platform for the immunodiagnosis of echinococcosis. Their data showed that a single peptide cannot provide sufficient diagnostic sensitivity, whereas pooling several peptide antigens improved the assay. Indeed, the sensitivity of mixes would likely increase by continuous search for new peptides with improved diagnostic properties to reach a sensitivity comparable to the crude parasite extract.

We conclude that the availability of new small antigens by peptide microarrays and the current application of baculovirus OBs as carrier particles appear as promising technology to highly produce and simply purify antigens with potential use for diagnosis.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain studies with human participants performed by any of authors. The protocol for animal handling and venipuncture was performed following the guidelines of the Institutional Committee for the Use and Care of Experimentation Animals (protocol approval No. 025/2011). All samples came from privately owned herds and were sampled with the approval of the owners.

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