Journal of Animal Production, 32 (2), https://revistas.reduc.edu.cu/index.php/rpa/article/view/e3475

Animal Health

Original

Determination of Paramphistomosis in Slaughter Cattle in the Province of Camagüey, Cuba

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Received: March 2020; Accepted: May, 2020; Published: June, 2020.

ABSTRACT

Background: Paramphistomosis is an emerging parasitosis. In Cuba, previous reports of *Paramphistomum sp* focused mainly on the description of circulating species. **Aim:** To determine the presence of paramphistomosis in slaughter cattle in Camagüey.

Methods: The data was collected from 550 slaughtered animals from six municipalities of the province, between March and May 2017. The independent variables of the study were municipalities and infested organs.

Results: The prevalence in slaughter cattle was 68.54%. The rumen-reticulum was found to have a significant difference with respect to rumen and reticulum, independently. In spite of the high *Paramphistomum* sp infestation density, only 9.4% showed a greater frequency than the highest infestation level (over 1000 paramphistomids). The lowest prevalence was found in the municipality of Najasa, with significant differences ($p<0.05$), compared to the mean prevalence observed. The 18S sequence was 99.7% identical to *Cotylophoron cotylophorum*. The phylogenetic analysis was done using the Neighbor-Joining method, with 1000 bootstrap replicates, which revealed that the *Paramphistomum* species found has an 85% likelihood of pertaining to the same clade of *C. cotylophorum*.

Conclusions: In Camagüey, there is a low infestation intensity of *Paramphistomum sp* in slaughtered animals, with no specificity over a particular organ.

Key words: Cattle, *Paramphistomum*, parasitology, PCR, slaughterhouse (Source: *MeSH)*

How to cite (APA)

León Companioni, Y., Arenal Cruz, A., Tamayo Escobar, Y., Vázquez Montes de Oca, R., Casado Simón, E., & Arias Borrero, N. (2020). Determination of Paramphistomosis in Slaughter Cattle in the Province of Camagüey Journal of Animal Production, 32(2) //revistas.reduc.edu.cu/index.php/rpa/article/view/e3475

INTRODUCTION

Paramphistomosis is an emerging parasitic disease with endemic areas in tropical and subtropical regions. It is present in Asia, Europe, the Americas, and Australia (Hayider, Mekuria, and Mekibib, 2018). The prevalences of this parasitosis in cattle vary, with 1.2% (some locations in Australia), 32.17% (India), 39.10% (the Americas), and 55.9% (north of Spain) (Siben, Domatsky, Nikonov, and Beletskaya, 2018).

It is caused by different species of trematodes (*Digenea*), belonging to superfamily *Paramphistomoidea,* which includes the main species of interest in veterinary disease. Some of them are *Paramphistomum cervi*; *P. microbothrioides, Calicophoron daubneyi, Calicophoron, and Cotylophoron cotyloforum*. They have an indirect biological cycle, in which fresh water mollusks are the intermediate hosts (Tandon, Roy, Shylla, and Ghatani, 2014; Sargison, Shahzad, Mazeri, and Chaudhry, 2019). Particularly, *Galba cubensis* behaves as an efficient intermediate host in the Americas, especially in Cuba (Vázquez, Sánchez, Alba, Rodríguez, and Pino, 2013; Abreu, Piña, Diéguez, Vázquez, and Fimia, 2019).

Paramphistomosis has two clinical manifestations: an acute intestinal form caused by immature forms, and a ruminal chronic form caused by adult parasites. The larval forms adhere to the mucosa, causing an atrophy of intestinal villi, gastroenteritis, dehydration, and severe loss of body condition, due to a reduction in the voluntary ingestion of foods and nutrient absorption efficiency (Sanguankiat *et al.*, 2016; Ploeger *et al.*, 2017).

The ruminal fluid, especially lipids and fatty acids, are despoiled by adult amphistomids. As a result, paramphistomosis causes a decline in the production of volatile fatty acids (VFA), microbial proteins in the rumen, and by-pass proteins. Consequently, there is a decrease in food conversion and a reduction of milk production (Casas, 2018; Forbes, 2018).

In Cuba, the climatic conditions of precipitation, relative humidity, and temperature are favorable (Palacio, Bertot, Molento, Vázquez, Izquierdo, and Arenal, 2017). This enables development and survival of larval stages in the grass and the intermediate host. The aim of this research is to determine the presence of paramphistomosis in slaughter cattle, in Camagüey.

MATERIALS AND METHODS

The data for the study were collected through *post-mortem* visual examination of 550 slaughtered bovines, during the March-May 2017 period, in slaughterhouses owned by the food industry of Camagüey.

The animal samples were collected in 6 of the 13 municipalities of Camagüey, distributed as follows: 105 in Camagüey, 92 in Jimaguayu, 60 in Minas, 135 in Najasa, 49 in Sibanicu, and 109 in Vertientes.

Analysis of prevalence

Helminthological necropsy examination

The rumen and reticulum were systematically inspected during the *post-mortem* examination, to detect the presence or absence of *Paramphistomum* sp, and the intensity of infestation. Each day, all the slaughtered animals were inspected. Upon slaughtering, the pre-stomachs were ligated at the ends to prevent the loss of parasites and content, and then were removed. The rumen was dissected on the dorsal and ventral ridges, and the dissection of the reticulum was made at the major and minor ridges. The objective was to keep paramphistomids from being exposed.

The prevalence was calculated by municipalities. Four levels were established to classify paramphistomid infestation intensity: Null (no presence), low (1-500), mid (501-1000), and high (more than 1000).

Molecular identification of *Paramphistomum*.sp

DNA collection

A number of 10 parasites were picked from the rumen of animals in the municipality of Vertientes. The parasites were placed in a vial containing 70% ethanol.

The DNA was collected with 100 µg/ml of K proteinase (Boeringer Manheim, Germany), in lysis buffer (Tris-HCl 10 Mm, pH 8.0, EDTA 100 mM, SDS al 0.5 %), for 5-8 h, at 60 ºC, with slow shaking. The samples were treated three times with phenol, and once with chloroform. Then, the aqueous phase was treated with 20 µg/ml of RNase A (Sigma, US A), during 1 h, at 37 ºC. The DNA was purified by successive extraction with phenol: chloroform and chloroform, and it was precipitated with two volumes of ethanol in 0.3 M sodium acetate, at -20 ºC, for 2 h. After centrifugation, the DNA sediments were dried and resuspended in 200 µl of water sterilized twice.

Polymerase Chain Reaction

The ribosomal 18S RNA (rRNA) was amplified with 27 F universal forward primers (5´-CCAGAATTCAGAGTTTGATCMTGGCTCA-3´), and 1492 reverse primers (5´-ACCAAGCTTTACGGYTACCTTGTTAGGACTT-3´), in thermocycler (Eppendorf, Hamburg, Germany), under these conditions: initial denaturing (95°C for 5 min), followed by 34 cycles at 94 °C, for 1 min, 58 °C for 1 min, and 72 °C, for 3 min each, and then, a final extension step at 72 °C, for 7 min. The PCR products were analyzed by electrophoresis in agarose gel (1 %, w/v).

The amplicons were eluted, and the purified DNA products were sequenced in Myleus (MG, Brazil). The nucleotide sequences were analyzed using the search engine of local basic alignment (BLAST). *Paramphistomum* determination was done using MEGA 6, for probabilistic DNA analysis by Neighbor-Joining, with 1000 bootstrap replications. Each species included in the analysis contains a GenBank reference number,

Statistical analysis

A quantification of variables municipalities and infested organs was performed (rumen, reticulum, and rumen-reticulum).

STATGRAPHICS Centurion XV, 20.2.06, 2007 was used to make a contingency table of variables infestation intensity and municipalities. The proportions of prevalence in the municipalities with respect to the mean, were compared. A mean proportion comparison of infestation intensity based on the infested organs, was conducted as well. In all the cases, the Chi-square test ($p<0.05$) was performed.

RESULTS

The prevalence in all 550 animals analyzed was 68.54%.

The predominant level of infestation intensity in all the municipalities was low, with 46.18% (Figure 1). Of them, Vertientes and Camagüey (9.82% and 9.45% of cases, respectively) showed the highest values, whereas Sibanicu (4.73%) had the lowest infestation intensity.

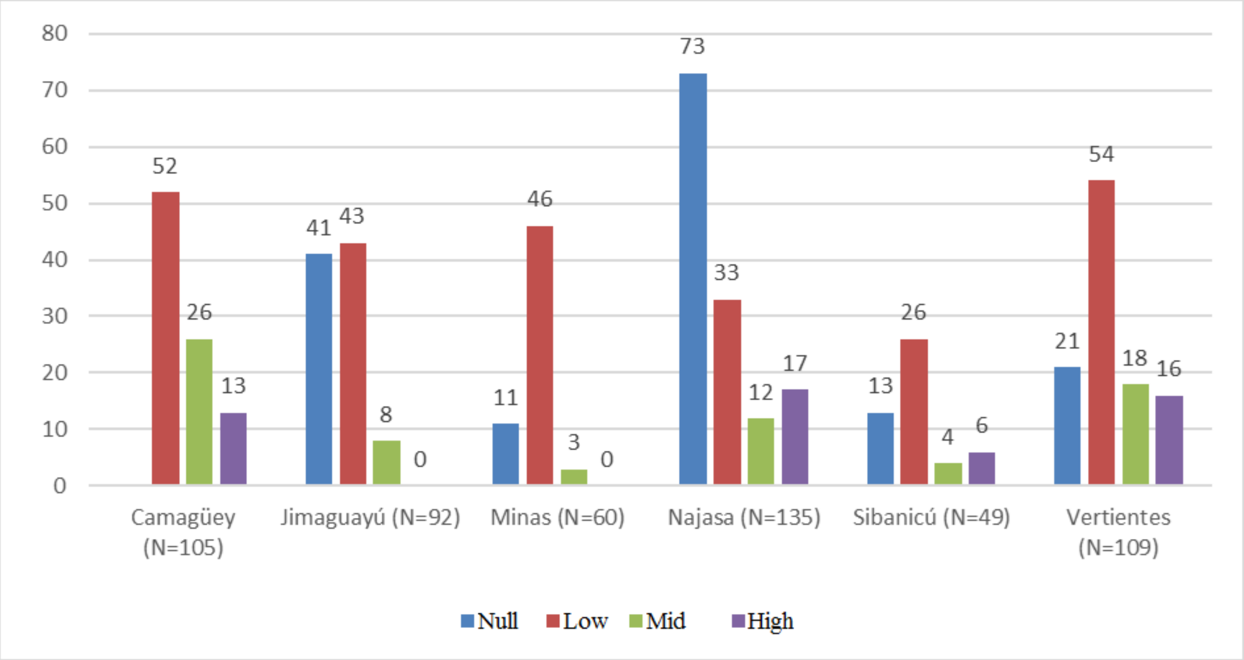


Figure 1. Results of the Contingency Table of infestation intensity, by municipality

Note: the values presented correspond to the frequency observed in each municipality, according to the infestation intensity.

The proportion comparison analysis evidenced significant differences of the mean in the municipality of Najasa, in relation to the other municipalities (Figure 2). The proportion observed in the other municipalities studied, regarding frequency, was within the set limits.

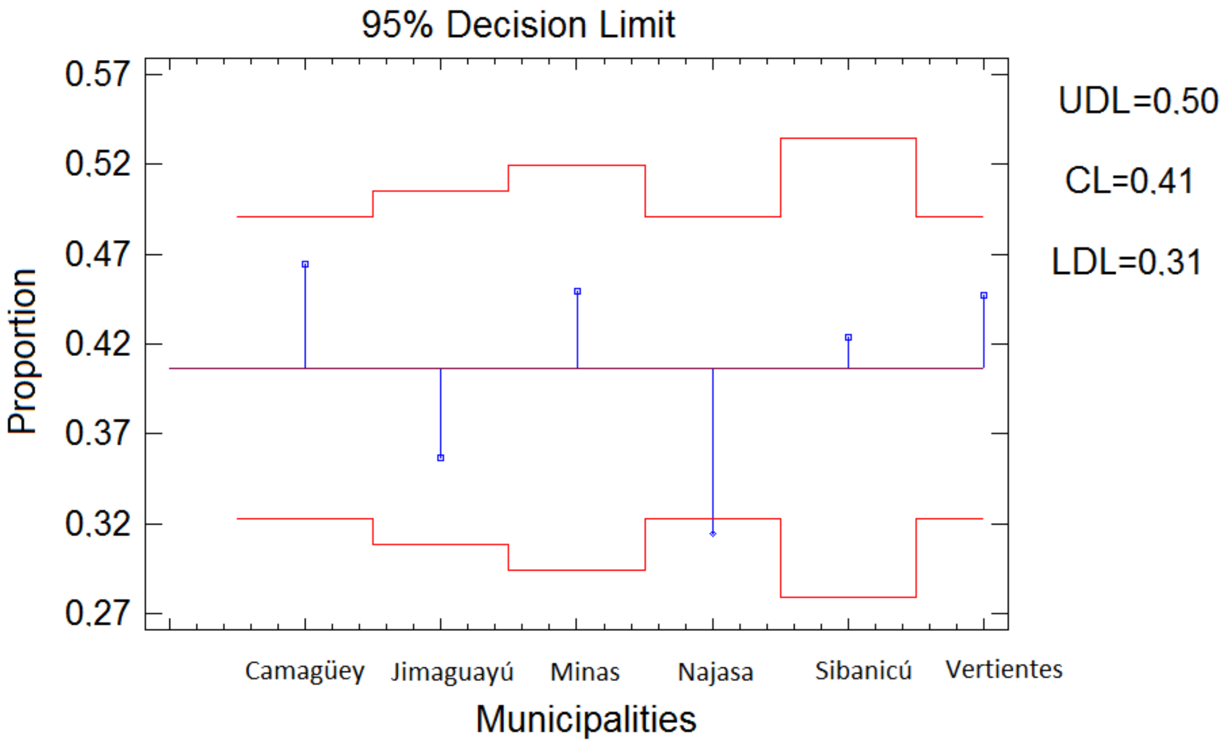


Figure 2. Comparison of proportions of prevalence in the municipalities in relation to the mean UDL: Upper Decision Limit CL: Central Line LDL: Lower Decision Limit.

Following analysis of proportions (95% confidence) between infestation intensity and the infested organs, the rumen-reticulum showed significant differences compared to rumen and reticulum (Figure 3).

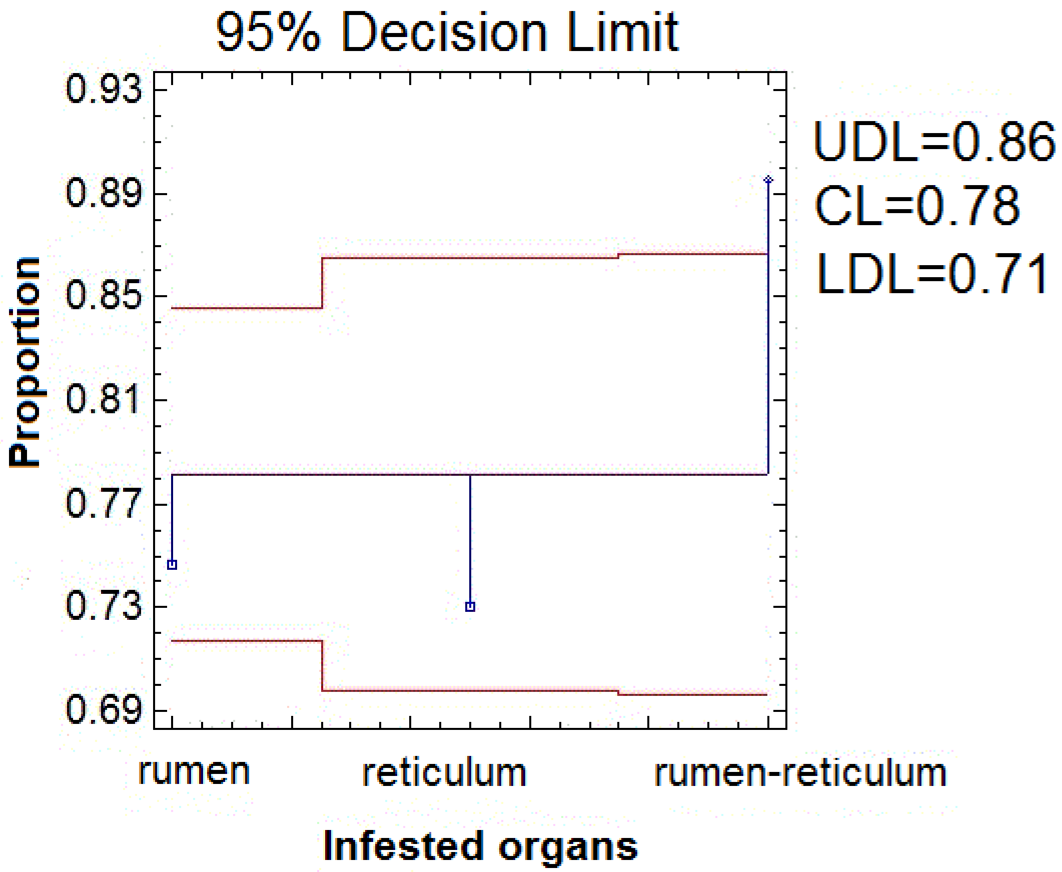
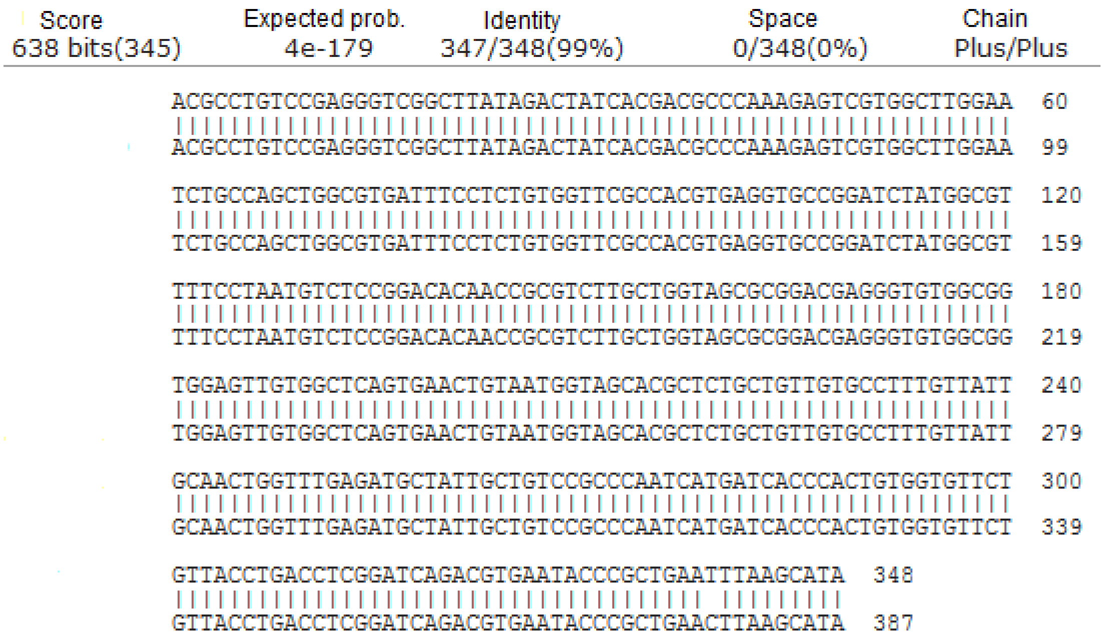


Figure 3. A mean proportion comparison of infestation intensity, according to the infested organs. UDL: Upper Decision Limit CL: Central Limit LDL: Lower Decision Limit.

A number of 348 nitrogenated bases from the ribosomal 18S intergene region were sequenced The individuals found in the province of Camagüey were 99.7% identical to *Cotylophoron cotylophorum*.

The best sequence alignment, according to BLAST, is shown in Figure 4. The only change observed was a transition of thiamine to cytosine. No space was observed in the sequence alignment (E 3e-175). Other species were 93-99% identical, with less sequence coverage.



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 Score             Expected prob.    Identity         Space         Chain
638 bits(345)        4e-179        347/348(99%)     0/348(0%)     Plus/Plus

ACGCCTGTCCGAGGGTCGGCTTATAGACTATCACGACGCCCAAAGAGTCGTGGCTTGGAA  60
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
ACGCCTGTCCGAGGGTCGGCTTATAGACTATCACGACGCCCAAAGAGTCGTGGCTTGGAA  99

TCTGCCAGCTGGCGTGATTTCCTCTGTGGTTCGCCACGTGAGGTGCCGGATCTATGGCGT  120
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
TCTGCCAGCTGGCGTGATTTCCTCTGTGGTTCGCCACGTGAGGTGCCGGATCTATGGCGT  159

TTTCCTAATGTCTCCGGACACAACCGCGTCTTGCTGGTAGCGCGGACGAGGGTGTGGCGG  180
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
TTTCCTAATGTCTCCGGACACAACCGCGTCTTGCTGGTAGCGCGGACGAGGGTGTGGCGG  219

TGGAGTTGTGGCTCAGTGAACTGTAATGGTAGCACGCTCTGCTGTTGTGCCTTTGTTATT  240
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
TGGAGTTGTGGCTCAGTGAACTGTAATGGTAGCACGCTCTGCTGTTGTGCCTTTGTTATT  279

GCAACTGGTTTGAGATGCTATTGCTGTCCGCCCAATCATGATCACCCACTGTGGTGTTCT  300
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
GCAACTGGTTTGAGATGCTATTGCTGTCCGCCCAATCATGATCACCCACTGTGGTGTTCT  339

GTTACCTGACCTCGGATCAGACGTGAATACCCGCTGAATTTAAGCATA  348
|||||||||||||||||||||||||||||||||||||| |||||||||
GTTACCTGACCTCGGATCAGACGTGAATACCCGCTGAACTTAAGCATA  387
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Figure 4. Best sequence alignment according to BLAST

The phylogenetic analysis indicated that the species found in the sample analysis was 85% likely to belong to the same clade of *Cotylophoron cotylophorum* (Figure 5). However, *Bacillus licheniformis* has no possibility of belonging to this group, which confirms that this analysis is correct.

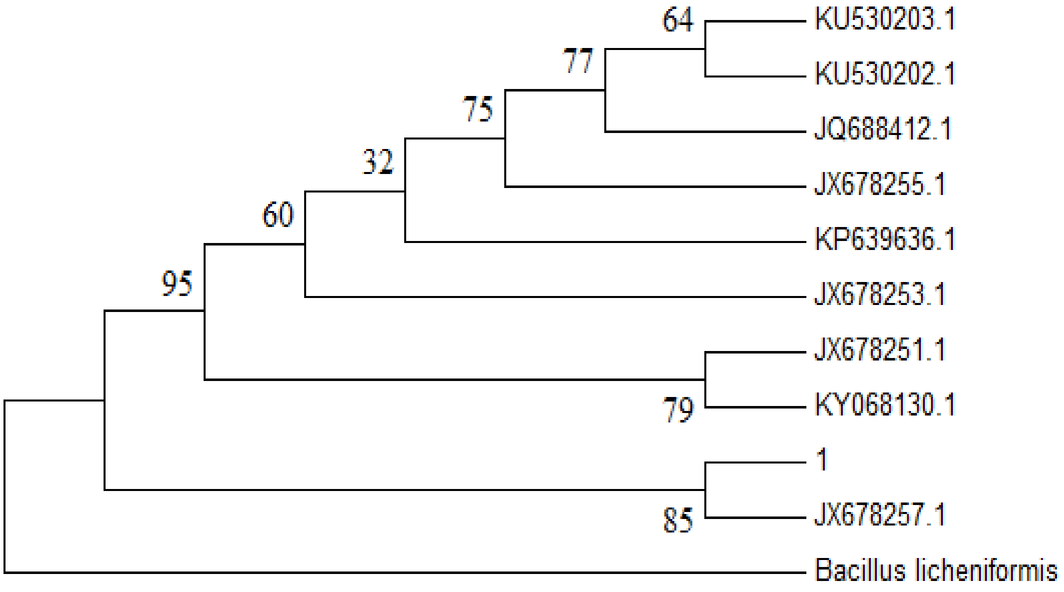

Figure 5. Phylogenetic analysis according to Neighbor-Joining (1000 bootstrap replications). Sequence references shown by species (10), and their respective GenBank access numbers *Cotylophoron cotylophorum*

JX678257.1, *Calicophoron shillongensis,* JX678251.1, *Orthocoelium* JX678255.1, *Fischoederius elongatus* KU530203.1, *Fischoederius cobboldi* KU530202.1, *Orthocoelium orthocoelium* JX678255.1, *Olveria bosi* JX678253.1, *Gastrothylax crumenifer* JQ688412.1, *Carmyerius dollfusi* KP639636.1, *Explanatum explanatum* KY068130.1. The KT924442.1 sequence from *Bacillus licheniformis* was used as external group.

DISCUSSION

An analysis of the prevalence observed showed that the values recorded were similar (68.54%) to the values reported by Cauquil *et al.* (2016) in the French Melanesian Archipelago, New Caledonia. They were able to determine 70% prevalence of paramphistomosis in adult cows. The authors noted that those levels might be produced because this parasitosis is not considered a clinical or production problem in New Caledonia, and treatment directed to this parasite is improbable, which corresponds to the Cuban scenario. This high level of prevalence is also influenced by the characteristics of the ecosystem in Camagüey. It consists of flat low fields, which are typically found in flooded savannas during the rainy season, thus contributing to greater dispersion of the intermediate host.

The prevalence in Italy, reported by Sanna *et al.* (2016), was 55.9% in adult cows, which can be considered high, since over 50% of the animals are affected with paramphistomosis. Perhaps, this was caused by the presence of intermediate hosts in the area, and the existence of water reservoirs, lakes, and rivers in the area. The same situation also takes place in Ethiopia, where prevalences of 51.82% were determined (Ayalew, Tilahun, Aylate, Teshale, and Getachew, 2016). Probably, this happens as a result of favorable environmental factors, which are similar to the factors reported in Cuba that contribute to mollusk reproduction and development in the area.

The difference between the proportions of prevalence in the municipalities may have been conditioned by the characteristics of ecosystems, the precipitations, and temperature of each region. Additionally, it may even be associated to productive forms of cattle raising in areas requiring the construction of dams to ensure stable water supply to animals. All the above can be translated into proper growing and developing conditions for intermediate hosts.

The animals inspected showed higher infestation in the rumen-reticulum than single-organ infestation (rumen or reticulum). This proves that there is no topic specificity of flukes over the rumen or the reticulum, though from an anatomical standpoint, the reticulum does not have the same conditions as the rumen for establishment.

In Galicia, Spain, a proportion of 94.3% was observed in the rumen, and only 5.7% in the reticulum (González-Warleta *et al.*, 2013). The authors inferred that a lesser burden in the latter may be related to the absence of papillae, the accumulation of CO_2, and the existing methane. Moreover, the form of papillae in the ruminal atrium may have a protective effect on trematodes, and the presence of a fluid layer may favor the ingestion of nutrients by the parasite.

The molecular results observed confirmed the results of Kotrla and Prokopic (1973), who, according to its morphology, determined the circulation of *Cotylophoron cotylophorum* in Cuba. Likewise, they proved that it was the most abundant of the species found in Camagüey. However, during this research, no other species previously detected by those authors, were found. Hence, a specific molecular study of these varieties may be required.

The confirmation of the presence of the species is in keeping with one of the genuses (*Cotylophoron*) determined by Alarcón and Velásquez (2009) in tropical and subtropical regions with the highest impact on cattle production in the Americas. These researchers demonstrated that *C. cotylophorum* is one of the species circulating in Colombia. Furthermore, Morales, Pino, and Moreno (2015) reported its presence in the Venezuelan states of Aragua, Zulia, and Bolivar. In southeast Iran, it was the second species with the highest prevalence (20%) detected in cattle slaughterhouses (Khedri *et al.*, 2015).

CONCLUSIONS

Paramphistomosis was detected in slaughter cattle in three slaughterhouses in Camagüey; differences were observed among the municipalities.

The low infestation intensity was demonstrated in the slaughter animals, with no signs of specificity for a particular organ.

Species *Paramphistomum*, which circulates among slaughter cattle, belongs to the same clade as *Cotylophoron cotylophorum*.

The high prevalence of this species evidences that paramphistomosis is a neglected disease in Cuban cattle populations.

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AUTHOR CONTRIBUTION

Conception and design of research: YLC, AAC, YTE, RVM, ECS, NAB; data analysis and interpretation: YLC, AAC, YTE, RVM; redaction of the manuscript: YLC, YTE.

CONFLICT OF INTERESTS

The authors declare no conflict of interests.