FULL ARTICLE

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Isolation and immunological characterization of rhoptries from *Neospora caninum*

Isolamento e caracterização imunológica de roptrias de Neospora caninum

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ABSTRACT

Neospora caninum is a parasite that causes considerable loss in cattle production. Understanding its infection mechanisms is essential to comprehend its impact on herds. Cell fractionation and characterization of the fractions could contribute to the selection of proteins and organelles with immunogenic characteristics. Thus, the present study aimed to isolate rhoptry and subcellular fractions of *N. caninum* using a sucrose gradient and characterize their immunogenicity in mice. Tachyzoites of the Nc-1 strain were cultured in VERO cells, fractionated by glass beads, and ultracentrifuged in a sucrose gradient ranging from 0.25–1.8 M. The fractions were characterized using transmission electron microscopy. Each fraction was inoculated with Quil-A adjuvant (20 µg) into mice to produce polyclonal antibodies for immunofluorescence cell culture and Western blotting. Ultracentrifugation resulted in three distinct fractions (F1, F2, and F3) and a pellet. Fraction one (F1) at 1.0 M concentration contained parasite membranes, F2 at 1.4 M contained rhoptry and conoid, F3 at 1.6 M contained mitochondria, and the pellet at 1.8 M fraction contained cell debris. The four fractions exhibited the same bands with molecular weights of 50, 51, 52, and 62 kDa. Only F2 showed rhoptry structures and a 54 kDa protein resembling NcROP2. This study successfully separated subcellular fractions of *N. caninum* through processing and ultracentrifugation, identified rhoptry structures, and determined specific protein weights of each fraction.

Keywords: *Neospora caninum*. Neosporosis. Organelles. Rhoptries. Fractionation.

RESUMO

Neospora caninum é um parasita que causa perdas consideráveis na pecuária bovina. Compreender os seus mecanismos de infecção é essencial para compreender o seu impacto nos rebanhos. O fracionamento celular e a caracterização das frações poderão contribuir para a seleção de proteínas e organelas com características imunogênicas. Assim, o presente estudo teve como objetivo isolar frações roptrias e subcelulares de *N. caninum* utilizando gradiente de sacarose e caracterizar sua imunogenicidade em camundongos. Taquizoítos da cepa Nc-1 foram cultivados em células VERO, fracionados por esferas de vidro e ultracentrifugados em gradiente de sacarose variando de 0,25 a 1,8 M. As frações foram caracterizadas por microscopia eletrônica de transmissão. Cada fração foi inoculada com adjuvante Quil-A (20 µg) em camundongos para produzir anticorpos policlonais para cultura de células de imunofluorescência e western blotting. A ultracentrifugação resultou em três frações distintas (F1, F2 e F3) e um pellet. A fração um (F1) na concentração de 1,0 M continha membranas do parasita, F2 a 1,4 M continha roptria e conoide, F3 a 1,6 M continha mitocôndrias e o sedimento na fração de 1,8 M continha restos celulares. As quatro frações exibiram as mesmas bandas com pesos moleculares de 50, 51, 52 e 62 kDa. Apenas F2 apresentou estruturas de roptria e uma proteína de 54 kDa semelhante a NcROP2. Este estudo separou com sucesso frações subcelulares de *N. caninum* através de processamento e ultracentrifugação, identificou estruturas de roptria e determinou pesos específicos de proteínas de cada fração.

Palavras-chave: *Neospora caninum*. Neosporose. Organelas. Roptrias. Fracionamento.

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Introduction

Neospora caninum is an obligate intracellular Apicomplexa protozoan responsible for neosporosis, a disease of great importance in the cattle industry and dogs (Dubey et al., 2007). The parasite is known for its efficient transplacental transmission in cattle, which may occur in successive pregnancies (Dubey & Schares, 2011; Horcajo et al., 2023).

Neosporosis in cattle results in significant financial losses, estimated to be approximately 546 million USD in the United States (Reichel et al., 2013). In addition to the value of aborted fetuses, the economic impact occurs due to the increase in lactation time, decreases in milk production, reduction in the productive life of the cow, increased culling and replacement of cows, cost of artificial insemination/embryo transfer/fertilization in vitro, uterine infections, veterinary assistance, and diagnostic techniques (Trees et al., 1999; Barros et al., 2021).

The proteins of the apical complex, such as rhoptry and micronemes, in addition to dense granules and surface proteins, are commonly used to develop diagnostic methods and vaccines as these organelles are directly related to the process of cell invasion, formation of the parasitophorous vacuole and parasite evasion mechanisms (Abdelbaky et al., 2018). Rhoptries are the second organelle orchestrated in the cell invasion process. This organelle is delimited by a membrane with two distinct regions, a bulbous basal portion and a narrowing that differ in structure and protein composition (Barylyuk et al., 2020). The narrow part called the neck is made up of proteins classified as rhoptry neck proteins (RONs), while the constituents of the bulbous part are described as rhoptry neck proteins (ROPs) (Lamarque et al., 2012).

Among the different diagnostic methods, indirect immunofluorescence and indirect enzyme-linked immunosorbent assay (ELISA) are prominent (Sinnott et al., 2017). These methods can use whole or fractionated antigens and specific proteins. For this selection, subcellular fractionation is performed in apicomplexan parasites, mainly to select and characterize the antigenicity of these proteins. A previous study using subcellular fractionation in *N. caninum* obtained eight new potential components of rhoptries similar to those previously described in *Toxoplasma gondii* (Marugán-Hernández et al., 2011). One of the concerns about serological tests is the low specificity and cross-reaction against other Apicomplexa parasites (*T. gondii, Babesia spp.,* and *Cryptosporidium spp*.) (Gondim et al., 2017). Subcellular fractionation is performed by different techniques, including the use of percoll or different concentrations of sucrose gradient subjected to ultracentrifugation (Dubremetz et al., 1989; Machado et al., 1993), and this use is a possibility for the identification of specific antigens for and improvement of serodiagnosis and detection of antigens useful for vaccine prevention.

Thus, the present study aimed to fractionate and separate subcellular fractions of *N. caninum*, identify rhoptry structures, determine specific protein weights of each fraction, and evaluate their immunogenicity in mice.

Material and Methods

Preparation of N. caninum tachyzoite suspension

To obtain fractions of *N. caninum*, tachyzoites of the Nc-1 strain were obtained from cultivation in VERO cells maintained at 37° C and 5% CO2 from a 75 cm3 culture flask, maintained in RPMI medium with serum equine 2%. To obtain the ideal concentration and subsequent isolation of fractions by sucrose gradient, 15 flasks were inoculated at a concentration of 105 cells/mL and quantified in a Neubauer chamber. The inoculation of *N. caninum* was carried out in a 4:1 ratio. After four days of incubation, the entire growth area was removed with the aid of a cell scraper in order to remove the cells. This process was performed after practically all VERO cells had ruptured. The material was passed through a 26 G needle coupled to a 10 mL syringe to rupture the infected cells that eventually had not been ruptured. The obtained content was centrifuged at 3,500 × g for 10 min at 4° C, the supernatant was discarded, and the sediment was homogenized in a 0.85% NaCl solution. Then, the number of tachyzoites/mL was counted in a Neubauer chamber to adjust the concentration to 1010 tachyzoites/mL for material processing.

Separation of subcellular fractions of N. caninum

The separation of *N. caninum* fractions was performed according to a previously described methodology (Garcia et al., 2004). After obtaining *N. caninum* tachyzoites (Nc-1 strain), standardization was performed for 1010 tachyzoites/mL, and then this material was subjected to two washes with TES (sucrose 250 mM; EDTA 1 mM; triethanolamine 5 mM - HCl pH 7.5) and centrifuged at 1,250 × g for 10 min. The precipitate obtained was then homogenized in 2 mL of TES and broken into glass beads (0.2 mm) for 1 min in a vortex. After the disruption of tachyzoites, according to (Garcia et al., 2004), centrifugation was performed at 20,000 × g for 30 min. The sediment obtained was homogenized in 5 mL of TES, distributed in a 16 × 102 mm polycarbonate tube (UltraClear™ - Beckman Coulter®, Thermo Fisher Scientific, Waltham, MA, USA) containing a sucrose gradient ranging from 0.25–1.6 M, over a 1.8 M base, and ultracentrifuged at 120,000 × g for 3 h (Optima XE-90 Ultracentrifuge - Beckman Coulter®, Thermo Fisher Scientific). The visible bands were collected, and each band was individually diluted in TES and subsequently centrifuged at 120,000 × g for 1 h. The sediment obtained was collected in 1 mL of TES and used for visualization by electronic microscopy, protein dosage, and mice sensitization to produce polyclonal antibodies.

Electron microscopy of N. caninum fractions

The pellet fragments obtained from the ultracentrifugation with approximately 1.0 mm thickness were fixed in a 2% glutaraldehyde solution in 0.1 M phosphate buffer for 2 h at 25° C and then passed to a 1% osmium tetroxide solution for 1 h washed three times in a 0.9% NaCl solution and then kept in 0.5% aqueous uranyl acetate overnight (Junqueira & Salles, 1975). Next, the fragments were dehydrated in increasing concentrations of acetone P.A. and placed in an Araldite 502 (Luft, 1961). Ultrathin cuts were performed using a Leica Ultracut UCT microtome (Leica, Wetzlar, Germany). The contrast of the ultrathin cuts was provided with 2% uranyl acetate and lead citrate (Reynolds, 1963; Watson, 1958). The images were obtained using a transmission electron microscope (JEOL JEM-1400Plus, JEOL, Tokyo, Japan) with a maximum voltage of 120 kV and recovered in a digital camera with an 8-megapixel CCD.

Production of polyclonal antibodies against N. caninum fractions

The animal procedure was approved by the ethics committee for the use of animals at the State University of Londrina (registration number 12443.2019.78).

Sixteen female Swiss mice, approximately 45 days old, were randomly distributed into four groups. All groups received 50 µg of the antigen associated with 20 µg of Quil-A. Changing the type of antigen between the groups refers to the fractions of 1.0 M in group one, 1.4 M in group two, 1.6 M in group three, and 1.8 M in group four. The animals were inoculated with 100 µL of antigen associated with QuilA subcutaneously on days 0, 7, 14, and 21. Blood was collected to obtain serum 42 days after the first inoculation. During collection, all animals were euthanized by halothane inhalation and cervical dislocation.

Indirect fluorescent antibody test for cell culture

Indirect Fluorescent Antibody Test (IFAT) was performed using *N. caninum* tachyzoites and VERO cells in 24-well plates with a 13-mm sterile coverslip and kept in an oven at 37° C with 5% CO2. A concentration of 1 × 104 cells/mL was added to each well, and after 12 h of growth, the cells were inoculated with 5 × 104 tachyzoites/mL of *N. caninum*. Coverslips with cells and tachyzoites were removed after 12, 24, and 48 h of growth, washed in PBS (pH 7.4), fixed with P.A. methanol (-20° C) for 10 min, and treated with P.A. acetone (-20° C) for 1 min. After fixation, the coverslips were frozen at -20° C until IFAT. Coverslips were washed twice in PBS (pH 7.4) for 5 min and blocked with PBS containing 1% BSA for 10 min at 25° C. After drying the slides at 25° C, the mouse sera, at 1:16 dilutions in PBS with 1% BSA, were added and kept at 37° C for 1 h. Subsequently, two washes were performed with PBS (pH 7.4) and incubated with the secondary antibody labeled with fluorescein isothiocyanate (Sigma-Aldrich®, Saint Louis, MO, USA). Then, it was diluted in PBS (pH 7.4) with 1% BSA at a concentration of 1:150 and kept at 37° C for 30 min. Two more washes were performed in PBS (pH 7.4), followed by drying the slides and reading under an inverted microscope (EVOS™ FL Color, Thermo Fisher Scientific). Samples were considered negative when there was no fluorescence in the tachyzoite structures.

Electrophoresis and western blotting

The antigens were separated by electrophoresis in a 12.5% SDS-PAGE gel (Laemmli, 1970). For the visualization of total proteins and fractions, the gel was fixed in trichloroacetic acid (10%) for 1 h, subsequently stained with Coomassie Blue at 37° C for 30 min, and bleached with acetic acid and methanol until the protein bands were visualized. As molecular weight standards, weights of 10, 15, 25, 35, 40, 55, 70, 100, 130, and 180 kDa were used.

Proteins separated on SDS-PAGE were electrophoretically transferred from the gel to a nitrocellulose membrane using a commercial kit following the manufacturer's recommendations (iBlot® Gel Transfer Stacks PVDF, Regular - Thermo Fisher Scientific).

After transfer, the membrane was stained with Ponceau to evaluate the transfer efficiency and bleached with distilled water. The remaining protein sites were blocked with a saturation buffer (Na2HPO4 0,007M; NaH2PO4 0,003 M; NaCl 0,14M; 5% of dried milk; pH 7.2) for 1 h at 25° C under slow agitation. The nitrocellulose paper was then cut into strips for incubation with the sera at a ratio of 1:100 for 1 h and 30 min and after being washed three times in a washing buffer containing Tween 20 (0.05%) with an interval of 5 min between each change of buffer.

The peroxidase-labeled anti-mouse IgG conjugate was diluted in the washing buffer and placed on the strips at a concentration of 1:2500. Following incubation for 1.5 h at 25° C under light agitation. The strips were washed in the same way as previously described after serum incubation. The development was performed using TMB (3,3',5,5'-tetramethylbenzidine) (TMB Solution for Blotting, Thermo Fisher Scientific). The reaction was stopped with distilled water, and the strips were stored in the dark until photographed. A nitrocellulose membrane strip was used to react with negative serum as a reaction control.

Results and Discussion

The ultracentrifugation of the antigen in sucrose gradient allowed the visualization of three distinct bands in the material and the pellet, with fraction one (F1) referring to a concentration of 1.0 M, fraction two (F2) with 1.4 M, fraction three (F3) with 1.6 M and the pellet in the 1.8 M fraction (F4). The images obtained in transmission electron microscopy allowed the observation of parasite membranes in F1, rhoptry, and conoid in F2, mitochondria in F3, and cellular remains in F4 (Figure 1). Similar results were observed for *T. gondii* (Garcia et al., 2004). This protozoan and other Apicomplexans, in which rhoptry was isolated, were used for comparisons and discussions, considering their similarities and the limited number of studies on this model with *N. caninum* (Machado et al., 1993; Garcia et al., 2004; Marugán-Hernández et al., 2011)

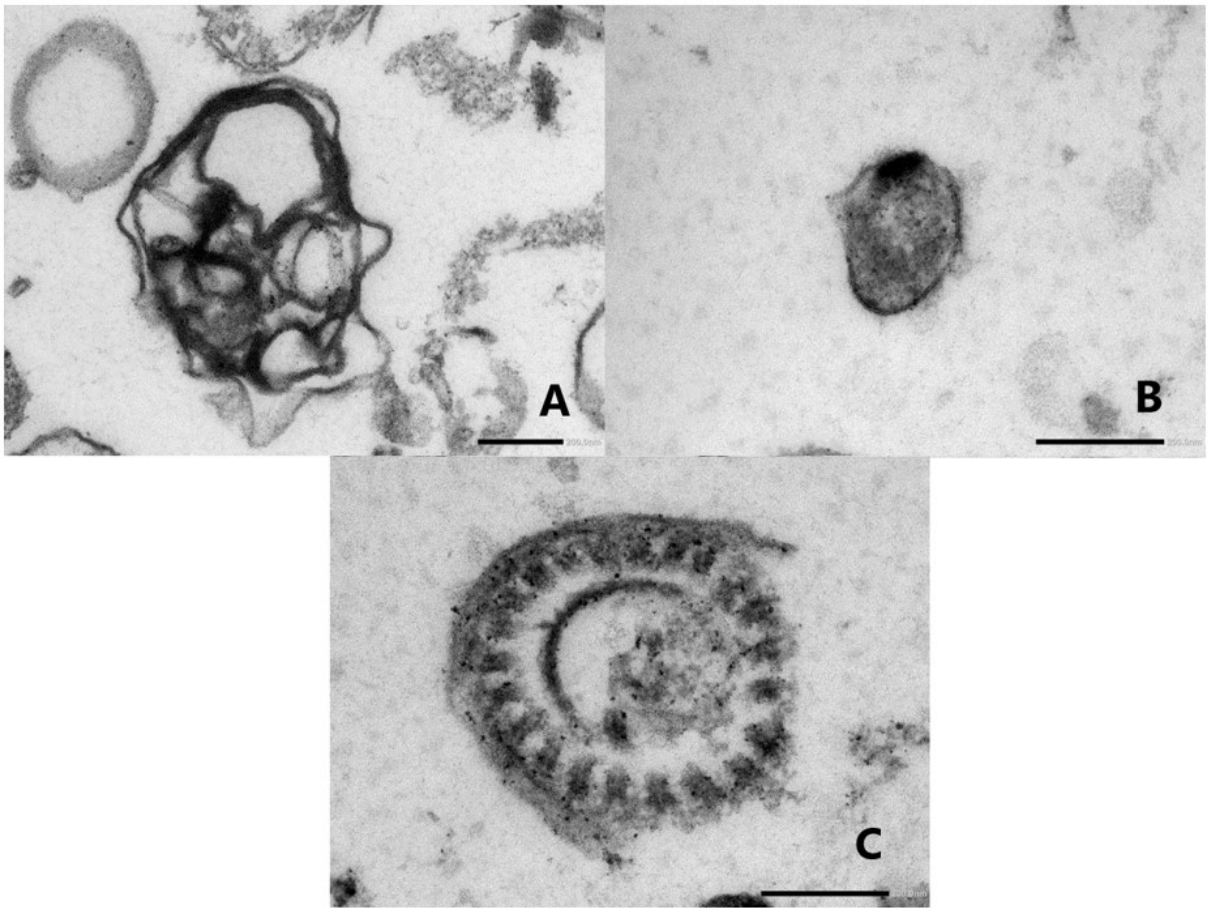

Figure 1 – Transmission electron microscopy of subcellular fractions of Neospora caninum (Nc-1 strain) shows A. membranes at 1.0M fraction, B. rhoptry at 1.4M fraction, and C. conoid at 1.4M fraction. Bar = 0.2µm.

Only F2 presented structures compatible with rhoptry in electron microscopy, and a 54 kDa protein was identified through Western blotting. This size is compatible and similar (54 kDa) to that described for NcROP2 (Debache et al., 2008; Marugán-Hernández et al., 2011). This fraction is the most appropriate for protein analysis of this organelle, contributing to discovering new molecular mechanisms involved with different aspects of the disease pathology, including the parasite-host interactions, which are intrinsic to the infectious process. Furthermore, subcellular fractionation allows the detection of proteins in low concentrations in the parasite, which is impossible with intact parasites in proteomics studies (Marugán-Hernández et al., 2011; Rico-San Román et al., 2020).

The conoid was detected as a contaminant in the fraction containing rhoptry. However, these contaminants are common in these isolations and have been previously described (Etzion et al., 1991; Garcia et al., 2004). Barylyuk et al. (2020) observed rhoptry-specific proteins in all subcellular fractions of *T. gondii*. Further, mitochondria-specific proteins were identified in all fractions except the supernatant.

Contamination between the fractions can explain proteins with equal weights described in different fractions. Furthermore, (Marugán-Hernández et al., 2011), using subcellular fractionation to select *N. caninum* rhoptry protein, noticed that all fractions were reactive when using anti-NcROP2. However, the fraction with the highest intensity was selected as 0.8 M. This reinforces the theory that even with the visualization of the organelles in the fractions by electron microscopy, proteins can be identified in other fractions.

In the present study, the parasite membrane was identified in the 1.0 M fraction, and a complex pattern of proteins was visualized between 10 and 130 kDa. Similar results were previously obtained for *N. caninum*, specifically identifying and characterizing the complex of membranes and plasmalemma in subcellular fractionation with dominant bands between 29, 40, 50, 65, and 110 kDa (Lei et al., 2005).

All four fractions exhibited the same bands with molecular weights of 50, 51, 52, and 62 kDa (Figure 2). Except for F1, the others showed a band at 32 kDa. The sizes of each band observed in the different fractions were determined using prediction calculations based on the patterns.

The electrophoretic analysis demonstrated that the parasite membranes identified in F1 contained proteins with compatible weights. The main surface protein of *N. caninum* tachyzoites is NcSAG1, which has an apparent molecular weight of 29 kDa (Howe et al., 1998). Proteins at this height are seen on electrophoresis, but when subjected to Western blotting, they show no labeling in fractions F1 and F4. These findings suggest that even with a complex pattern of proteins in the fractions, only some are associated with the production of antibody responses in mice.

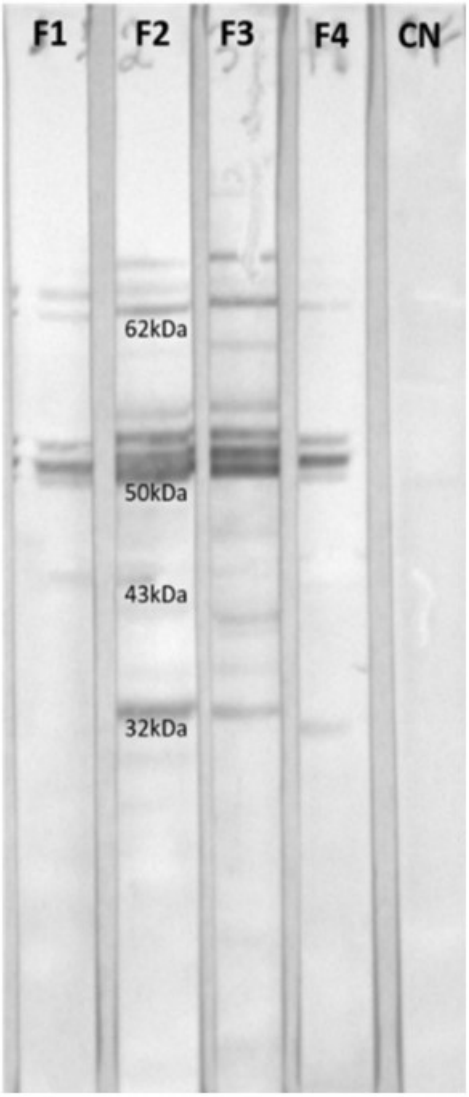

Figure 2 – Western Blotting from sucrose subcellular fractions of Neospora caninum (Nc-1 strain). The fractions obtained were F1=1.0M, F2=1.4M, F3=1.6M, and F4=1.8M. Western Blotting from subcellular fractions of N. caninum using mouse polyclonal antibodies. CN= negative control.

For this study, native proteins of fractionated antigens of *N. caninum* were used to produce polyclonal antibodies. However, the literature describes culture immunofluorescence, mainly with secondary antibodies specific to recombinant proteins (Ma et al., 2017; Pastor-Fernández et al., 2016; Yoshimoto et al., 2015). Thus, the comparison of the results of this technique is limited and does not allow the prediction of a specific response pattern between the different fractions under the established conditions for immunofluorescence.

Structures similar to those of the dense granules and micronemes were not observed. However, a previous study described that distinguishing parts of rhoptry from dense

granules and micronemes in randomly selected cross-sections can often be difficult (Blackman & Bannister, 2001).

Conclusion

In conclusion, this study showed that the processing and ultracentrifugation of the tachyzoite lysate allowed the separation of subcellular fractions of *N. caninum* and the identification of rhoptry. Immunofluorescence analysis of the culture did not reveal any discernible response pattern with the polyclonal sera. However, further studies using 2D Western blotting will contribute to determining specific protein weights associated with each fraction. Our data may contribute to future proteome studies to detect *N. caninum*-specific antigens for vaccine candidates and chronic and acute serological diagnosis in neosporosis.

Conflict of Interest

The authors declare no conflict of interest.

Ethics Statement

The ethics committee for the use of animals at the State University of Londrina approved this work under number 23340.2018.90.

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References

Abdelbaky HH, Fereig RM, Nishikawa Y. Identification of the antigenic region of *Neospora caninum* dense granule protein 7 using ELISA. Parasitol Int. 2018;67(6):675-8. http://doi.org/10.1016/j.parint.2018.06.012. PMid:29959092.

Barros LD, Bogado ALG, Furlan D, de Melo Jardim A, Okano W, da Silva LC, Pereira CES, Bronkhorst DE, Cardim ST, Garcia JL. Effects of *Neospora caninum* on reproductive parameters in dairy cows from a closed herd in Brazil. Vet Parasitol Reg Stud Rep. 2021;23:100524. http://doi.org/10.1016/j.vprsr.2020.100524. PMid:33678379.

Barylyuk K, Koreny L, Ke H, Butterworth S, Crook OM, Lassadi I, Gupta V, Tromer E, Mourier T, Stevens TJ, Breckels LM, Pain A, Lilley KS, Waller RF. A comprehensive subcellular Atlas of the Toxoplasma proteome via hyperLOPIT provides spatial context for protein functions. Cell Host Microbe. 2020;28(5):752-66.e9. http://doi.org/10.1016/j.chom.2020.09.011. PMid:33053376.

Blackman MJ, Bannister LH. Apical organelles of Apicomplexa: biology and isolation by subcellular fractionation. Mol Biochem Parasitol. 2001;117(1):11-25. http://doi.org/10.1016/S0166-6851(01)00328-0. PMid:11551628.

Debache K, Guionaud C, Alaeddine F, Mevissen M, Hemphill A. Vaccination of mice with recombinant NcROP2 antigen reduces mortality and cerebral infection in mice infected with *Neospora caninum* tachyzoites. Int J Parasitol. 2008;38(12):1455-63. http://doi.org/10.1016/j.ijpara.2008.04.001. PMid:18495126.

Dubey JP, Schares G, Ortega-Mora LM. Epidemiology and control of neosporosis and *Neospora caninum*. Clin Microbiol Rev. 2007;20(2):323-67. http://doi.org/10.1128/CMR.00031-06. PMid:17428888.

Dubey JP, Schares G. Neosporosis in animals: the last five years. Vet Parasitol. 2011;180(1-2):90-108. http://doi.org/10.1016/j.vetpar.2011.05.031. PMid:21704458.

Dubremetz JF, Ferreira E, Dissous C. Isolation and partial characterization of rhoptries and micronemes from *Eimeria nieschulzi* zoites (Sporozoa, Coccidia). Parasitol Res. 1989;75(6):449-54. http://doi.org/10.1007/BF00930971. PMid:2755931.

Etzion Z, Murray MC, Perkins ME. Isolation and characterization of rhoptries of *Plasmodium falciparum*. Mol Biochem Parasitol. 1991;47(1):51-61. http://doi.org/10.1016/0166-6851(91)90147-X. PMid:1857385.

Garcia JL, Gennari SM, Navarro IT, Machado RZ, Sinhorini IL. *Toxoplasma gondii*: isolation of tachyzoites rhoptries and incorporation into Iscom. Exp Parasitol. 2004;108(1-2):40-6. http://doi.org/10.1016/j.exppara.2004.07.002. PMid:15491547.

Gondim LFP, Mineo JR, Schares G. Importance of serological cross-reactivity among *Toxoplasma gondii, Hammondia* spp., *Neospora* spp., *Sarcocystis* spp. and *Besnoitia besnoiti*. Parasitology. 2017;144(7):851-68. http://doi.org/10.1017/S0031182017000063. PMid:28241894.

Horcajo P, Coronado M, Pastor-Fernández I, Collantes-Fernández E, Rico-San Román L, Reyes-Palomares A, Ortega-Mora L-M. Whole-transcriptome analysis reveals virulence-specific pathogen-host interactions at the placenta in bovine neosporosis. Front Immunol. 2023;14:1198609. http://doi.org/10.3389/fimmu.2023.1198609. PMid:37520552.

Howe DK, Crawford AC, Lindsay D, Sibley LD. The p29 and p35 immunodominant antigens of *Neospora caninum* tachyzoites are homologous to the family of surface antigens of Toxoplasma gondii. Infect Immun. 1998;66(11):5322-8. http://doi.org/10.1128/IAI.66.11.5322-5328.1998. PMid:9784539.

Junqueira LCU, Salles LMM. Artefatos de microscopia eletrônica aplicada a material biológico. Cien Cult. 1975;27:461-471.

Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 1970;227(5259):680-5. http://doi.org/10.1038/227680a0. PMid:5432063.

Lamarque MH, Papoin J, Finizio A-L, Lentini G, Pfaff AW, Candolfi E, Dubremetz J-F, Lebrun M. Identification of a new rhoptry neck complex RON9/RON10 in the Apicomplexa parasite *Toxoplasma gondii*. Moreno SN. PLoS One. 2012;7(3):e32457. http://doi.org/10.1371/journal.pone.0032457. PMid:22427839.

Lei Y, Birch D, Davey M, Ellis JT. Subcellular fractionation and molecular characterization of the pellicle and plasmalemma of *Neospora caninum*. Parasitology. 2005;131(Pt 4):467-75. http://doi.org/10.1017/S003118200500805X. PMid:16174411.

Luft JH. Improvements in epoxy resin embedding methods. J Biophys Biochem Cytol. 1961;9(2):409-14. http://doi.org/10.1083/jcb.9.2.409. PMid:13764136.

Ma L, Liu J, Li M, Fu Y, Zhang X, Liu Q. Rhoptry protein 5 (ROP5) is a key virulence factor in *Neospora caninum*. Front Microbiol. 2017;8:370. http://doi.org/10.3389/fmicb.2017.00370. PMid:28326073.

Machado RZ, McElwain TF, Suarez CE, Hines SA, Palmer GH. *Babesia bigemina*: isolation and characterization of merozoite rhoptries. Exp Parasitol. 1993;77(3):315-25. http://doi.org/10.1006/expr.1993.1089. PMid:8224087.

Marugán-Hernández V, Álvarez-García G, Tomley F, Hemphill A, Regidor-Cerrillo J, Ortega-Mora LM. Identification of novel rhoptry proteins in *Neospora caninum* by LC/MS-MS analysis of subcellular fractions. J Proteomics. 2011;74(5):629-42. http://doi.org/10.1016/j.jprot.2011.02.004. PMid:21315855.

Pastor-Fernández I, Regidor-Cerrillo J, Jiménez-Ruiz E, Álvarez-Garciá G, Marugán-Hernández V, Hemphill A, Ortega-Mora LM. Characterization of the *Neospora caninum* NcROP40 and NcROP2Fam-1 rhoptry proteins during the tachyzoite lytic cycle. Parasitology. 2016;143(1):97-113. http://doi.org/10.1017/S0031182015001511. PMid:26521890.

Reichel MP, Ayanegui-Alcérreca, MA, Gondim LFP, Ellis JT. What is the global economic impact of *Neospora caninum* in cattle: the billion dollar question. Int J Parasitol. 2013;43(2):133-42. http://doi.org/10.1016/j.ijpara.2012.10.022. PMid:23246675.

Reynolds ES. The use of lead citrate at high ph as an electron-opaque stain in electron microscopy. J Cell Biol. 1963;17(1):208-12. http://doi.org/10.1083/jcb.17.1.208. PMid:13986422.

Rico-San Román L, Horcajo P, Regidor-Cerrillo J, Fernández-Escobar M, Collantes-Fernández E, Gutiérrez-Blázquez D, Hernáez-Sánchez ML, Saeij JPJ, Ortega-Mora LM. Comparative tachyzoite proteome analyses among six *Neospora caninum* isolates with different virulence. Int J Parasitol. 2020;50(5):377-88. http://doi.org/10.1016/j.ijpara.2020.02.003. PMid:32360428.

Sinnott FA, Monte LG, Collares TF, Silveira RM, Borsuk S. Review on the immunological and molecular diagnosis of neosporosis (years 2011-2016). Vet Parasitol. 2017;239:19-25. http://doi.org/10.1016/j.vetpar.2017.04.008. PMid:28495191.

Trees AJ, Davison HC, Innes EA, Wastling JM. Towards evaluating the economic impact of bovine neosporosis. Int J Parasitol. 1999;29(8):1195-200. http://doi.org/10.1016/S0020-7519(99)00093-4. PMid:10576571.

Watson ML. Staining of tissue sections for electron microscopy with heavy metals. J Cell Biol. 1958;4(6):727-30. http://doi.org/10.1083/jcb.4.6.727.

Yoshimoto M, Otsuki T, Itagaki K, Kato T, Kohsaka T, Matsumoto Y, Ike K, Park EY. Evaluation of recombinant *Neospora caninum* antigens purified from silkworm larvae for the protection of *N. caninum* infection in mice. J Biosci Bioeng. 2015;120(6):715-9. http://doi.org/10.1016/j.jbiosc.2015.04.002. PMid:25935502.

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