Cloning and expression of a chimera containing ROP2 of Neospora caninum fused with OprI lipoprotein from Pseudomonas aeruginosa

Clonagem e expressão de quimera contendo a proteína ROP2 de Neospora caninum fusionada a lipoproteína OprI de Pseudomonas aeruginosa

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ABSTRACT

Neospora caninum is the etiologic agent of neosporosis, is one of the main responsible for abortion in cattle herds, causing economic losses to Cattle-raising. Vaccination of cattle would be an important alternative; however, the lack of effective vaccines prevents the application of this control method. The proteins present in rhoptries (ROPs), due to their importance in cell infection and their antigenic and immunogenic characteristics, became excellent candidates for vaccine antigens. The bacterial lipoproteins as an Oprl from Pseudomonas aeruginosa have received particular attention as an adjuvant carrier molecule. The aims of this study were to clone and expressing a chimera containing NcROP2 fused with OprI lipoprotein from P. aeruginosa for the future development of a recombinant vaccine against N. caninum. We cloned and expressed, in Escherichia coli Rosetta (DE3) pLysS, the region of NcROP2 described between amino acids 191 and 359, fused OprI producing the chimera rROP2/OprI, showed an expected size of ~50 kDa. and characterized its antigenicity. The protein was purified and characterized by Western blot with anti-histidine monoclonal antibodies and their antigenicity recognized by sera from animals naturally infected by N. caninum. The chimera rROP2/OprI was recognized by antibodies anti-N. caninum reviling common antigenic determinants of the recombinant protein and its native form, suggesting its use for developing a recombinant vaccine.

Keywords: ROP2, OprI, N. Caninum, P. Aeruginosa, Recombinant Protein, Chimera.

RESUMO

Neospora caninum é o agente etiológico da neosporose, uma das principais doenças responsáveis por abortos em rebanhos bovinos, causando assim perdas econômicas a pecuária. A vacinação dos bovinos poderia ser uma alternativa interessante, porém a falta de vacinas eficazes impede a aplicação desse método de controle. As proteínas presentes nas róptrias (ROPs), devido sua importância na infecção de células e características imunogênicas e antigênicas, tornam-se excelentes candidatas como antígenos vacinais. Lipoproteínas bacterianas como a OprI de Pseudomonas aeruginosa tem recebido particular atenção como moléculas adjuvantes carreadoras. Os objetivos deste estudo foram clonar e expressar a quimera contendo NcROP2 fusionada a lipoproteína OprI de P. aeruginosa para o futuro desenvolvimento de uma vacina recombinante contra N. caninum. Nós clonamos e expressamos, em Escherichia coli Rosetta (DE3) pLysS, a região de NcROP2 entre os aminoácidos 191 e 359, fusionado a OprI na quimera rROP2/OprI, demonstrando o tamanho predito de ~50 kDa e caracterizando sua antigenicidade. A proteína foi purificada e caracterizada por Western blot com anticorpos monoclonais anti-histidina e sua antigenicidade reconhecida por soros de animais naturalmente infectados por N. caninum. A quimera rROP2/OprI foi reconhecida por anticorpos anti-N. caninum, revelando determinantes antigênicos comuns entre a proteína recombinante e a forma nativa de NcROP2, o que sugere a possibilidade de seu uso no desenvolvimento de uma vacina recombinante.

Palavras-Chave: Rop2, Opri, N. Caninum, P. Aeruginosa, Proteína Recombinante, Quimera.

1 INTRODUCTION

The parasite Neospora caninum is the main cause of abortion in cattle in many countries around the world and causes important economic losses to the cattle industry (Dubey, 2003; Cerqueira-César et al., 2017; Majewski et al., 2020). In the last years several subunit vaccines based on proteins involved in adhesion, invasion, and intracellular proliferation of N. caninum, and immunodominant antigens have been evaluated as targets for vaccine development (Debache et al., 2009; Monney et al., 2011, Pastor-Fernández et al., 2015). Rhoptry proteins, such as NcROP2, are involved in a variety of cellular functions related to parasite-host cell interaction (Alaeddine et al., 2013; Dubremetz, 2007; Saeij et al., 2007), and has been regarded as a promising antigen candidate for use in vaccines.

The lipoprotein, OprI is the most abundant component from outer membrane of Pseudomonas aeruginosa, and there is a large body of evidence that the innate immune system recognizes it by Toll-like receptor -2 (TLR-2) (Kawa and Akira, 2009). The use of OprI as an adjuvant fusion protein has been reported (Myzuno and Kageyama 1978; Cornelis et al., 1996; Basto et al., 2012; 2015). Cote-Sierra et al., (2002), reported the use of OprI fused with a protein from Leishmania major was able to induce immune response towards a protective T helper (Th)1 profile. More recently, it was shown that OprI was fused to the chimeric N. caninum antigen Mic3-1-R was able to induce an immune response with mixed Th1 and Th2 properties in mice (Aguado-Martínez et al., 2016).

The aims of this study were to clone and expressing a chimera containing NcROP2 fused with OprI lipoprotein from P. aeruginosa for the future development of a recombinant vaccine against N. caninum.

2 MATERIALS AND METHODS

2.1 NEOSPORA CANINUM STRAIN AND DNA EXTRACTION

Neospora caninum isolate NC-1 used in this study was provided by the laboratory of parasitic diseases, Federal University of Santa Maria. The parasite was propagated in Vero cells using Dulbecco's modified essential medium supplemented with 10% fetal calf serum, at 37 ºC in a humidified atmosphere of 5% CO_2. When 80% of the Vero cells

had been destroyed by the tachyzoites, the monolayer cells were removed and washed twice with phosphate buffered saline (PBS) solution and centrifuged at 1000×g for 10 minutes. The genomic DNA from tachyzoites was isolated according to the method described by Sambrook and Russell (2001).

2.2 PRIMERS, PCR, CLONING, AND DNA SEQUENCING

Oligonucleotide primers to the ROP2 from Neospora caninum (NcROP2) used in this study, were described previously by Monney et al. (2011), however, for correct reading frame fusion with the Pseudomonas aeruginosa OprI subunit, cleavage sites for EcoRI and XhOI were added in forward (3'-ACGAATTCCTGCGACCAGGCCA-5') and reverse (3'-CTCATCCGGCGTGTTAGTCGGG-5') primers. PCR was performed with a final volume of 25 µl containing a solution containing approximately 40 ng of the extracted DNA, 100 mM of dNTP, 2 units of Taq DNA polymerase, 10× reaction buffer, 50 mM $MgCl_2$, 0.01 pmol of each primer. The PCR amplification consisted in 30 cycles of 94 ºC for 1min, 58 ºC for 1min, and 72 ºC also for 1 min.

After DNA amplification, the PCR product obtained was purified with NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel). PCR products and pOLT7 expression vector, which containing OprI gene (Basto et al., 2012), were digested with restriction enzymes EcoRI and XhOI (New England Biolabs), and ligated with T4 DNA ligase (New England Biolabs). The resulted product (pOLT7/rROP2/OprI) was used to transform Escherichia coli strain TOP10F by heat shock method and transformants were selected in Luria Bertani (LB) plates (1% tryptone, 0.5% yeast extract, 0.5% NaCl, and 2% agar) supplemented with kanamycin (30 µg/ml). Bacterial clones were screened for recombinants and plasmid DNA was extracted using NucleoSpin® Plasmid kit (Macherey-Nagel). Recombinant clones were characterized by endonuclease digestion and confirmed by colony PCR with the above primers. Also, one of these recombinant clones was propagated and plasmid DNA was sequenced to confirm that the sequence of the insert was as expected. The sequencing was performed in ABI prism® 3500 Genetic Analyzer (Applied Biosystems).

2.3 EXPRESSION OF RECOMBINANT CHIMERA RROP2/OPRI

The recombinant vector pOLT7/rROP2/OprI was transformed into E. coli Rosetta BL21 DE3 pLysS (Novagen) by heat shock method. Transformed cells were grown in LB broth supplemented with 30 µg/mL of kanamycin and 34 µg/mL of chloramphenicol

in an orbital shaker maintained at 200 rpm, overnight at 37 °C. The Cultures were diluted at 1:20 (v/v) in fresh medium, and the incubation proceeded at the same conditions until the mid-log growth phase (OD_{600} = 0.8−1.0). Recombinant protein expression was induced for 3 h by the addition of isopropyl β-D-1 thiogalactopyranoside (IPTG) to a final concentration of 1 mM. The cells were harvested by centrifugation at 5,500×g for 20 min at 4 °C.

2.4 PREPARATION OF OUTER MEMBRANES, DEPYROGENATION AND LIPOPOLYSACCHARIDE EXTRACTION

The bacterial outer membranes were prepared by differential solubilization with N-lauroylsarcosine sodium salt solution (sarkosyl) and outer membrane depyrogenation was performed by hot phenol/water extraction as previously described (Basto et al., 2014) with minor modifications. Briefly, the pellet cells were washed with PBS, and resuspended in 25 mL of lysis buffer (25 mM Tris–HCl pH 8.0, 50 mM glucose, 10 mM EDTA), with 2.5 mg/mL of lysozyme and 1 mM phenylmethylsulfonyl fluoride (PMSF). After 30 min of incubation on ice, equal volume of 2% (w/v) sarkosyl was added, the mixture was homogenized and then ultra-sonicated. Finally, the cell lysate was ultra-centrifuged at 40,000 × g for 4 h at 4 ºC to obtain an insoluble outer membrane pellet. The outer membrane pellets were resuspended in 10 mL of PBS after an equal volume of 65 ºC heated phenol (Sigma) was added, then the samples were heated at 65 ºC for 15 min and vortexed intermittently for 15 min, followed by centrifugation at 13,400 × g for 15 min at 4 ºC. The upper lipopolysaccharide (LPS) containing aqueous phase was removed and the phenol phase was treated, twice, with 65 ºC heated deionized water, in the same conditions, followed by centrifugation. The phenol phase was transferred to new tubes, 5-volumes of pre-chilled 0.1 M ammonium acetate in methanol were added and proteins were allowed to precipitate overnight at -20 ºC. The protein precipitates were centrifuged as above and the pellets were sequentially washed in pre-chilled 0.1 M ammonium acetate in methanol, 100% ethanol and acetone, and finally air-dried at room temperature.

2.5 PROTEIN PURIFICATION BY AFFINITY CHROMATOGRAPHY

The recombinant protein purification was performed by affinity chromatography with Ni^{2+} under denaturing conditions as previously described by Basto et al. (2012), but with minor modifications. Briefly, the protein precipitates were resuspended in 10 mL of

binding buffer (20 mM NaH_2PO_4, 0.5 M NaCl, 20 mM imidazole, 6 M guanidine hydrochloride, 2% (v/v) Triton X-100, 20 mM β-mercaptoethanol, and protease inhibitor cocktail (Complete, Mini, EDTA-free, Roche), pH 7.4), and incubated with soft agitation, overnight, at room temperature. The followed day, samples were centrifuged at 20,000×g for 15 min at 4 ºC, after the pH were adjusting to 7.4, and the supernatants were incubated with Ni Sepharose 6 Fast Flow (GE Healthcare) at the ratio 1 mL protein sample/100 µL resin, during 2 h at room temperature. After the samples were transferred to an empty PD10 column (GE Healthcare), the chromatographic columns were washed with 20 resin-bed volumes of buffer W1-TX100 (20 mM NaH_2PO_4, 0.5 M NaCl, 20 mM imidazole, 8 M urea, 2% (v/v) Triton X-100, pH 7.4). The detergents were removed from the columns with 20 resin-bed volumes of buffer W2 (20 mM NaH_2PO_4, 0.5 M NaCl, 8 M urea, pH 7.4) and the elution was performed with elution buffer (20 mM NaH_2PO_4,0.5 NaCl M, 8 M urea, pH 4.5) in acidic conditions. The eluates were dialyzed against PBS, aliquoted in Protein LoBind tubes (Eppendorf), and stored at −80 ºC. The concentration of recombinant protein was measured by the BCA™ Protein Assay kit (Pierce) according to the manufacturer's instructions.

2.6 SDS-PAGE AND WESTERN BLOTTING

Purified proteins were boiled in SDS-PAGE loading buffer and separated on 15% separating gel in Mini-PROTEAN electrophoresis system (Bio-Rad). The gel was stained with Coomassie Brilliant Blue R250. For Western blotting, proteins were transferred onto a nitrocellulose membrane using Bio-Rad Mini Trans-Blot Cell. The membrane was blocked with 5% non-fat dry milk and antigenic proteins were detected by incubating membrane with the MAb Anti-6xHis HRP conjugated (1:5,000) or sera (1:100) from naturally infected animals (cattle and sheep) with N. caninum. Membranes were then incubated anti-bovine or anti-sheep (1:10,000) immunoglobulins HRP conjugated. The recombinant protein was detected when the membrane is placed in DAB solution (0.6 mg diaminobenzidine, 0.03% nickel sulfate, 50mM Tris–HCl pH 8.0, and hydrogen peroxide 30 vol.) until a colored reaction appeared.

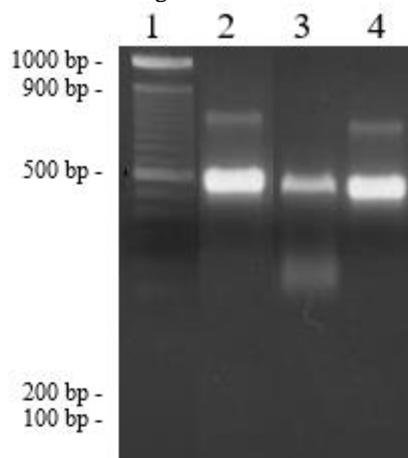
3 RESULTS

3.1 PCR, AMPLIFICATION OF ROP2 FRAGMENT, AND CLONING

The expected 522 bp of the parasite DNA gene fragment that encodes for NcROP2 protein (aminoacid 191 to 359) was amplified by PCR (Fig. 1). Amplified fragments were

purified and cloned into the pOLT7 expression vector, which containing OprI gene, resulting expression construct, pOLT7/rROP2/OprI. The E. coli TOP10F transformed with this expression construct resulted in several kanamycin-resistant colonies, which were screening and confirmed by colony PCR (Fig. 1). One of these clones was propagated in liquid culture and plasmid DNA was extracted and sequenced. A BLAST (Basic Local Alignment and Search Tool) was performed showing a perfect alignment (100% identity) with the original sequence of NcROP2 deposited on GenBank (Accession No. HM587954).

Figure 1. Electrophoresis in 1% agarose gel. Amplification of the NcROP2 gene through the PCR technique. 1. DNA Ladder (pb); 2. Amplified NcRop2 gene; 3. Vector pOLT7 cloned with the NcROP2 gene; 4. Topo vector cloned with the NcROP2 gene.



3.2 EXPRESSION AND PURIFICATION OF THE RECOMBINANT PROTEIN

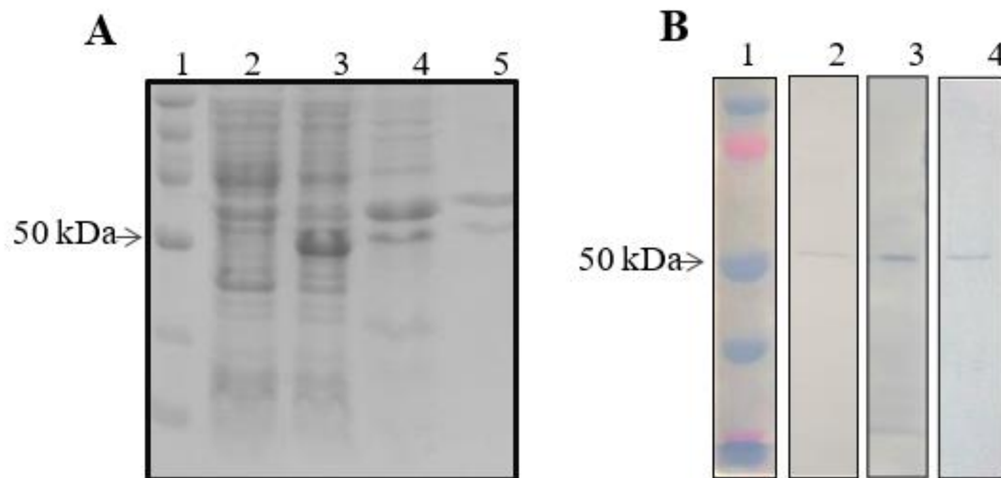
The E. coli Rosetta BL21 DE3 pLysS transformed with the expression plasmid pOLT7/rROP2/OprI, in the presence of IPTG, expressed a recombinant protein of approximately 50 kDa. Also, through the protocol of preparation of the outer membrane and extraction of the LPS used in this study, it was possible to purify the recombinant chimera rROP2/OprI through affinity chromatography, using Ni^{+} resin (Fig. 2A). The protein yield obtained with this process was approximately 534mg/L.

3.3 CHARACTERIZATION OF THE CHIMERA RROP2/OPRI BY WESTERN BLOTTING

The characterization of the rROP2/OprI chimera was performed using the Western Blot analysis. The reaction using MAb Anti-6xHis (Fig. 2B), showing a band of approximately 50 kDa, this indicating the presence of histidine tail in the N-terminal

portion of the chimera constituted by the fusion of NcROP2 with OprI. The rROP2/OprI was recognized by sera from cattle and sheep naturally infected with N. caninum, showing his antigenicity (Fig. 2B).

Figure 2. Protein expression and characterization. A) SDS-PAGE (15%) staining with Coomassie Blue. 1. Protein Ladder; 2. Transformed E. coli Rosetta (DE3) pLysS50 before induction with IPTG; 3. Transformed E. coli Rosetta (DE3) pLysS50 after the induction with IPTG; 4. Samples after the preparation of outer membranes; 5. Purified rRop2/Oprl; B) Western blotting analysis of recombinant purified chimera rROP2/OprI. 1. Protein Ladder; 2. Anti-6xHis monoclonal antibody; 3. Sera from cattle naturally infected with N. caninum; 4. Sera from sheep naturally infected with N. caninum.



4 DISCUSSION

Neospora caninum is the main parasite cause of abortion in cattle around the world, leading to an important animal health problem with high economic losses. So, a vaccine is a rational approach for the control of the disease (Hemphill et al., 2016; Pinheiro et al., 2018). Rhoptry proteins, such as NcROP2, are involved in a variety of cellular functions related to parasite–host cell interaction, such as cell invasion and formation of the parasitophorous vacuole (Alaeddine et al., 2013; Dubremetz, 2007; Saeij et al., 2007). Different studies reported that NcROP2 antigen are a promisor candidate to a subunit recombinant vaccine (Debache et al., 2008; 2009; Monney et al., 2011; Pastor-Fernández et al., 2015). In addition, it was shown that OprI fused to the chimeric N. caninum antigen Mic3-1-R was able to induce an immune response with mixed Th1 and Th2 properties (Aguado-Martínez et al., 2016).

In this study, we demonstrated the successful expression of the recombinant chimera composed by the fusion of a NcROP2 from N. caninum with a lipoprotein OprI from P. aeruginosa. The histidine tail in the N-terminal portion of the recombinant protein present in the expression vector (Basto et al., 2012), make possible to detect the chimeric

protein expression by Western blotting using MAb Anti-6xHis. Noteworthy, was the antigenic characterization of chimera rROP2/OprI, confirmed by immunoblotting using sera from cattle and sheep naturally infected with N. caninum, showing that the rROP2/OprI retained native NcROP2 characteristics. The present study has some limitations, first we did not evaluate the immunogenicity of the chimera, as well as we did not perform any vaccination-challenge study. But the chimeric obtained of OprI fused with NcROP2 achieved in this study, can be a promising alternative for the formulation of the recombinant vaccine against N. caninum.

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