Genetic Detection of *Neospora caninum* in the Blood of Dairy Cattle from Boyacá, Colombia

Detección genética de *Neospora caninum* en sangre de ganado lechero de Boyacá, Colombia

Detecção genética de *Neospora caninum* no sangue do gado leiteiro de Boyacá, Colômbia

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Abstract

*Neospora caninum* is a parasite of the phylum Apicomplexa that causes significant economic losses for cattle husbandry worldwide. Despite its relevance, information regarding infection prevalence in endemic areas of Colombia is scarce. Previous studies have reported a high seroprevalence in dairy cattle from Boyacá, which suggests a significant risk factor for active transmission of neosporosis. However, there is no available data concerning the infection stage or the presence of said parasite in the peripheral blood of dairy cattle. In this study, genetic detection of *N. caninum* was carried out using a nested PCR with an *Nc-5* marker on peripheral blood samples from dairy cows in the municipalities of Paipa, Toca, and Tuta. Four positive samples were sequenced through the Sanger method, which were then edited, aligned, and compared to sequences available in the GenBank database. The parasite’s DNA was detected in 23 out of 170 analyzed blood samples. A qualitative detection limit was estimated (~64 parasites per volume of blood sampled). This is the first report of DNA detection of *N. caninum* through PCR in blood from Colombian dairy cattle. Thereupon, further studies about *N. caninum* molecular detection and population genetics in cattle peripheral blood could be very useful for the early diagnosis of neosporosis and creating more effective epidemiological surveillance strategies.

Keywords: dairy cattle; genetics; molecular detection; nested PCR; neosporosis.
Resumen
Neospora caninum es un parásito del filo Apicomplexa que genera grandes pérdidas económicas para la ganadería a nivel mundial. A pesar de su importancia, la información sobre la prevalencia de infección en áreas endémicas de Colombia es escasa. Estudios previos han reportado una alta seroprevalencia en ganado lechero de Boyacá, que sugiere un factor de riesgo significativo para la transmisión activa de la neosporosis. Sin embargo, no hay datos disponibles sobre el estado de infección o la presencia del parásito en la sangre periférica del ganado lechero. En este estudio, se realizó la detección genética de N. caninum por PCR anidada del marcador Nc-5 en muestras de sangre periférica de vacas lecheras de los municipios de Paipa, Toca y Tuta. Se secuenciaron cuatro muestras positivas por el método de Sanger, que se editaron, alinearon y compararon con secuencias disponibles en la base de datos GenBank. Se detectó el ADN del parásito en 23 de las 170 muestras de sangre analizadas. Se estimó un límite cualitativo de detección en las muestras (~64 parásitos por volumen de sangue muestreada). Este es el primer reporte de detección de DNA de N. caninum a través de PCR en sangue de gado leiteiro colombiano. A partir de estas evidencias, estudios adicionales sobre la detección molecular de N. caninum e genética populacional en sangue periférico del gado podrían ser muy úteis para o diagnóstico precoce da neosporose e para a criação de estratégias de vigilância epidemiológica mais eficazes.

Palavras-chaves: detecção molecular; gado leiteiro; genética; neosporose; PCR aninhado.

Introduction
Neospora caninum is a cyst-forming obligate intracellular parasite belonging to the phylum Apicomplexa (subclass: Coccidia), which infects a wide variety of domestic and wild hosts worldwide (Dubey, 2003). This parasite is the etiological agent for neosporosis, a serious disease in cattle that causes causing major reproductive problems such as abortion and stillbirth (Dubey and Schares, 2006). Studies about N. caninum infection prevalence aimed at reducing the impact on livestock production are considered essential, given that this condition causes huge economic losses in dairy cows/livestock farming worldwide (Dubey et al., 2007).

Serological-based methods and immunohistochemical staining are the most common techniques for detecting N. caninum infections in cattle and several hosts, as evidenced by an increasing amount of studies in countries from Africa (Amdouni et al., 2018), Asia (Pagmadulam et al., 2018; Yao et al., 2009; Yu et al., 2007), and Europe...
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Studies in Latin American countries have been carried out in Argentina (Moore et al., 2014), Brazil (da Silva et al., 2017; de Magalhães et al., 2014; García-Melo et al., 2009), Ecuador (Changoluisa et al., 2019), Peru (Serrano-Martínez et al., 2019), and Venezuela (Lista-Alves et al., 2006). However, in Colombia, information about the status of neosporosis status is scarce, and its transmission dynamics are uncertain.

Some reports have indicated a highly heterogeneous seroprevalence throughout Colombian regions with different cattle production purposes, i.e., 33% reported for the department of Córdoba (Cardona et al., 2015) and 78% for Caquetá (Motta-Giraldo et al., 2012), where livestock production is mostly for beef; and 54% for Cundinamarca, 28.3% for Antioquia (Llano et al., 2018), and 64% and 52% for Boyacá (Cruz-Estupiñán et al., 2019; Pulido-Medellín et al., 2016), where production mainly concerns milk. According to these reports, Boyacá has the second highest N. caninum seroprevalence in Colombia, and one of the highest for the entire Andean region.

Serology is the most commonly used method for diagnosing neosporosis in live animals (Dubey et al., 2007), but assays can be limited by false-negative results in early or chronic cases of infection (Yao et al., 2009) because of specific antibody (Ab) fluctuation sometimes coming below serological test detection limits (Álvarez-García et al., 2003). PCR-based methods have the ability to amplify N. caninum DNA in adult and aborted fetus tissues, as well as and in body fluids (blood, milk, and semen) (Collantes-Fernández et al., 2002; Ferre et al., 2005; Yao et al., 2009).

N. caninum DNA detection offers valuable information about infection, even in the absence of Abs (McInnes et al., 2006; Ramos et al., 2017) and co-infection (Li et al., 2014). Besides, it enables studying the genetic diversity and phylogenetic relationships of N. caninum from multiple hosts and geographical origins (Amdouni et al., 2018; Li et al., 2014; Nardoni et al., 2019; Rocchigiani et al., 2017). It has been used for the direct demonstration of parasite DNA in tissues and body fluids such as peripheral blood (Okeoma et al., 2004; Yao et al., 2009), serum (Bârburaș et al., 2019; McInnes et al., 2006), and semen (Amdouni et al., 2019; Ferre et al., 2005).

The Nc-5 gene has been widely validated and used for the detection of N. caninum through PCR because it is specific for the parasite, and its sequence is repeated several times in the genome (Okeoma et al., 2004; Okeoma et al., 2004; Müller et al., 1996; Yao et al., 2009).

According to the Colombian livestock census of 2017, the dairy industry produces 24.3% of the country’s gross domestic product (ICA, 2017). Boyacá is considered the backbone of Colombia’s dairy industry, as specialised dairy systems are usually located in the colder climates of central and western provinces (Gobernación de Boyacá, 2019).

Although the region of Boyacá is one of the most relevant for the dairy industry in Colombia, the knowledge about active N. caninum infection of dairy cattle remains poor, due to the fact that the area has been understudied. Nested PCR and sequencing for the Nc-5 single copy marker were used on samples collected from three relevant dairy farming areas located near the municipalities of Paipa, Toca, and Tuta in Boyacá’s central province to support evidence regarding N. caninum infection in the peripheral blood of dairy cattle.

Materials and methods

Animals and study sites

The study involved a sample of 170 non-pregnant cows that were 15 months and older, distributed in eight dairy herds from the municipalities of Paipa (n = 50), Toca (n = 50), and Tuta (n = 70) in Boyacá’s central province (Figure 1). Dairy herds were selected according to the number of productive heads of cattle (more than 30) that had recent abortions according to owners.
Sample collection and DNA extraction

Between March and October of 2018, whole blood samples were collected from the coccygeal vein of each animal and placed into sterile tubes containing ethylenediaminetetraacetic acid (EDTA). Samples were transported in an ice box and immediately transferred to the laboratory, where they were stored at -20°C until DNA extraction and processing. Genomic DNA was extracted from 200 μl of blood from each sample (after previous spinning for 2 min at 3000 rpm) using the RealiPrep Blood gDNA Miniprep System (Promega), according to the manufacturer's instructions. DNA integrity was checked on 2% agarose gel, stained with ethidium bromide and quantified using an Epoch Microplate Spectrophotometer (BioTek). A pGEM-T easy vector (Promega) containing an Nc-5 region 350 bp fragment amplified from the N. caninum Bahía strain (Ramos et al., 2017) was serially 10-fold diluted in sterile human blood (ranging from 10^{-1} to 10^{-4} ng) and extracted using the RealiPrep Blood gDNA Miniprep System (Promega) as a control for nested amplification of a ~220 bp fragment, according to what was reported elsewhere (McInnes et al., 2006). Each PCR reaction was carried out in a 30 μl volume containing 2 μl template DNA (~40 ng/μl) for the primary PCR and 1.5 μl amplicon for secondary PCR, 1X PCR buffer, 0.2 mM dNTP, 0.5 μM of each primer, 2 mM MgCl₂, and 1.25 U Taq DNA polymerase (ExcelTaq, SMOBIO Technology, Inc.). The conditions for both PCR reactions were initial denaturation at 94 °C for 4 min, followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 53 °C for 30 s, and extension at 72 °C for 30 s, with a final extension at 72 °C for 5 min. A negative control (Human DNA) and a positive control (plasmid) were also included in both primary and secondary reactions.

Nested PCR products were visualised on 2% agarose gel and stained with ethidium bromide. Nc5-PCR amplicon products from four randomly selected individuals and plasmid DNA were sent to Macrogen Inc. in Korea to be purified and sequenced in both directions.

Nc-5 sequence analysis

Partial Nc-5 forward and reverse sequences were adjusted and aligned to obtain a consensus sequence for each individual; multiple sequences were aligned using ClustalX default parameters (Thompson et al., 1997) and kept in BioEdit v.7.0.9.0 (Hall, 1999). Partial Nc-5 sequences were compared to
available conspecific sequences from several hosts and geographical origins obtained from GenBank (Table 1). MEGA v10.1.7 (Kumar et al., 2018) was used for calculating K2p-based matrix genetic distances, and the UPGMA-based algorithm was used for drawing heatmaps in R (v1.0.12).

RESULTS

Plasmid Nc-5 and N. caninum DNA detection

Initial Nc-5 plasmid concentration was 4 ng/µl DNA, and detectable amplification was observed until 10⁻⁴ ng (0,004 ng/µl) dilution for outer primers (Figure 2A). The qualitative detection limit of N. caninum DNA was ~64 parasites per volume of blood analyzed (200 µl), based on N. caninum’s genome size, i.e., 61 Mb (Reid et al., 2012), and according to interconvertible estimation from DNA picograms to base pairs (where 1 Mb = 1,022 x 10⁻³ pg), as reported elsewhere (Dolezel et al., 2003).

A total 23 out of 170 samples tested had a ~220 bp amplicon after inner-PCR (Figure 2B); ten were from Paipa, seven from Toca, and six from Tuta. The 23 cows that tested positive through nested PCR were over 30 months of age. However, in the range of 15 to 29 months old, N. caninum DNA was not detected. The prevalence of parasite DNA in blood was 13.5% (23/170; 95% C.I. 7-18), ranging from 6.2% in herd 3 to 37.5% in herd 2 (both from Toca). Nevertheless, no N. caninum DNA was found in herd 5 of Toca and herd 7 of Tuta (Table 1).

Comparing N. caninum Nc-5 sequences

Partial Nc-5 from four randomly selected samples was compared to plasmid and an available sequence data for the Nc-5 gene (GenBank accession no. X84238) (Figure 3). The plasmid sequence was almost identical to the reference sequence. Sequences obtained from Paipa and Tuta showed clear differences against the reference sequence and plasmid, as well as between them (Figure 3). Colombian samples shared two nucleotide substitutions in positions 751 and 784, although eight indels were only observed in the Paipa03 sample (Figure 3). Distance-based heatmaps including Nc-5 publicly available sequences showed that most of the included sequences were roughly similar, ranking from 0 to 0.1, except for some pig and bird sequences from China, which had values above 0.15 (Figure 4).

Colombian N. caninum Nc-5 sequences had slight differences, and no conclusive divergence

Table 1. N. caninum-DNA origin, number of samples analyzed and prevalence in blood from herds in Boyacá, Colombia (C.I. is the confidence interval)

| Locality | Number in Figure 1 | Animals examined | PCR positive | Prevalence (%) | 95% C.I.  |
|----------|--------------------|-----------------|--------------|----------------|----------|
| Paipa    | 1                  | 50              | 10           | 20             | 10.5 - 34.1 |
|          | 2                  | 8               | 3            | 37.5           | 10.2 - 74  |
|          | 3                  | 16              | 1            | 6.2            | 4.9 - 46.3 |
|          | 4                  | 13              | 3            | 23.1           | 6.1 - 54   |
|          | 5                  | 13              | 0            | 0              | 0         |
| Toca     | 6                  | 17              | 2            | 11.8           | 2 - 37.7   |
|          | 7                  | 20              | 0            | 0              | 0         |
|          | 8                  | 33              | 4            | 12             | 3.9 - 29   |
| Tuta     | 1                  | 50              | 10           | 20             | 10.5 - 34.1 |
|          | 2                  | 8               | 3            | 37.5           | 10.2 - 74  |
|          | 3                  | 16              | 1            | 6.2            | 4.9 - 46.3 |
|          | 4                  | 13              | 3            | 23.1           | 6.1 - 54   |
|          | 5                  | 13              | 0            | 0              | 0         |
|          | 6                  | 17              | 2            | 11.8           | 2 - 37.7   |
|          | 7                  | 20              | 0            | 0              | 0         |
|          | 8                  | 33              | 4            | 12             | 3.9 - 29   |
| Total    | 170                | 23              | 13.5         | 7 - 18         |

Source: Authors
could be observed within the main cluster that included most sequences from diverse hosts and countries worldwide (Figure 4). Due to the short fragments used on the study and the limited Nc-5 information, no phylogenetic or phylogeographic assumptions could be made; instead, further studies are needed which must include additional genetic and biological samples.

**DISCUSSION**

Neosporosis is thought to involve low parasitaemia, mostly during non-gestational periods. However, some reports for parasites circulating in blood and other body fluids have increasingly become available in scientific publications (Bărbaruș et al., 2019; Dubey et al., 2007; Ferre et al., 2005; Yao...
Figure 4. Distance-based cluster and heatmaps for *N. caninum* Nc-5 partial sequences from different hosts and regions worldwide (details in Table S1)

Source: Authors

Table 2. Origin and access codes of *Neospora caninum* Nc-5 sequences used in the K2p-based distance analysis.

| Country       | Biological origin | Accession number  |
|---------------|-------------------|-------------------|
| Argentina     | Goat              | MG973171-72       |
| Australia     | Wolf              | GU194961-65       |
| China         | Bird              | MK570535-90       |
| Iran          | Sheep             | KR106181-85       |
|               | Bird              | KP702735-37       |
|               | Dog               | KY124528-29       |
|               | Cat               | KY124530-31       |
|               | Cow               | MH884746-47       |
| Israel        | Crow              | KR858302-03       |
| Poland        | Bison             | HM031965-66       |
|               | Cow               | EF463098-99       |
| Romania       | Buffaloes         | MK012391-95       |
| United States | Wolf              | KF649844-48       |

Source: Authors
et al., 2009). This study has explored and revealed, for the first time, *N. caninum* in peripheral blood of non-pregnant cattle in Colombia.

*N. caninum* in blood could indicate two scenarios: evidence of acute infection involving tachyzoites circulating in the blood flow, or latent infection involving released bradyzoites differentiating into circulating tachyzoites to form new cysts (Okeoma et al., 2004; Yao et al., 2009). The PCR-based method used here did not support either scenario. However, qualitative analysis of our data indicated that the samples contained at least 64 parasites per volume of peripheral blood analyzed (200 μl). This would suggest that *N. caninum* parasitaemia was higher than what was initially thought, and this highlighted the need for developing more sensitive molecular-based methods for neosporosis diagnosis and surveillance.

Previous studies have found 10 parasites/ml in infected bull semen. However, this was different from what was observed in cow blood, as well as between animals (Ferre et al., 2005). Variable parasitaemia was suggested because *N. caninum* DNA was found in cell fractions due to blood providing a tachyzoite transport medium between host tissues (Okeoma et al., 2004). Identifying parasite DNA in seropositive cattle blood samples was thus intermittent (Collantes-Fernández et al., 2002), which indicates fluctuating parasitaemia as time elapsed, possibly due to immunological action, few circulating parasites, or a short in-host period (Ferre et al., 2005; Yao et al., 2009). We would thus suggest that the heterogeneous prevalence estimated for herds responds to the samples’ parasitaemia dynamics, as well as other extrinsic factors.

The loop-mediated isothermal amplification-based (LAMP) method has recently been suggested for detecting *N. caninum* DNA in fetal tissue from bovine abortion, as well as in canine feces (Ramos et al., 2017). The LAMP technique is ideal in future research to test this approach on peripheral blood, given that it could offer valuable information for the timely management of infection, thereby replacing post-mortem diagnosis.

Results gave 13.5% overall parasite DNA prevalence (95% C.I. 7-18) in dairy cattle in six out of eight herds sampled from the municipalities of Paipa, Tuta, and Toca in Boyacá. Age was found to be an important factor that influences seroprevalence values, as seropositivity has been seen to increase with it (Dubey et al., 2007). *N. caninum* DNA was detected here in 23 cows over 30 months of age. This finding suggests that there is not enough evidence of recent displacement or passive transportation from other possible endemic zones.

Active neosporosis infection can be assumed in Boyacá livestock. Further genetic-based studies concerning neosporosis transmission epidemiological dynamics in this area are still needed to provide opportune evidence to better design infection surveillance strategies.

Previous studies in Colombia have indicated that neosporosis infection in cattle is heterogeneous and very high in some places; a 54% seroprevalence has been reported in Bogotá and the department of Nariño (Zambrano et al., 2001), 78% in Caquetá (Motta-Giraldo et al., 2012), 33% in Córdoba (Cardona et al., 2015), and 64% and 52% in Boyacá (Cruz-Estupiñán et al., 2019; Pulido-Medellín et al., 2016). This study recorded 6.2 to 37.5% of *N. caninum* prevalence ranking in several herds from Boyacá (Table 1), which indicates variable infection dynamics on both micro (herd) and regional (departments) scales. This picture supposes that transmission scenarios are driven by local factors involved in heterogeneous biological and epidemiological traits (namely immunological status, intermediate hosts, owner practices, and parasite diversity) which are yet to be further defined.

Several studies have been aimed at understanding *N. caninum* genetic diversity regarding different geographical and host-related origins. Internal transcribed spacer 1 (ITS1) sequence analysis has suggested moderate to high differentiation among *N. caninum* isolates from different regions, and different biological origins (Gondim et al., 2004).
Similar results were found when microsatellite markers were analysed in several populations from American and European countries (Regidor-Cerrillo et al., 201). This study compared four Colombian N. caninum partial Nc-5 sequences, thus constituting initial evidence of potentially high genetic differences between them, as well as those from GenBank (Table 2).

Although limited phylogenetic information was available for the Nc-5 fragment used, a genetic distance of more than 10% was identified between sequences analysed from Paipa and Toca, and non-conclusive genetic clustering was observed within the entire dataset. This would suggest remarkable genetic diversity regarding N. caninum strains circulating in Colombia. Further population and phylogeographic studies about Colombian N. caninum must be carried out to supply additional information about the evolution and biological traits involved in neosporosis-related epidemiological dynamics.

CONCLUSIONS

This is the first report of PCR-based identification of N. caninum in dairy cattle blood in Colombia, and it indicated active infection in livestock from Boyacá. Further studies about the molecular detection of N. caninum in cattle peripheral blood are essential (such as those considering more sensitive PCR-based approaches, i.e. loop-mediated isothermal amplification) for the early diagnosis of neosporosis, as well as introducing more effective cattle/dairy farming-related epidemiological surveillance strategies in Colombia.

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