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Molecular characterization of *Eurytrema coelomaticum* in cattle from Paraná, Brazil

Caracterização molecular de *Eurytrema coelomaticum* em bovinos do estado do Paraná, Brasil

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Abstract

This study investigated the occurrence of *Eurytrema* spp. in cattle by analysis of the partial 18S rRNA gene sequence. Trematodes from 44 bovine pancreas were collected and classified based on typical morphological features. PCR assay and sequence analyses of amplified products confirmed that the trematodes classified as *Eurytrema coelomaticum* were phylogenetically distinct from those identified as *E. pancreaticum*. The results of this study represent the first molecular characterization of *E. coelomaticum* within the Americas, and provide an efficient method to differentiate digenetic trematodes of domestic animals.

Keywords: Bovine trematode, *Eurytrema* spp., PCR, phylogeny.

Resumo

Este estudo avaliou a ocorrência de *Eurytrema* spp. em bovinos por meio da análise da sequência parcial do gene 18S rRNA. Trematódeos procedentes de 44 pâncreas bovinos foram coletados e classificados com base em aspectos morfológicos típicos. A técnica de PCR e a análise da sequência de nucleotídeos dos produtos amplificados confirmaram que os trematódeos classificados como *Eurytrema coelomaticum* eram filogeneticamente distintos daqueles identificados como *E. pancreaticum*. Os resultados deste estudo representam a primeira caracterização molecular de *E. coelomaticum* nas Américas e disponibiliza um método eficiente para diferenciar trematódeos digenéticos em animais domésticos.

Palavras-chave: Trematoda bovino, *Eurytrema* spp., PCR, filogenia.

Introduction

Eurytrema spp. is a fluke that parasitizes the pancreatic ducts and rarely the bile ducts of cattle, buffaloes, camels, sheep, and goats (BASCH, 1966; SOULSBY, 1982; ISHII et al., 1983; MATTOS JUNIOR; VIANNA, 1987). Parasitism frequently results in epithelial hyperplasia, hypertrophy of pancreatic ducts, and periductal fibrosis that lead to eurytrematosis (TANG, 1950; MATTOS JUNIOR; VIANNA, 1987; HEADLEY et al., 2009). Although bovine eurytrematosis is more frequently associated with *Eurytrema coelomaticum* in Brazil (TRAVASSOS et al.,

1969), *E. pancreaticum* has been described in studies that have not examined the morphological characteristics of the trematode (CAMPOS et al., 1974; BUSETTI et al., 1983).

Most investigations relative to *Eurytrema* spp. in Brazil have been based on morphological characterization (TRAVASSOS et al., 1969; YAMAMURA et al., 1995; PINHEIRO et al., 2012), biological cycle (BRANDOLINI; AMATO, 2001), epidemiological trends (AZEVEDO et al., 2004; BASSANI et al., 2006), and pathological manifestations (YAMAMURA et al., 1995; HEADLEY, 2000; HEADLEY et al., 2009). Additionally, there are also descriptions of *Eurytrema* spp. associated with chronic wasting disease (RACHID et al., 2011) and simultaneous intoxication due to chronic seneciosis (HEADLEY et al., 2004) in cattle. However, there are no studies relative to the molecular characterization of *Eurytrema* spp. from Brazil.

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Recent studies have demonstrated that molecular analysis is an important tool for the identification (OTRANTO et al., 2007) and phylogenetic analyses (CAI et al., 2012) of trematodes. Moreover, partial fragments of 18S rRNA gene were successfully used to design phylogenetic trees and study the different orders of digenean trematodes (FERNANDEZ et al., 1998; ZHENG et al., 2007). This study determined the species of *Eurytrema* present in cattle from northern Paraná, Brazil by the molecular characterization of the partial fragment of the 18S rRNA gene of the digenean trematode.

Materials and Methods

Study location, trematodes sampling, collection, and morphological identification

All trematodes were collected from individual cows submitted for slaughter at a municipal abattoir located in northern Paraná, southern Brazil. All cattle were of different breeds, sex, and ages, and originated from different geographical regions of the state of Paraná, including Campo do Tenente (25° 58' 41" S; 49° 40' 58" W), Borrazópolis (23° 56' 28" S; 51° 35' 15" W), and Jaboti (23° 44' 36" S; 50° 04' 33" W), and Jandaia do Sul (23° 36' 11" S; 51° 38' 36" W).

All pancreas were evaluated for the presence of trematodes, after which pools of two trematodes were used for DNA extraction and subsequent PCR analysis. The number of parasites used for molecular investigation was determined by the sample weight required for DNA extraction. From each pool of parasites, an equal number of trematodes were prepared for identification based on typical morphological characteristics (TRAVASSOS et al., 1969; YAMAMURA et al., 1995).

DNA extraction, PCR, sequencing, and phylogenetic analyses

Trematode DNA was extracted by using the Easy-DNA Kit-Life (Invitrogen Corp. Carlsbad, CA, USA), and then used in a PCR assay designed to amplify the partial 18S rRNA gene of *Eurytrema* spp. (ZHENG et al., 2007); positive controls were included in all PCR assays. Nuclease free water (Invitrogen Corp. Carlsbad, CA, USA) was used as the negative control. The PCR assay was performed as described (ZHENG et al., 2007), with slight adaptation. Briefly, the PCR protocol consisted of an initial denaturing cycle of 94°C for 5 min, 35 cycles of 45 s at 94°C, 45 s at 55°C, 2 min at 72°C followed by 72°C for 10 min. All PCR products were separated by electrophoresis in 2% agarose gels, stained with ethidium bromide, and examined under ultraviolet light.

The amplified PCR products were then purified (illustra GFX PCR DNA and Gel Band Purification Kit, GE Healthcare, Little Chalfont, Buckinghamshire, UK) and submitted for direct sequencing using the forward and reverse primers. The obtained sequences were examined for quality analysis of chromatogram readings by using the PHRED software (<http://asparagin.cenargen.embra.br/phph>); sequences were only accepted if base quality was equal to or greater than 20. Consensus sequences were then generated by the CAP3 program (<http://asparagin.cenargen.embra.br/cgi-bin/phph/cap3.pl>). The partial nucleotide sequences obtained were initially compared by the BLAST (<http://www.ncbi.nlm.nih.gov/BLAST>) program with similar sequences deposited in GenBank. Phylogenetic tree and sequence alignments based on the 18S rRNA gene of the digenean trematodes family were then created by using MEGA 5.2 (TAMURA et al., 2011), constructed by the neighbor-joining method, based on 1,000 bootstrapped data sets; distance values were calculated by using the Kimura 2 parameter model. *Fasciola gigantica* was used as the out-group to provide stability to the generated tree. The sequence identity was generated by using the software BioEdit (HALL, 1999).

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Results

Trematodes and morphological classification

The pancreas of 44 cows from the cities of Campo do Tenente ($n=4$), Jandaia do Sul ($n=7$), Borrazópolis ($n=15$), and Jaboti ($n=18$) contained trematodes, resulting in 13 samples that were used for molecular analyses. All trematodes collected were classified as *E. coelomaticum* based on typical morphological features (TRAVASSOS et al., 1969; YAMAMURA et al., 1995).

Molecular characterization, phylogenetic analysis, and sequence identities

The PCR assays amplified the 528 bp fragment of the 18S rRNA gene of *E. coelomaticum* from all pools of trematodes; three of the 13 samples were sequenced and used in phylogenetic evaluation. Initial BLAST analyses revealed that these sequences (GenBank accession number KJ010808, KJ010809, and KJ010810) demonstrated 100% similarity with sequences deposited in GenBank. The phylogenetic analyses revealed that the isolates from this study formed a cluster that contained *E. coelomaticum*, *E. pancreaticum* (DQ401034), and *Lyperosomum collurionis* (AY222143). However, these isolates were more closely related to *E. coelomaticum* (DQ401035) and *E. pancreaticum* (DQ401034). The nucleotide sequences used for phylogenetic analyses during this study are given in Figure 1. Additionally, the generated matrix identity (data not show) revealed that the isolates from this study demonstrated 99.8% similarity with *E. coelomaticum* but 99.4% similarity with *E. pancreaticum*.

Discussion

This study confirmed at the molecular level that all trematodes previously identified by morphological characteristics are *E. coelomaticum*; similar studies have been done in other geographical locations (ZHENG et al., 2007). Additionally, these findings represent the first characterization of *Eurytrema* spp. in the Americas by molecular techniques. The methods used during this investigation are more efficient to characterize trematodes, can

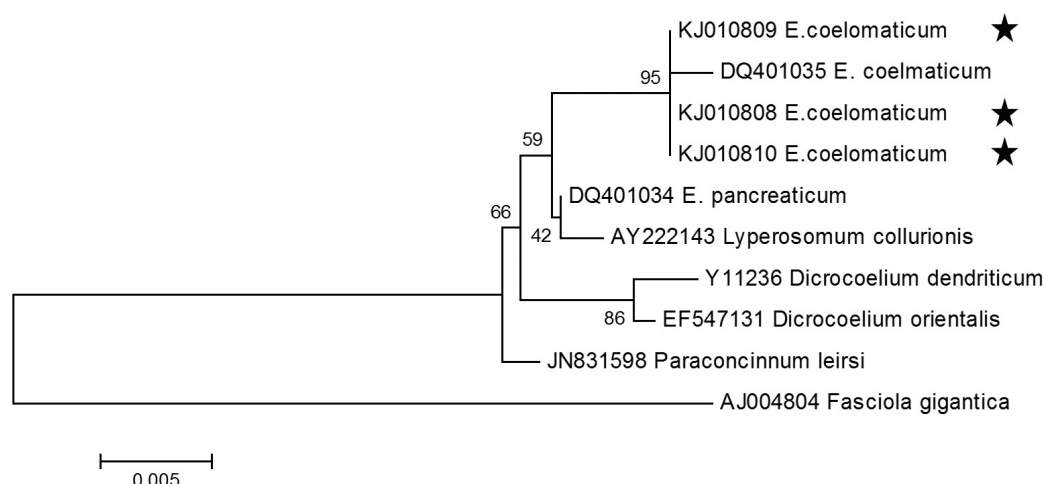


Figure 1. Phylogenetic tree based on the 18S rRNA gene sequences of trematodes generated by MEGA 5.2. *Fasciola gigantica* was used as the out-group. The sequences derived from this study are highlighted (star).

demonstrate genetic variation between different species originated from different geographical regions, and reduce errors that could occur when morphological characteristics are being identified.

When the data identify matrix derived from this study was analyzed, it was shown that the sequences derived from this study were 99.8% and 99.4% similar to those of *E. coelomaticum* and *E. pancreaticum*, respectively, observed in China (ZHENG et al., 2007). Moreover, these sequences clustered with other closely related members of the Dicrocoeliidae family, but were distant from *Dicrocoelium dendriticum* and *D. orientalis*, while these trematodes formed a cluster that was phylogenetically distant from *F. gigantica*; similar results were described (ZHENG et al., 2007). Unfortunately, the sequences of a recent phylogenetic investigation that analyzed the differences of *Eurytrema* spp. (CAI et al., 2012) by the 18S rRNA gene were not located in GenBank, and consequently not included in this study.

In addition, the results of the polygenetic analysis suggest that the trematode *E. coelomaticum* from distinct geographical regions might be closely related, considering that the isolates from this study and that from China (ZHENG et al., 2007) clustered together. Alternatively, these results suggest that there is phylogenetic difference between *E. coelomaticum* and *E. pancreaticum*, since these trematodes were grouped in different branches, indicating that these are two different parasites; similar results were described when trematodes from different host were analyzed phylogenetically (CAI et al., 2012). However, there seems to be phylogenetic differences between the same species of *Eurytrema* spp. from the different hosts and geographical locations (CAI et al., 2012).

Since bovine eurytrematosis is endemic in the state of Paraná (AZEVEDO et al., 2004; BASSANI et al., 2006), and has been described in the states of Minas Gerais (RACHID et al., 2011), Rio de Janeiro (MATTOS JUNIOR; VIANNA, 1987), Mato Grosso do Sul (YAMAMURA et al., 1995), efforts are being made to obtain examples of pancreatic trematodes of ruminants from these regions to effectively characterize the species of *Eurytrema* existent in Brazil.

Conclusion

The results from this study confirmed that trematodes of cattle from southern Brazil were morphologically and phylogenetically consistent with *E. coelomaticum*.

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