MOLECULAR IDENTIFICATION OF DIFFERENT RUMEN FLUKE SPECIES

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Abstract

Rumen flukes are widely distributed digenetic trematodes belong to the superfamily Paramphistomoidea which hit domestic and wild ruminants and cause a parasitic disease known as paramphistomiasis or paramphistomosis. The economic importance of paramphistomes is globally underestimated because of their proliferation and abundance among ruminants. Substitutional approaches to improving species identification are required. The paramphistomes scrutinised during this present study were adult flukes. Our five samples include four samples of paramphistomes from cattle and only one from sheep were identified according to the ITS-2 sequencing as followers of the genera *Paramphistomum*, *Calicophoron*, and *Gastrothylax*. The DNA of each worm was isolated, and the ITS-2 rDNA was amplified, purified, and sequenced. Then, the sequences were uploaded to GenBank, which allocated them the following accession numbers: OK216189, OK216190, OK216191, OK216192, and OK216193. MEGA X has been used to execute the phylogenetic analysis on these sequences. This result reveals that ITS-2 is an excellent molecular marker, and can be beneficially used for paramphistomes identification to determine the affinity of samples among the different genera of the superfamily Paramphistomoidea. The current investigation will help build a scope to make the identification of paramphistomes very straightforward and, eventually, the command of this underappreciated cosmopolitan digenean group.

Keywords: Paramphistomes, Rumen fluke, Molecular identification, PCR, ITS-2 sequencing.

1. Introduction

Rumen flukes, also called paramphistomes, are parasitic trematodes that infect the digestive tracts of ruminants, for instance, cattle, sheep, buffaloes, and goats. Around 70 fluke species are classified as paramphistomes, which belong to the superfamily Paramphistomoidea (*Firdausy et al., 2019*). Paramphistomes are found all over the world, with a variety of unique species in various locations and host species. Different rumen fluke species were recognized in some countries, and sometimes paramphistomes were found as co-infections in the same host rumen. Some species are reported in distinct host species in geographically distant regions (*Zintl et al., 2014*).

Many serious economic losses result from the rumen fluke infection in regard to treatment costs, diminished production, management improvement, and may lead to mortality in severe cases (*Matebesi-Ranthimo et al., 2014*).
Paramphistomiasis disease causes severe symptoms during the migration of immature worms and the consumption of intestinal epithelial cells. As a result of this ailment, musty diarrhea, anaemia, anorexia, wide edema, electrolyte, and protein loss were reported. Paramphistomiasis is frequently associated with bodily state deterioration, coarse hair, lethargy, disability, loss of appetite, intestinal ulcers, and intermandibular edema in ruminants (Sanabria and Romero, 2008).

On the other hand, infection with the adult rumen fluke results in a mild disease that includes ulcerative rumenitis, leanness of ruminal papillae, and emaciation. In ruminants, mortality rates from juvenile flukes may range from 80 to 90 percent, which may be related to the variety of paramphistome species or the host's nutritional status, health, immunity, and genetic designation (Hotessa and Kanko, 2020). The elevated prevalence of paramphistomes in some countries makes it necessary to investigate the genetic diversity of different species. The realistic morphological identification of rumen flukes is relatively challenging because it necessitates median transverse sections through thick, enormous bodies in order to visualize the internal features (Lotfy et al., 2010).

However, because paramphistomes have morphological similarities, reliable identification requires molecular methods. In order to address the issue of defining novel species or strains based on phenotypic traits, several genetic tools are being combined with traditional diagnostic procedures (Rinaldi et al., 2005). Regardless of the difficulties, morphological characterization of paramphistomes is still important in taxonomic research. Morphological identification approaches can be used in conjunction with molecular studies to aid in the identification of paramphistomes.

Histology, flattening, and electron microscopy have been utilized in some investigations, while histological and molecular characterization have been used in others. A thorough histological study is necessary for molecular characterization of paramphistomes. In the industrialized world, the use of molecular techniques for paramphistome identification is becoming more common, but it is still limited in Africa (Radwan et al., 2014; Ichikawa et al., 2013; Panyarachun et al., 2013).

This present study was investigated to provide a molecular phylogenetic framework for paramphistomes within which their biodiversity can be evaluated. Furthermore, by combining location and host data concurrently, a database may be built that can subsequently be utilized to analyze the rumen fluke host-parasite relationships and biodiversity. These investigations will also result in improved methods for identifying adult paramphistomes and a much more detailed grasp of their evolutionary associations. The above-mentioned framework's ultimate goal is to provide baseline information that will aid in the control of such parasites in both animals and humans.
2. Materials and Methods

2.1. Collection of Specimens

In this study, the rumens of slaughtered sheep and cattle were examined by regular visits to the different slaughterhouses in the subtropical regions between September 2020 and August 2021. A total of 370 sheep and 410 cattle were examined after slaughtering. About 10-15 adult worms were gathered from the rumen of each naturally infested ruminant and separately placed in clean containers.

The collected flukes were washed several times in tap water to remove the debris and ruminal content, then washed with PBS (phosphate-buffer saline) and placed in 70% ethanol. Five adult flukes (one sample from sheep and four samples from cattle) were kept at -20 °C until being processed in the biotechnology unit of the Animal Health Research Institute (AHRI), Egypt.

2.2. Morphological Identification

One fluke from each container was removed from ethanol to make a section in it by hand that was then stained for morphological identification based on the technique established by Eduardo (1982). All specimens included in this molecular study were initially examined and identified as paramphistomes with respect to the morphological features.

2.3. Extracting DNA

Paramphistomes were washed five times with PBS for genomic DNA isolation. The nucleic acids from the tested paramphistomes were purified by the QIAamp DNA Mini Kit using silica membranes. The spin-column procedure did not require mechanical homogenization, so it took roughly 20 minutes to complete the preparation.

The internal transcribed spacer regions 2 (ITS-2) of the specimens were amplified and sequenced. The ITS-2 regions were amplified using a specific primer, ITS-2 F 5’-GGTACCGGTTGATCAGCTCGTG-3’ and R 5’-GGGATCCTGTTAGTTTCTTTTCCTCCGC-3’ (Arya et al., 2016). The polymerase chain reaction (PCR) cycles were performed by the usage of the Eppendorf Mastercycler epigradiant machines. The thermocycler was set to vary at a rate of 1 C/s, with one cycle of 95 °C for 1 minute, 55 °C for 2 minutes, and 74 °C for 1 minute 30 seconds, followed by 30 cycles of 95 °C for 30 seconds, 55 °C for 30 seconds, and 74 °C for 1 minute 30 seconds, plus a final extension step of 7 minutes. Using an Applied Biosystems 3100 automated sequencer, purified PCR products were sequenced on both strands using PCR Microcon columns. The samples were photographed under UV light (Figure 1).

2.4. Sequence alignment and phylogenetic analysis

ITS-2 sequences have been aligned by MUSLE using the Alignment Explorer in MEGA X (Kumar et al., 2018). This study's sequences were submitted to GenBank (Tab. 1) and then compared to the previously registered sequences. For the outgroup taxa, we used the ITS-2 sequences of Cryptocotyle lingua. In the same manner, phylogenetic analyses were done using MEGA X.
3. Results

Out of 370 sheep and 410 cattle, which were examined in abattoirs, only 34 sheep (9.2%) and 128 cattle (31.2%) were infected with paramphistomes. Five randomly selected species (one from sheep and four from cattle) were morphologically examined by light microscopy to be accurately confirmed as paramphistomes.

The PCR products ran on electrophoresis produced a product size of 539 bp (Fig. 1). By sequence analysis of amplicons acquired from adult paramphistomes, *Paramphistomum cervi* was identified from one sheep and two cattle, *Calicophoron microbothrium* and *Gastrothylax crumenifer* was identified from one cattle. All of the samples' ITS-2 sequences were successfully improved, and the sequences generated were submitted to GenBank. Accession numbers of GenBank registered species are highlighted in figure 2.

Because all of the samples analysed belonged to the Paramphistomoidea, the current phylogenetic study comprised samples from two families in NCBI-GenBank: Paramphistomatidae and Gastrothylacidae. Moreover, BLAST showed the ITS-2 regions of *P. cervi* to have 100% homology with the *P. cervi* isolated from sheep in China (KJ459936 and KJ459935) and 97.1% with *P. epiclitum* isolated from *Bos grunniens* in India (KX840345). It was observed, in terms of nucleotide sequences, the *P. cervi* isolates from cattle and sheep were found to be 100% identical. In the comparative nucleotide sequences of the ITS-2 gene region of its isolates, no genetic differences were observed between *P. cervi* isolates from cattle and sheep (Tab. 2).

In this present study, the ITS-2 rDNA sequence of *C. microbothrium*, isolated from cattle, showed 96.9-100% identity with *C. microbothrium* isolated from cattle in southern Africa (KP639632, KP639634, KP639638 and KP639631). Moreover, *G. crumenifer* showed 97.1-100% identity with that isolated from cattle in India (KU530204, MN371811, HM159382, JQ688412, and JX518955).

However, the sequence divergence values in table 2 (given as a percentage of the distance estimation) revealed that the interspecific variances in ITS-2 among members of the Paramphistomatidae family ranged from 0.2 to 3%. As a result, the ITS-2 sequence is a useful identifier for species-level taxonomic research of the superfamily Paramphistomoidea.

**Tab. (1):** Samples of paramphistomes included in the present study.

<table>
<thead>
<tr>
<th>SAMPLE CODE</th>
<th>MOLECULAR IDENTIFICATION</th>
<th>HOST</th>
<th>ACCESSION NUMBER</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td><em>Paramphistomum cervi</em></td>
<td>Sheep</td>
<td>OK216189</td>
</tr>
<tr>
<td>S2</td>
<td><em>Paramphistomum cervi</em></td>
<td>Cattle</td>
<td>OK216190</td>
</tr>
<tr>
<td>S3</td>
<td><em>Calicophoron microbothrium</em></td>
<td>Cattle</td>
<td>OK216192</td>
</tr>
<tr>
<td>S4</td>
<td><em>Paramphistomum cervi</em></td>
<td>Cattle</td>
<td>OK216191</td>
</tr>
<tr>
<td>S5</td>
<td><em>Gastrothylax crumenifer</em></td>
<td>Cattle</td>
<td>OK216193</td>
</tr>
</tbody>
</table>
Tab. (2): Pairwise estimates of distances, expressed as percentage evolutionary divergence values, between ITS-2 sequences of different paramphistomes species.

Fig. (1): PCR amplification of rDNA ITS-2 gene region in paramphistomes species found in sheep and cattle (Amplicon length 539 bp). S1: PCR amplicons of adult parasites from sheep. S (2-3-4-5): PCR amplicons of adult parasites from cattle.
Fig. (2): Analysis of ITS-2 rDNA sequences of paramphistomes using the maximum likelihood composite method, supporting bootstrap values from 1000 replicates. Using *Cryptocotyle lingua* (Accession number: MW544138) as outgroup. GenBank accession numbers are followed by the species name and values that were indicated at each node.

4. Discussion

Although paramphistomiasis is a widely common disease that is influenced by a multifactorial approach that combines parasitic agents, hosts, transmission processes, and environmental factors (*Javed et al., 2006*), its importance is underestimated globally as it is a serious hurdle for resource-poor farmers in tropical areas of the world raising viable livestock, with a high frequency among ruminants (*Sanabria and Romero, 2008*). The rumen fluke species may also impact the pathology and severity of paramphistomiasis (*Zintl et al., 2014*). Thus, the accurate identification of paramphistome species is totally necessary for the understanding of disease epidemiology and how to control it.

Rumen flukes were collected from the rumen of naturally infected sheep and cattle for the current study. Based on morphological traits described by *Yamaguti (1952)*, *Foreyt (2001)*, and
Eduardo (1937), the collected worms were found to belong to the superfamily Paramphistomidea. Due to the uncertain morphological boundaries of distinct genera and species of paramphistomes (Huson et al., 2017; Khan et al., 2019), genetic approaches were required for precise identification.

Despite the fact that histological examination appears to be more accurate than morphological identification (Chaouhary et al., 2015), both are flawed because variability within species can be observed due to a variety of factors such as host species, rumen fluke age, fixation condition, and the section plane used in slide preparation. Furthermore, because the immature flukes that cause the sickness are difficult to detect, exact identification is even more challenging (Ichikawa et al., 2013). To address these challenges, a variety of molecular biology approaches, particularly ITS2 sequencing, can be used to aid in species identification.

GenBank is a public data store with a history of data inaccuracies (Bouadjenek et al., 2017). As a result, creating a globally maintained consensus reference library to address this issue would be beneficial. The use of ITS2 sequencing for definite identification of paramphistomes has been demonstrated to be useful. As a consequence, the ITS2 primers used in this study to amplify a shorter area appear to identify a smaller part of the ITS2 sequence.

The most common species found in the current collection was Paramphistomum cervi. Three adult Paramphistomum, which were isolated from cattle (two flukes) and sheep (only one fluke), were identified morphologically as Paramphistomum cervi, also clustered together in the phylogenetic tree with identical ITS2 sequences. The present phylogenetic analysis of the ITS2 region of P. cervi, C. microbothrium, and G. crumenifer separately forms clades with flukes from other countries, which may possibly explain parasite transmission by infected hosts moving about (Mohapatra et al., 2013). These resemblances also suggest that they may have shared a common evolutionary history (Blair et al., 1996).

Many researchers from all over the world have reported ITS2 sequence variation across the same species from different hosts (Zheng et al., 2014; Ma et al., 2015; Cauquil et al., 2016). The present three ITS2 trimmed sequences of P. cervi showed 100% homology, i.e., there was no grade of variance that was consistent with the work of Rinaldi et al. (2005) and Zheng et al. (2014).

5. Conclusion and Recommendation

The current study's findings demonstrate that ITS region 2 is an excellent molecular marker for paramphistome identification and can be utilized to assess sample affiliation within Paramphistomoidea groups. Furthermore, the findings of this study may serve as a foundation for the development of a necessary framework to aid in the identification and, eventually, control of this underappreciated and taxonomically challenging digenean cosmopolitan group. The creation of a worldwide consensus reference database would be extremely useful in aiding accurate identification of rumen fluke species as well as research on their distribution, emergence, pathogenicity, and control. The generic ITS2 PCR is not recommended without sequencing as a diagnostic method for this specimen type.
6. Conflict of Interests
The authors are of the opinion that they have no competing interests.

7. References


