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Sarcocystis neurona, *Toxoplasma gondii*, and *Neospora caninum* infection in bovine fetuses from a slaughterhouse in southern Brazil[1]

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ABSTRACT.- Roman I.J., Tagarra L.G., Rodrigues F.S., Cargnelutti J.F., Sangioni L.A. & Vogel F.S.F. 2024. ***Sarcocystis neurona*, *Toxoplasma gondii*, and *Neospora caninum* infection in bovine fetuses from a slaughterhouse in southern Brazil.** *Pesquisa Veterinária Brasileira 44:e07504, 2025.* Laboratório de Doenças Parasitárias, Departamento de Medicina Veterinária Preventiva, Universidade Federal de Santa Maria, Av. Roraima 1000, Camobi, Santa Maria, RS 97105-900, Brazil. E-mail: fernanda.vogel@ufsm.br

This study aims to describe the molecular detection of *Sarcocystis neurona*, *Toxoplasma gondii*, and *Neospora caninum* in brain samples obtained from bovine fetuses at a slaughterhouse in South Brazil. Brain samples from 35 fetuses of asymptomatic pregnant beef cows underwent nested polymerase chain reaction (PCR) to amplify a fragment of the *18S* rRNA gene specific to *S. neurona*, *T. gondii*, and *N. caninum*. The amplicons were subjected to a two-step digestion process: first, with the restriction enzyme DdeI, which differentiates *N. caninum* from *T. gondii* and *S. neurona*; and subsequently, with the HpaII enzyme, to distinguish *S. neurona* from *T. gondii* and *N. caninum*. Of the 35 brain samples tested, 26 yielded positive PCR results for the *18S* rRNA gene. Of these, 23 were digested with restriction enzymes, yielding 17 positive samples for *S. neurona*, five for *N. caninum*, and one for *T. gondii*. Specific primers for *S. neurona*, *N. caninum*, and *T. gondii* were employed to confirm the restriction fragment length polymorphism results. DNA sequencing and phylogenetic analysis, based on the ITS-1 region, were conducted on a positive sample for *S. neurona*, confirming our surprising findings. The sequence from fetus 75 exhibited a high nucleotide identity (97.79%) and clustered with *S. neurona* sequences available in GenBank. Molecular analyses confirmed the unprecedented detection of *S. neurona*, a protozoan not previously reported in cattle, in bovine fetal brain samples, thereby underscoring the necessity for further research on Apicomplexa protozoan infections in cattle.

INDEX TERMS: *Sarcocystis neurona*, transplacental, bovine, fetal brain, reproductive losses.

RESUMO.- [**Infecção por *Sarcocystis neurona*, *Toxoplasma gondii* e *Neospora caninum* em fetos bovinos de um matadouro no Sul do Brasil.**] Este estudo tem como objetivo descrever a detecção molecular de *Sarcocystis neurona*, *Toxoplasma gondii* e *Neospora caninum* em amostras de cérebro obtidas de fetos bovinos em um matadouro no Sul do Brasil. Amostras de cérebro de 35 fetos de vacas gestantes assintomáticas foram submetidas à reação em cadeia da polimerase (PCR) aninhada para amplificar um fragmento do gene *18S* rRNA específico para *S. neurona*, *T. gondii* e *N. caninum*. Os amplicons foram submetidos a um processo de digestão em duas etapas: primeiro, com a enzima de restrição DdeI, que diferencia *N. caninum* de *T. gondii* e *S. neurona*; e posteriormente, com a enzima HpaII, para distinguir *S. neurona* de *T. gondii* e *N. caninum*. Das 35 amostras de cérebro testadas, 26 apresentaram resultados positivos na PCR para o gene *18S* rRNA. Destas, 23 foram digeridas com enzimas de restrição, resultando em 17 amostras positivas para *S. neurona*, cinco para *N. caninum* e uma para *T. gondii*. Primers específicos para *S. neurona*, *N. caninum* e *T. gondii* foram empregados para confirmar os resultados do polimorfismo de comprimento de

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fragmentos de restrição. Sequenciamento de DNA e análise filogenética, baseada na região ITS-1, foram realizados em uma amostra positiva para *S. neurona*, confirmando nossos resultados surpreendentes. A sequência do feto 75 apresentou uma alta identidade de nucleotídeos (97,79%) e se agrupou com sequências de *S. neurona* disponíveis no GenBank. As análises moleculares confirmaram a detecção inédita de *S. neurona*, um protozoário não anteriormente relatado em bovinos, em amostras de cérebro fetal bovino, destacando assim a necessidade de mais pesquisas sobre infecções por protozoários do filo Apicomplexa em bovinos.

TERMOS DE INDEXAÇÃO: *Sarcocystis neurona*, transplacentária, bovino, encéfalo fetal, perdas reprodutivas.

INTRODUCTION

Sarcocystis spp., *Neospora* spp., and *Toxoplasma gondii* are protozoans distributed globally, belonging to the phylum Apicomplexa (Lindsay & Dubey 2020) and infections caused by these agents have been confirmed in multiple species, including cattle. Cattle serve as intermediate hosts for certain species of *Sarcocystis* spp. and are susceptible to infections by other related or closely related species (Lindsay & Dubey 2020). They are also recognized as one of the primary intermediate hosts for *Neospora caninum*, although their epidemiological role in *T. gondii* infections remains to be fully elucidated (Lindsay & Dubey 2020).

N. caninum is acknowledged as one of the leading causes of reproductive disorders in cattle (Ribeiro et al. 2019). Given its high transmission rate and the persistence of the infection, this agent has been the subject of numerous studies focusing on reproductive losses in cattle (Bartley et al. 2012, 2013, Benavides et al. 2012).

T. gondii is capable of causing severe diseases in small ruminants and poses a significant economic burden on the livestock industry (Lindsay & Dubey 2020, Henker et al. 2022). In sheep and goats, primary maternal infections may lead to outcomes ranging from embryonic death and reabsorption to fetal death, mummification, abortion, stillbirth, and neonatal death (Henker et al. 2022); the disease manifests more severely in goats than in sheep. Moreover, cattle and water buffaloes exhibit greater resistance to acute clinical toxoplasmosis compared to other livestock species, and there are no confirmed cases of clinical toxoplasmosis in these animals (Lindsay & Dubey 2020).

In contrast to *N. caninum* and *T. gondii*, the genus *Sarcocystis* displays a broader range of definitive and/or intermediate hosts (Lindsay & Dubey 2020), with over 200 known species that infect mammals, marsupials, birds, and reptiles, following an obligatory two-host prey-predator life cycle. There are five known *Sarcocystis* species that affect cattle: *S. cruzi*, *S. heydorni*, *S. hirsuta*, *S. hominis*, and *S. rommeli* (Moré et al. 2011, Dubey & Rosenthal 2023). The definitive hosts for these species are canines (*S. cruzi*), felines (*S. hirsuta* and *S. rommeli*), and primate (*S. heydorni and S. hominis*). *S. cruzi* is the most pathogenic species affecting cattle, and clinical manifestations are typically observed during the development of second-generation schizonts in blood vessels in the acute phase (Gjerde 2013, Bräunig et al. 2016, Dubey & Rosenthal 2023). In pregnant animals, abortion may occur, and calf growth may be inhibited or even halted (Lindsay & Dubey 2020).

While *N. caninum* is a primary cause of abortion in cattle, the involvement of other Apicomplexa species cannot be ruled out (McAllister 2016). Given this scenario, this study seeks to present the findings of molecular detection of Apicomplexa protozoans in brain samples obtained from bovine fetuses, without clinical implications of abortion, at a slaughterhouse in South Brazil.

MATERIALS AND METHODS

Ethics approval. No approval of research ethics committees was required to accomplish the goals of this study because experimental work was conducted with samples from slaughterhouses.

Samples. Brain samples from 35 fetuses of non-clinical pregnant beef cows were obtained from an officially inspected slaughterhouse in Santa Maria (Rio Grande do Sul State – RS, South Brazil). All materials utilized for each tissue collection in the slaughterhouse were decontaminated with a 2.5% sodium hypochlorite solution followed by hydrogen peroxide (Santos et al. 2010) to prevent DNA cross-contamination. The slaughtering and evisceration processes were carried out in compliance with Brazilian legal protocols concerning ethics and animal welfare under the oversight of technicians from the Official Veterinary Inspection Service. The brain samples were individually packaged, refrigerated (+5 °C), and transported to the "Laboratório de Doenças Parasitárias" (Laboratory of Parasitic Diseases – LADOPAR) at the "Universidade Federal de Santa Maria" (UFSM). These 35 fetuses were part of a larger group of 100 samples collected to primarily isolate *Neospora caninum* for other experiments. Initially, serum samples from the 100 fetuses were examined using an indirect immunofluorescence assay to detect anti-*N. caninum* antibodies, employing a cut-off ratio 1:25 (Cadore et al. 2010). Only brain samples from fetuses that exhibited a positive antibody reaction (35/100) were subjected to agent detection by polymerase chain reaction (PCR) and cell culture inoculation (results not shown) (Dubey et al. 1988). Random brain tissue segments were stored at -20 °C until the DNA extraction.

DNA Extraction. Brain samples were subjected to DNA extraction using the Wizard genomic DNA purification kit (Promega, Madison/WI, USA), according to the manufacturer's protocol, but with modifications during the lysis phase (Bräunig et al. 2016).

PCR screening. DNA samples underwent nested PCR to amplify a fragment of the *18S* rRNA gene of *Sarcocystis neurona*, *Toxoplasma gondii*, and *N. caninum* following the protocol outlined by (Silva et al. 2009). An external primer pair was used for the first-round PCR: Tg18s48F (5'-CCATGCATGTCTAAGTATAAGC-3') and Tg18s359R (5'-GTTACCCGTCACTGCCAC-3'). For the second-round PCR, an internal primer pair was employed: Tg18s58F (5'-CTAAGTATAAGCTTTTATACGGC-3') and Tg18s348R (5'-TGCCACGGTAGTCCAATAC3'). The initial denaturation was set at 94 °C for 5 minutes, followed by 35 cycles of denaturation at 94 °C for 45 seconds, annealing at 55 °C for 45 seconds, and extension at 72 °C for 45 seconds. A final extension was carried out at 72 °C for 5 minutes. The final (second-round) PCR products, measuring 310 base pairs (bp), were analyzed through electrophoresis on a 2% agarose gel stained with Gel Red (Biotium, Hayward/CA, USA). Previously sequenced samples from the LADOPAR-UFSM sample bank were used as positive controls for *S. neurona*, *Sarcocystis cruzi*, *T. gondii*, and *N. caninum*, as well as a negative control using ultrapure water was included in the reactions.

Restriction fragment length polymorphism. The nested PCR products were subjected to a two-step restriction enzyme digestion following the protocol by Silva et al. (2009). Initially, the enzyme DdeI (Promega, Madison/WI, USA) was employed to differentiate between

N. caninum, *T. gondii*, and *S. neurona*. DdeI digested the *T. gondii* and *S. neurona* amplicons at one restriction site, producing fragments of 182 bp and 110 bp, respectively. *N. caninum* lacked a restriction site, yielding a product of 290 bp. Subsequently, the HpaII enzyme (Promega, Madison/WI, USA) was used to distinguish *S. neurona* from *T. gondii* and *N. caninum*. Here, *T. gondii* and *N. caninum* were cleaved into two fragments, measuring 173 and 119 bp and 173 and 120 bp, respectively. *S. neurona* was not cleaved, resulting in a single fragment of 290 bp. The restriction process was carried out at 37°C for 60 minutes. All digestion products were then electrophoresed on a 3% agarose gel stained with Gel Red (Biotium, Hayward/CA, USA).

PCR for *Neospora caninum*. Samples testing positive for *N. caninum* were subjected to conventional PCR using primers specific for *N. caninum*, as per the protocol described by (Müller et al. 1996). The specific primers Np21F (5'-CCCAGTGCGTCCAATCCTGTAAC-3') and Np6R (5'-CTCGCCAGTCAACCTACGTCTTCT-3') were incorporated into a PCR mix with a final volume of 25 µL. The mix underwent an initial denaturation at 95 °C for 5 minutes, followed by 35 cycles of 95 °C for 1 minute, 60 °C for 1 minute, and 72 °C for 1 minute. A final extension step was performed at 72 °C for 10 minutes, followed by refrigeration at 4 °C. PCR products were separated on a 1.5% agarose gel stained with Gel Red (Biotium, Hayward/CA, USA) and visualized under UV light after 40 minutes of electrophoresis at 60 V. The expected final product had a length of 328 bp. A previously sequenced sample of *N. caninum* served as a positive control, and ultrapure water was used as a negative control.

PCR for *Toxoplasma gondii*. Samples that tested positive for *T. gondii* were subjected to conventional CR using primers specific for *T. gondii*, based on the protocol by (Homan et al. 2000). The specific primers Tox4F (5'-CGCTGCAGGGAGGAAGACGAAAGTTG-3') and Tox5R (5'-CGCTGCAGACACAGTGCATCTGGATT-3') were added to a PCR mix with a final volume of 25 µL. The mixture was then subjected to an initial denaturation at 94 °C for 2 minutes, followed by 35 cycles of 94 °C for 1 minute, 60 °C for 1 minute, and 72 °C for 1 minute. A final extension step was performed at 72 °C for 10 minutes, followed by refrigeration at 4 °C. PCR products were run on a 1.5% agarose gel stained with Gel Red (Biotium, Hayward/CA, USA) and visualized under UV light after 40 minutes of electrophoresis at 60 V. The expected final product measured 529 bp. A previously sequenced sample of *T. gondii* was used as a positive control, and ultrapure water was employed as a negative control.

PCR for *Sarcocystis neurona*. Samples positive for *S. neurona* were subjected to hemi-nested PCR using primers specific for *S. neurona*, as outlined by (Valadas et al. 2016). The specific primers ITS-234F19 (5'-TCAACCATTGAATCCCCAA-3'), ITS-720R19 (5'-TCATTTTGAACATGTACCA-3'), and ITS-578R23 (5'-AGCACGCCTTCATATTATAAACC-3') were included in a PCR mix with a final volume of 25 µL. The mixture underwent an initial denaturation at 94 °C for 3 minutes, followed by 35 cycles of 94 °C for 45 seconds, 58 °C for 30 seconds, and 72 °C for 90 seconds. A final extension step was performed at 72 °C for 10 minutes, followed by refrigeration at 4 °C. PCR products were separated on a 2% agarose gel stained with Gel Red (Biotium, Hayward/CA, USA) and visualized under UV light after 40 minutes of electrophoresis at 60 V. The expected final product had a length of 367 bp. A previously sequenced sample of *S. neurona* was employed as a positive control, and ultrapure water was used as a negative control.

Nucleotide sequencing and phylogenetic analysis for *Sarcocystis neurona*. *S. neurona* PCR products were purified using a PureLink PCR Purification Kit (Invitrogen, CA, USA), following the manufacturer's instructions. Sequencing was conducted in duplicate by the Sanger method using the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, MA, USA). ACTGene Análises Moleculares (RS, Brazil) carried out the sequencing. Sequences were analyzed in the Staden Package software to obtain a consensus, which was then compared to sequences in the GenBank NCBI database using the BLAST search tool.

Alignment of the consensus sequence from a positive *S. neurona* sample (fetus 75) with 23 complete genomic sequences and six partial sequences from various *Sarcocystis* species from GenBank was performed using the ClustalW method (Thompson et al. 1994). This was facilitated by the BioEdit Sequence Alignment Editor software (version 7.0.5.3) (Hall 1999). Phylogenetic analysis was conducted using the Molecular Evolutionary Genetics Analysis software (MEGA X; version 10.2.4) (Kumar et al. 2018). The best-fit model for the analysis was determined by the jModelTest software (Posada 2008). The evolutionary history was inferred through the maximum likelihood method using 1,000 bootstrap replicates. Parameters for the maximum likelihood analysis of *Sarcocystis* sequences included: Genomic region: ITS-1, substitution model: Hasegawa-Kishino-Yano (HKY), Bayesian Information Criterion: 1983.6415, and Log-Likelihood: -831.17.

RESULTS

Of the 35 fetuses subjected to nested PCR, 26 (26/35) tested positive for DNA amplification using the TG18S primers. Among these, three (3/26) could not undergo restriction fragment length polymorphism (RFLP), while 23 (23/26) were subjected to RFLP analysis. This yielded 17 (17/23) samples positive for *Sarcocystis neurona*, five (5/23) for *Neospora caninum*, and one (1/23) for *Toxoplasma gondii*. Specific primers for *S. neurona*, *N. caninum*, and *T. gondii* were employed to confirm the RFLP results.

A sample from one fetus (fetus 75) underwent nucleotide sequencing, and its consensus sequence was analyzed using the BLAST tool for phylogenetic comparison. Fetus 75 was found to cluster with *S. neurona* sequences (Fig.1). Upon BLAST analysis, the consensus sequence from fetus 75 showed a 97.79% nucleotide identity with *S. neurona* (MN172273, MN822081, MN822080, MN822079, MN822078, MN822077, and MN822076). However, it had less than 92.82% nucleotide identity with sequences of *Sarcocystis dasypi* (AY082633) and 90.96% nucleotide identity with sequences of *Sarcocystis falcatula* (AF098246).

DISCUSSION

The role of agents belonging to the phylum Apicomplexa in reproductive disorders among production animals is significant. For instance, *Neospora caninum* is a leading cause of abortion in bovines, *Toxoplasma gondii* is commonly associated with reproductive losses in sheep and goats, and *Sarcocystis* spp. have been occasionally linked to abortion in sheep (Pescador et al. 2007, Lindsay & Dubey 2020, Dorsch et al. 2021).

The histopathological diagnosis often arises from the similarity in clinical signs and histopathological findings related to these protozoa, usually focusing on the intermediate host species rather than the specific pathogen involved. Consequently, histopathological lesions observed in bovine fetal tissues are often attributed to *N. caninum* infections (Dorsch et al. 2021).

While this association is generally accurate, the absence of further investigation can result in missed opportunities for detecting other protozoal agents responsible for abortions in cattle. Lesions in the central nervous system are not genus- or species-specific, and the presence of replicative structures such as tachyzoites does not always permit differentiation between *N. caninum*, *T. gondii*, and *Sarcocystis* spp. The absence of tissue cysts, particularly during the acute phase of infection, further complicates the matter (Pescador et al. 2007, Dorsch et al. 2021, Henker et al. 2022). For these reasons, molecular techniques are commonly employed to identify infectious agents, underscoring the importance of incorporating molecular analyses in studies of bovine abortus.

In contrast to *N. caninum*, the role of toxoplasmosis in cattle remains uncertain, and its impact on productivity has been poorly investigated (Dubey et al. 2020). Nonetheless, seroprevalence studies are abundant, as the detection of antibodies suggests persistent infection by *T. gondii*, and direct agent detection using molecular methods is still relatively rare (Dubey et al. 2020). Our data reinforce the likelihood of vertical transmission of *T. gondii*, confirming the widespread dissemination indicated by serological studies (Maia et al. 2021).

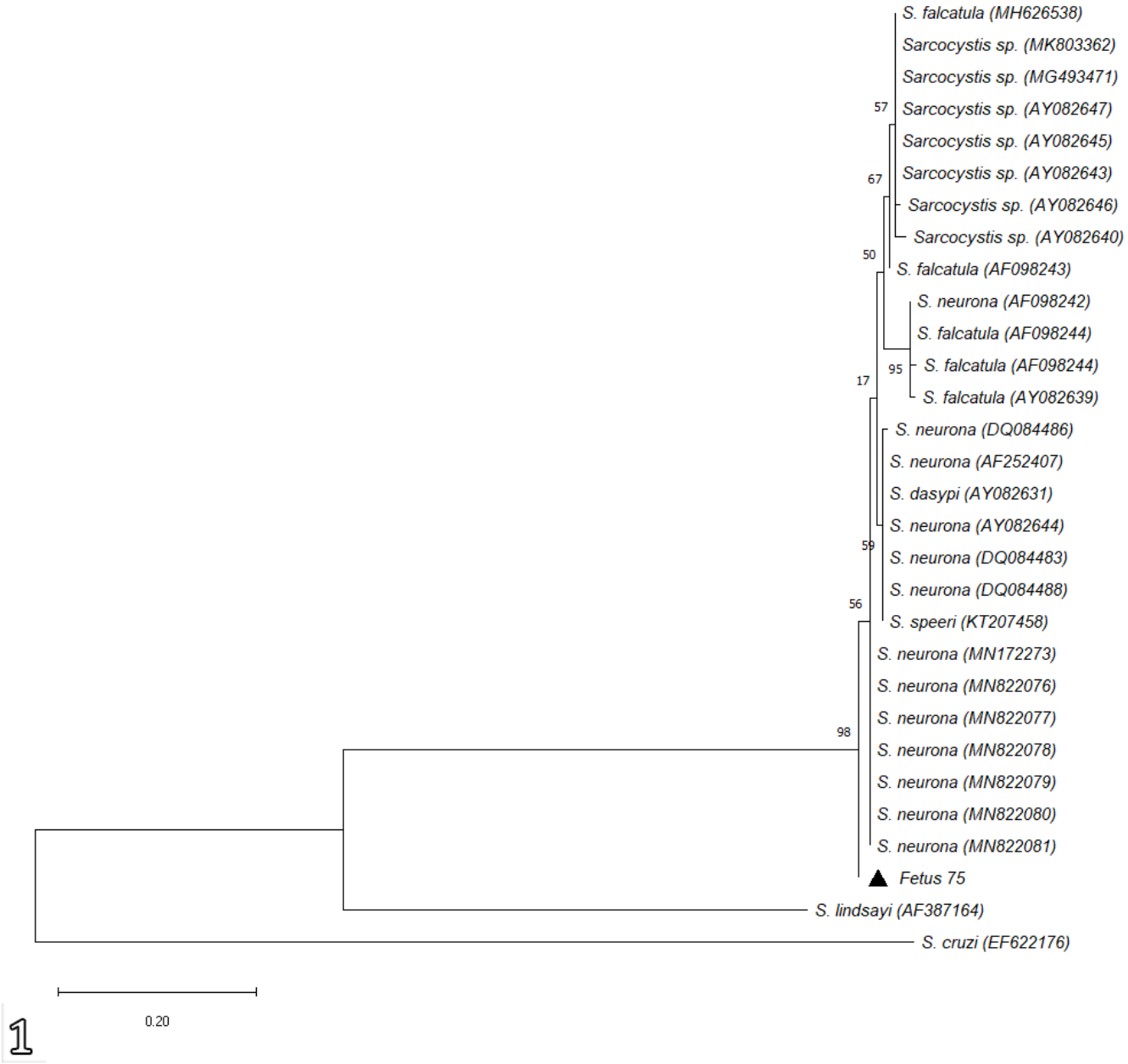

Fig.1. Phylogenetic tree based on the partial sequence of the ITS-1 obtained using primer sets F19 and R19/R23. Analyses were performed using the maximum likelihood method in the MEGA X software (version 10.2.4). Bootstrap values were calculated based on 1,000 replicates. The sequence indicated by the completed triangle corresponds to the one identified and analyzed in this study. One sequence (*Sarcocystis cruzi* – EF622176) was used as an out-group sequence.

Our study's method successfully detected *Sarcocystis neurona* in bovine fetal brain samples from South Brazil. To our knowledge, this is the first report indicating the presence of *S. neurona* in cattle, demonstrating the protozoan's ability to cross the transplacental barrier. Previous research has shown that the maternal-fetal interface structures in equine species are permissive to infection by *S. neurona* (Cabral et al. 2022). While detection does not confirm the pathogen's potential to cause gestational losses, it does stress the need to include *S. neurona* among the agents under investigation for bovine abortions.

The diagnosis of *S. neurona* was made using a comprehensive approach combining PCR, RFLP, DNA sequencing, and phylogenetic analysis. The *18S* rRNA gene, having the highest copy number compared to other genetic targets, was selected for its heightened screening potential. We employed PCR targeting the *18S* region and RFLP as a screening method. This combination offers advantages in sensitivity, efficiency, and cost-effectiveness compared to individual tests and sequencing (Silva et al. 2009). Nevertheless, due to the conserved nature of the *18S* rRNA gene, the results should be interpreted cautiously and, where possible, corroborated by using other genetic targets, such as species-specific primers (Cupolillo et al. 1995, Beltrame-Botelho et al. 2005, Kompalic-Cristo et al. 2005, Valadas et al. 2016).

The infection of *S. neurona* in cattle was validated through positive PCR amplification targeting the ITS-1 region, using specific primers as suggested by Valadas et al. (2016). The ITS-1 region exhibits higher variability among *Sarcocystis* species, enabling confirmatory outcomes (Hammerschmitt et al. 2020, Henker et al. 2020, De Santi et al. 2023). *S. neurona* belongs to a unique subgroup within the *Sarcocystis* genus, with opossums (*Didelphis albiventris* and *Didelphis virginiana*) acting as definitive hosts. In this *Sarcocystis* subgroup, other species (e.g., *S. speeri*, *S. lindsayi*, and *S. falcatula*) have also been identified (Gondim et al. 2021). The species in this group display high genetic similarities, particularly when opossums serve as their definitive hosts (Gondim et al. 2021). *In silico* analyses confirm that the primers recommended by Valadas et al. (2016) can cover all species in this *Sarcocystis* subgroup. Consequently, phylogenetic analysis is essential to explore the relationships and sequence similarities within the group.

Our constructed phylogenetic tree incorporated reference sequences from this subgroup, sequences from uncharacterized *Sarcocystis* derived from opossums, and sequences of *S. neurona* retrieved from animals displaying myeloencephalitis and geographically proximate to the samples analyzed in this study (Hammerschmitt et al. 2020, Henker et al. 2020, De Santi et al. 2023). BLAST analysis revealed that our sample shares higher genetic similarity with *S. neurona* than other *Sarcocystis* species for which opossums are the definitive hosts.

In addition to horizontal transmission, vertical transmission is also plausible for the spread of *S. neurona* in cattle. Contact between *D. albiventris* (the definitive host) and cattle is unsurprising given the shared or similar habitats between cattle and horses, coupled with the dispersion of the definitive host (Hoane et al. 2006, Cazarotto et al. 2016). Similar to the case in equines, cattle can be viewed as accidental hosts, as the genetic material identified does not show the characteristic presence of tissue cysts (Cabral et al. 2022). The ramifications of this infection on cattle production are yet to be fully elucidated; however, our findings highlight the need for awareness that protozoans other than *N. caninum* and *T. gondii* might play a role in bovine fetal infections.

CONCLUSION

The utilization of a suite of molecular techniques enabled the detection of *Sarcocystis neurona* and other Apicomplexa DNA in brain samples of bovine fetuses from South Brazil. While the implications of *S. neurona* infection on fetuses or pregnant cattle remain undefined, our findings emphasize the importance and necessity of further research to characterize the infection of *S. neurona* in cattle and its potential impact on bovine production.

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