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ORIGINAL

Seroprevalence and risk factors associated to *Neospora caninum* in dairy cattle herds in the municipality of Pasto, Colombia

Seroprevalencia y factores de riesgo asociados a *Neospora caninum* en ganado lechero en el municipio de Pasto, Colombia

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ABSTRACT

Objective. To determine seroprevalence and risk factors associated to *Neospora caninum* in non-vaccinated dairy cattle against infectious agents of reproductive syndrome in the municipality of Pasto, Colombia. **Materials and methods.** Farms over 2527 meters over sea level were selected, a total of 238 serum samples of Holstein cows were collected and analyzed using the indirect ELISA test to determine *N. caninum* seropositivity. An epidemiological survey was realized in each herd which included variables related to health and management measures of cattle. A multivariate analysis of binary logistic regression was used with a confidence interval of 95% ($p<0.05$) using SPSS19® program. **Results.** The estimated prevalence of *N. caninum* was 76.9%. The risk factors associated to neosporosis infection in the analyzed farms are as follows: residues of abortions generally left outdoors and not buried (OR 3.81, 95% CI 1.5 - 9.6); dogs fed with leftovers (OR 15.44 IC 95% 1.94-123.22) and bulls allowed to mate with cows (OR 19.68, 95% CI 2.34 - 165.52). **Conclusions.** The high prevalence of *N. caninum* and the low abortion rate in dairy herds of the municipality of Pasto corroborated no existence of the disease in all animals serologically positive, but it did suggest that at some point in their lives they were exposed to *N. caninum*. From the identified risk factors in this study, recommendations can be provided for an effective control of reproductive diseases like Neosporosis present in this region.

Key words: Abortion, fetal death, parasites, serology (*Source: CAB*).

RESUMEN

Objetivo. Determinar la seropositividad de *N. caninum* en bovinos no vacunados contra el síndrome reproductivo y los factores de riesgo asociados a la presentación de esta enfermedad en los hatos lecheros del municipio de Pasto, Nariño. **Materiales y métodos.** Se seleccionaron fincas sobre los 2527 msnm. Se analizaron muestras de suero de 238 vacas Holstein mediante la prueba de ELISA indirecta para determinar la seropositividad a *N. caninum* y se realizó una encuesta epidemiológica incluyendo variables sobre el ganado como: medidas sanitarias y de manejo. Se realizó un análisis multivariado mediante regresión logística binaria con un intervalo de confianza del 95% ($p<0.05$) utilizando el programa SPSS19®. **Resultados.** La prevalencia estimada para *N. caninum* fue de 76.9 %. Los factores de riesgo asociados a Neosporosis en las fincas estudiadas son los residuos de abortos, que no se entierran y se dejan a la intemperie (OR 5.49; IC 95% 1.7-17.7), alimentar los perros con desperdicios (OR 15.44 IC 95% 1.94-123.22) y la monta directa (OR 14.62 IC 95% 1.55-137.53). **Conclusiones.** La elevada prevalencia de *N. caninum* y la tasas bajas de abortos en el municipio de Pasto, confirma que la enfermedad no se presenta en todos los animales positivos serológicamente, pero sugiere que en algún momento de su vida fueron expuestos al agente causal. A partir de los factores de riesgo identificados en este trabajo se pueden establecer recomendaciones para un control efectivo de enfermedades reproductivas como la Neosporosis presentes en la región.

Palabras clave: Aborto, muerte fetal, parásitos, serología (*Fuente: CAB*).

INTRODUCTION

Bovine neosporosis is a parasitic disease caused by the protozoan *Neospora caninum* (*N. caninum*) and it is considered one of the main causes of abortion in cattle worldwide (1). Abortions due to Neosporosis infection have been reported as a substantial financial loss in livestock industry (2). The transmission from an infected cow to its offspring has been identified as the main route of infection. Elimination of *N. caninum* in aborted fetuses is considered one of the most relevant ways to maintain the infection in herds (3).

Dogs have been identified as definitive hosts, where the sexual stage of protozoa develops in the gut forming oocysts eliminated with the feces and then ingested by cattle (4). The asexual phase starts in cows with the formation of tissue cysts and tachyzoites in the intermediate host or fetus. The completion of the epidemiological cycle occurs when dogs ingest infected tissues- aborted fetus or placenta tissue- forming oocysts all over again (5). Drinking water and food, contaminated by dogs or any other carriers not yet identified can cause this infection (6,7).

Abortions can occur sporadically or in outbreaks at any time of gestation (8), but it is more common between the 4th and 6th month of pregnancy (6). It has been reported that there is a higher prevalence in dairy cattle than in beef cattle because of management factors facilitating the spread of the disease (9).

Seroprevalence of *N. caninum* has been reported as follows: in Argentina 64.5% in cows with clinical history of abortion (10), in Paraguay a seroprevalence of 29.8% was detected by ELISA and it was identified as the major cause of abortions in meat and milk cattle (11). In Parana, Brazil 15.1% was found in dairy Holstein breed (12). In Aguascalientes, México 57.5% seroprevalence was found in cows with a clinical history of abortion (13), similar to those found in dairy herds in Tulcán-Ecuador 51.64 % (14). In northwestern United States a 24% of seroprevalence was found; which increases during the winter, attributed to the high density of cattle (15).

In Colombia, 54.1% of seroreactivity was reported in 357 animals with a history of abortion (16). In Nariño, there have been no epidemiological studies on patterns of *N. caninum*; therefore the objective of this study was to determine the prevalence and risk factors associated with this parasite.

MATERIALS AND METHODS

A cross-sectional study was conducted to determinate the seroprevalence of *N. caninum* in the municipality of Pasto. The difference among exposed animals and non-exposed animals with the analyzed variables was found as a risk factor.

Study site. This research was conducted in 10 dairy farms in the rural municipality of Pasto, located nearby the Galeras Volcano, which belongs to an ecosystem of Lower Mountain according to the Holdridge classification. The ecosystem is characterized by 700 mm annual precipitation, average temperature of 13.3°C and humidity of 60% to 88%. These farms were extensively managed; cows remain most of the time within fenced pastures, with no physical separation between heifers and adult cows.

Cattle population. The sample size was determined by a simple random sampling strategy, based on cow population in dairy farms according to the 2010 Census Foot and Mouth disease (FMD) vaccination in the municipality of Pasto:

$$n = \frac{\mathbf{N} * \mathbf{Z_2} * \mathbf{P} * (\mathbf{1} - \mathbf{P})}{\mathbf{N} * \mathbf{e_2} + \mathbf{Z_2} * \mathbf{P} * (\mathbf{1} - \mathbf{P})}$$

Where:
N: Number of dairy cows in the municipality (3489)
P: Expected prevalence (40%)
e: Accepted error (5%)
Z: Confidence level (90%)

A total of 238 Holstein cows were sampled, these animals had never been vaccinated against Neosporosis and other reproductive diseases. These animals were in small size herds with and a moderate level of milk production (15 kg/cow/day), that corresponds with traditionally managed herds. These farms have information systems about reproductive events and animal identification. The inclusion criteria were: nursing cows (>2 years old) with more than 6 months of permanence in the farms.

Variables. Epidemiological data were collected through a structured survey obtained by a direct interview with the cattle farmer. The included variables were: 1. Reproductive Management— synchronization, culling, and type of reproduction (natural or artificial insemination). 2. Animal Health— annual abortion, deworming, and vaccination; 3. Pasture Management— organic fertilizer and manure as fertilizer. 4. Origin of Replacements Cows (external, same farm or mixed). 5. Biosecurity— water source, elimination of fetuses and placentas. 6. Presence of animals— sheep, horses, pigs, cats, and dogs.

Sample collection and serological examination. Blood samples (10 ml) were collected by venipuncture of coccygeal vein using sterile tubes without anticoagulant (Vacutainer) which were subsequently taken to the Clinical Veterinarian Diagnostic Laboratory at "Universidad de Nariño". These blood samples were centrifuged (1500 rpm / 5 min) in order to obtain the serum which was finally stored at -20°C until it was analyzed.

The presence of antibodies against *Neospora caninum* were tested using a commercial indirect Enzyme-linked Immunosorbent Assay (ELISA) kit (Uppsala, Sweden, Svanova Biotech®), following the manufacturer's specifications. The ELISA equipment for the kit was: 1. a lector STAT FAX 3200, 2. Washing equipment STAT FAX 2600 and 3. Incubator Stat Fax 2200.

The *N. caninum* iscom ELISA Kit is designed to detect bovine Neospora-specific antibodies (Immunoglobulin G-IgG) in serum. The kit procedure is based on a solid phase indirect Enzyme Linked Immunosorbent Assay (ELISA). In this procedure, serum samples were exposed to noninfectious Neospora antigen incorporated into iscoms coated onto wells of micro titer strips.

Neospora antibodies (if present in the serum sample) bind parasite antigen in the wells. The HRP conjugate added subsequently forms a complex with these *N. caninum* antibodies. Unbound material is removed by rinsing before the addition of substrate solution. Subsequently a blue color develops which is due to the conversion of the substrate by the conjugate. A positive result is indicated by development of a blue color. The reaction is stopped by addition of the stop solution; the color changes to yellow. The result can be read visually or by a microplate photometer, where the optical density (OD) is measured at 450 nm.

The sensitivity and specificity of the test was 99% and 96% respectively. The plates were read at 450nm and the results were given in optical density values expressed in percentage of positivity for antibodies to *N. caninum*. The used formula was:

$$(\mathbf{pp})\%\ \mathbf{positivity\ values} = \frac{\text{sample or negative control (OD corrected)}}{\text{positive control (OD corrected)}} \mathbf{x100}$$

The control values were within the following limits: positive control optical density (OD)>0.8 and OD negative control <0.15. To ensure validity, the duplicate of the OD values should not differ more than 25% from each other. This information was obtained from the Diagnostic Instructions Kit.

Data analysis. The prevalence of antibodies to *N. caninum* was estimated from the ratio of positive results to the total number of examined cattle (17). The association between seroprevalence and risk factors was quantified using a multivariate binary logistic regression with a confidence interval of 95%. The significance of the association was estimated by determining Odds Ratio (OR) of each factor with a $p<0.05$. The adjustment of the test was assessed with statistics Hosmer-Lemeshow. Calculations were performed using SPSS 19® with the next steps: Analyzer>regression>logistic binary; where the dependent variable was positive or negative to *N. caninum* and variables like risk factors were analyzed as the covariate.

RESULTS

Percentage of abortions in this study was 7%, but only 2% were attributed to *N. caninum* infection. The other animals which aborted were positive to other infectious agents such as *Brucella abortus*, *Herpesvirus bovino* tipo I and virus of *diarrhea bovine*.

Medical records of the farms reported placental retention and return to estrus after artificial insemination, increasing the mating per conception; which directly affects open days (140±20 days). These farms only had records of vaccination against FMD and brucellosis.

The prevalence for *N. caninum* in the municipality of Pasto was of 76.9% (183 cows seropositive). In nine farms, the seropositivity was over to 60% and it was lower only in one farm (Farm D.) The results of seroprevalence in each farm are described in table 1.

Table 1. Prevalence of antibodies (IgG) to *N. caninum* in Holstein cows in dairy farms in the municipality of Pasto.

Farm	Number of cows	Number of positive cows to Neospora	Number of negative cows to Neospora	Prevalence (%) Neospora
A	27	22	5	81.5
B	25	15	10	60.0
C	19	13	6	68.4
D	8	3	5	37.5
E	22	17	5	77.3
F	15	13	2	86.7
G	38	24	14	63.2
H	23	21	2	91.3
I	47	42	5	89.4
J	14	13	1	92.9
TOTAL	238	183	55	76.9

Three variables were associated with seropositivity to Neosporosis: residues of abortions generally left outdoors and not buried, dogs fed with leftovers and bulls allowed to mate with cows (Table 2).

Table 2. Risk factors associated with seropositivity of *Neospora caninum* in dairy farms in the municipality of Pasto.

Variable	[2]OR	[1]IC 95% Lower	Upper	P-Value
Bulls allowed to mate with cows	14.62	1.55	137.53	0.012
Abortion Residues	5.49	1.7	17.7	0.003
Feeding dogs with leftovers	15.44	1.94	123.22	0.006

[1] Confidence interval 95%, [2]Odds ratio, [3]P< 0.05

Other variables showed co linearity or low association such as: management and the serologic results of cattle, presence of other animals (sheep, horses, pigs, cats, and dogs), pasture management (organic fertilizer, manure as fertilizer), and origin of replacements cows (external, same farm or mixed).

DISCUSSION

The seroprevalence of *N. caninum* within this region is high compared to other parts of the country. In Monteria 10.2% seropositivity was found in cows with reproductive problems (18) Other studies in Colombia by Zambrano et al (16) reported 54.1% of seroreactivity in 357 animals with a history of abortion, using the same diagnostic technique ELISA in Antioquia, the prevalence found in Holstein cattle was of 39.9% and in Brangus cows was 2% (19).

Abortion is the most commonly reported event in cases of Neosporosis (20); however in this study, the abortion rate was 7% and it was related to the *N. caninum* infection. This research has questioned the association between infertility or sub fertility and seropositivity to the agent. The majority of these farms have eventually had reproductive problems. The most common issue was the high range of open days exceeding the estimated range and repetition of heats.

It also states that the fetal mummification is a common event in cases of neosporosis described in cases of natural and experimental infections (21), only one farm reported this situation.

The presence of dogs was common in all farms, which cannot be determined statistically as a risk factor because of the co-linearity. However, in a study in Brazil, no association between the presence of dogs in farms with *N. caninum* seropositivity was found (12).

Other authors describe a strong association between the existence of canine defecation in pastures, presence of street dogs and other wild animals (foxes) with the presentation of *N.*

caninum (22,23). Moreover, feeding dogs with leftovers and residues of abortions represent a risk factor, because the food is contaminated with tachyzoites; starting the cycle in definitive hosts (24).

The elimination of aborted material becomes a major risk factor in this sampled region; contrary to those reported in Aguascalientes, México (13) where no association was found between the provision of aborted fetuses and placental debris with seroprevalence. Fetuses and/or stillbirths, and poor provision of these materials in the farm are conducive to a proliferative environment of this parasite in pasture water and facilities. Animals found within the premises that entered in the parasite cycle may contaminate them because oocysts are environmentally resistant (25).

Mating among cows and bulls is a common practice in this region, which has become a risk factor even though, artificial insemination is allowed in cows during repeats estrus. Experiments conducted in the U.S. showed that venereal transmission is possible with a large number of tachyzoites (26). In this study, two out of three bulls sampled were seropositive for antibodies to *N. caninum*. Benavides et al, consider that there is a strong association between abortions and absence of drainage systems and septic tanks in the Department of Nariño, in regards to the management of wastewater and storm water because this improves the maintenance of *N.caninum* and Leptospira sp (27). However, in these farms there were no risk factors for the disease studied.

In terms of the replacements management of farm raised or purchased cattle, there were no risk factors associated with seropositivity of *N. caninum*. Farmers breeding their own replacement cattle are one relevant mechanism in order to maintain a high prevalence (28).

In conclusion, the results obtained in this research do not show the existing disease in animals that were positive to the serologic test. This suggests that these animals were eventually exposed to the causal agent causing the formation of specific antibodies to the parasite. Finally, it is important to mention that the poor management of aborted animals, feeding dogs with leftovers, and cattle mating are risk factors associated with the seropisivity of *N. caninum*

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