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RESEARCH ARTICLE

Molecular Characterization and Phylogenetic Analysis of *cox*1 and ITS 1 Gene Fragments of *Moniezia* Species Isolated from Sheep

Mohammad I Alberfkani[1], Amal JS Albarwary[2], Ghanem M Jaafar[1], Anias I Zubair[1] and Razan Y Abdullah[3]

[1]Department of Medical Laboratory Technology, College of Health and Medical Techniques, Duhok Polytechnic University, Duhok, Kurdistan Region, Iraq
[2]Department of Medical Laboratory Sciences, College of Health Sciences, University of Duhok, Kurdistan Region, Iraq
[3]Department of PCR, Central Public Health Laboratory, Duhok, Iraq
*Corresponding author: mohammad.said@dpu.edu.krd

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A B S T R A C T

Monieziasis caused by *Moniezia* species is considered the most common gastrointestinal disorder in sheep. The present study was performed to determine the prevalence of the tapeworms in the sheep's intestine in Duhok province, Kurdistan region, Iraq based on molecular techniques. A total of two hundred sheep were examined during the period from January to June 2022. Thirty-two (16%) sheep were harboring *Moniezia* species, while statistically local sheep were more susceptible to infection (32.3%) as compared to imported sheep (8.1%). Sampled tapeworms were analyzed morphologically and using conventional PCR for amplifying and sequencing of ITS 1 and *cox1* gene. The results of PCR confirmed the identity of tapeworms as *Moniezia* spp. with a product length of 743 base pairs for ITS 1 and 364 base pairs for *cox1* gene. The sequencing analysis using *cox1* gene revealed that; 25 *Moniezia expanse* and 7 *Avitellina centripunctata* while sequence analysis by using ITS 1 gene revealed 20 *Moniezia expanse* and 12 *Moniezia* spp. with 99.6-100% homology. This study confirms the prevalence of *Moniezia* spp. in Iraq by using the ITS 1 and *cox1* gene.

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INTRODUCTION

Monieziasis is a gastrointestinal disorder of domestic animals, especially sheep, caused by *Moniezia* species, which belong to the family Anoplocephalidae in the order Cyclophyllidea. Morphologically, the body of *Moniezia* differentiates into small unarmed scolex, neck and long chain strobila containing immature, mature and gravid proglottids (Diop *et al.,* 2015). Based on the morphological characteristics, twelve species of *Moniezia* have been identified, among those *Moniezia expansa* and *M. benedeni* are the most frequent tapeworm isolated from sheep and cattle, respectively (Diop *et al.,* 2015; Ohtori *et al*., 2015). The life cycle of *Moniezia* spp. is indirect in which sheep and cattle serve as the final host. The oribatids mites serve as intermediate hosts which live freely in soil and grass (Khadijah *et al*., 2014).

Domestic animals swallow the oribatids mites infected with the tetragonal and triangular eggs of *M. benedeni* and *M. expansa*, respectively. The eggs hatch and develop to infective larvae (cysticercoids) while remaining in the mite (Fox, 2018; Abdelhamid *et al.,* 2021). Domestic animals get infection by ingestion of the infected mites. Larvae of *Moniezia* move from the digestive tract to small intestine, where they attach to the walls with their suckers and mature into adult tapeworm. Although, the pathogenicity of monieziasis is low in adult livestock, but in sheep causes perforation, intestinal obstruction, perineal and hepatic abscess, appendicitis and cholecystitis (Al-Otaibi *et al*., 2021). Tapeworms may lead to poor body condition, weight loss, and reduces livestock production (Hilegiworgise *et al.,* 2019; Mafruchati, 2020). The infected animals with compromised immune systems are more likely to be vulnerable to other health issues, which leads to a higher mortality rate (Mehmood *et al*., 2013; Cedillo *et al*., 2015; Prchal *et al*., 2015).

In Arab countries such as Iraq, sheep is the most important livestock species and plays a role in supporting the country's food security. It was estimated that nearly eight million sheep are bred in Iraq which provides people with meat, milk and wool along with providing decent

jobs and income to rural communities (Jarjees and Al-Bakeri, 2012; Thweni and Yassen, 2015). In general, there are few reports about economic losses among animals infected with monieziasis. Nonetheless, as the population of livestock animals increased annually, the prevalence of infections should be estimated in order to determine factors affecting the economy. Hence, the object of the current research was to estimate the prevalence of *Moniezia* spp. in sheep in Kurdistan region, Iraq.

Several reports described the incidence of monieziasis in the domestic animals of Iraq (Fadl *et al*., 2011; Anisimova and Al-Fatlawi, 2012; Ali *et al.,* 2018, Ghanim *et al*., 2022). However, few of these studies include molecular analysis of the tapeworm by using *cox1* gene and ITS 1. Molecular technique such as PCR was applied for the differentiation of very close species of *Moniezia*. Hence, the current research study was conducted to estimate monieziasis among slaughtered sheep in Duhok city and confirmed the detection of *Moniezia* spp. by using PCR and molecular phylogenetic analysis.

MATERIALS AND METHODS

Samples collection: A total of two hundred whole small intestines were collected into 0.85% balanced saline solution from the *Ovis aries* sheep belonging to local sheep breed (Karadi) and imported sheep breed (Awassi) slaughtered at different abattoirs in Zakho city during the period from January to June 2022. A macroscopic examination of the animal intestine was carried out in the microbiology lab to check the presence of cestodes and small intestines of were also examined microscopically for the presence of cestodes. The adult tapeworms were collected by fine forceps and washed several times with 0.85% normal saline solution, and microscopically identified as *Moniezia* spp. depending on the description keys (Mellau *et al*., 2010). All identified tapeworms were stored in 70% ethanol and 4°C for DNA extraction.

DNA extraction and Primer used: The genomic DNA was extracted from homogenized tapeworm body by using Genomic DNA extraction Kit provided by AddBio (Korea) based on the guidelines. Nanodrop Instrument was used to estimate the concentration and purity of DNA. Conventional PCR was used for amplifying extracted DNA by targeting *cox1* gene and ITS 1 as DNA markers obtained from NCBI database. The mitochondrial gene (*cox1*) encoding cytochrome c oxidase subunit I (COI) was targeted for amplification by using two primers; Forward (*cox1*F): 5'TTTTTTGGGCATCCTGA GGTTAT'3 and Reverse (*cox1*R): 5'TAAAGAAAGAA CATAATGAAAATG'3) (Bowles *et al.,* 1992). The Internal Transcribed Spacer-1 (ITS 1) was amplified with a set of designed primers Forward (ITSF) 5'TGCTACC CGCATGATGTTGT'3 and Reverse (ITSR): 5'ACACAG TTGGCTGCACTCTT) (Wickström *et al*., 2005).

DNA amplification using Polymerase chain reaction: Single plex PCR was performed for amplification in a total volume of 40µl reaction tube containing a mixture of 20µl Crystal Hot Start DNA Master Mix (0.2 mM of dNTP, 1× Ex Taq Buffer and 2.0 mM of $MgCl_2$), 4 µl forward primer (10 pmol), 4µl reverse primer (10 pmol), 4µl of DNA samples as a template and 8 µl of nuclease free water for each of the primers. Thermocycler was used for amplification using the following setting for *cox1* gene: 94°C for 5 min followed by 30 cycles of 94°C for 1 min, 58°C for 1 min and 72°C for 1 min, and then final strand elongation at 72 °C was done for an additional 7 min. (Shalaby and Amer, 2012). While the PCR amplification for ITS 1 was as follows: 95°C for 5 min followed by 30 cycles of 95°C for 30 sec, 58°C for 30 sec and 72°C for 1 min, and then final strand elongation at 72°C was done for an additional 5 min. (Wickström *et al*., 2005). The PCR products were subjected to Agarose gel electrophoresis (1.5%) after staining with Red Safe Dye with green fluorescence (GeNet Bio, Korea). Electrophoresis running at 85 Volt for 45 min. DNA ladder with molecular weight (100-1000bps) was added for estimating the band size. Then ultra-violet transilluminator (Maestrogen, Taiwan) was used for visualization of amplified DNAs.

DNA sequencing: The PCR amplicons of ITS 1 (743 bp) and *cox1* gene (364 bp) were sequenced by using Sanger sequencing in both directions. Fifteen amplicons of *Moniezia* spp. were selected based on different sources (Local and imported) and were submitted to Macrogen Company (South Koera) for DNA sequencing. The obtained sequences were trimmed and clean-up by BioEdite application and analyzed using Basic Local Alignment Search Tool (BLAST) (http://www.ncbinlm.nih.gov/BLAST). The obtained nucleotide sequences were submitted to the GenBank to get the accession number.

For conducting phylogenic tree, the obtained specimens (accession number) and related sequences were analyzed and aligned using MEGA Software (version 10). The phylogenetic tree was built up using maximum Likelihood methods and aligned using Muscle method associated with nucleotide substitution models (JC: Jukes-Cantor) for ITS1 and (HKY: Hasegawa-Kishino-Yano) for cox1 gene. The bootstrap test percentages of 1000 replicates were used for conducting the phylogenic tree. Two cyclophyllidean cestodes, *Echinococcus canadensis* and *Anoplocephala magna* were used to join the tree as outgroup root for the phylogenetic tree of cox1 gene since the Basic Local Alignment Search Tool (BLAST) finds they were most similar to *Moniezia* spp.

RESULTS

Based on the morphological characteristics, all tapeworms collected belonged to the genus *Moniezia* species. These worms appeared as segmented long worm with length up to six meters. Each segment had single genital pore in the anterior third of lateral margin, clear cirrus sac, vitelline gland behind the ovary, several testes spread through the central proglottid and inter-proglottid-based glands on the middle of the segment. Out of 200 small intestines of sheep slaughtered at Zakho abattoir, only 32 (16%) infested intestine was diagnosed positive for *Moniezia species*. All infected sheep were male and no infection was detected among female with no significant differences between both sexes as shown in Table 1.

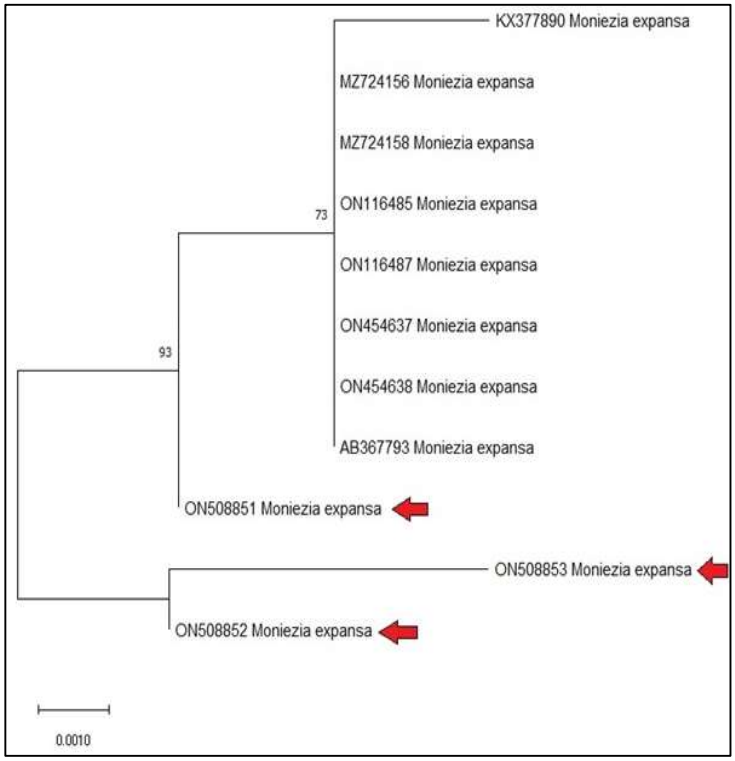

Fig. 1: Phylogenetic tree based on the ITS 1 gene partial sequencing of *Moniezia* spp. isolated from sheep (red arrows). Maximum Likehood tree was aligned by using Muscle method associated with nucleotide substitution models (JC: Jukes-Cantor) Tree was made by using MEGAX VERSION 10 with bootstrap value of 1000 replicates. Numbers on the nodes are bootstrap values.

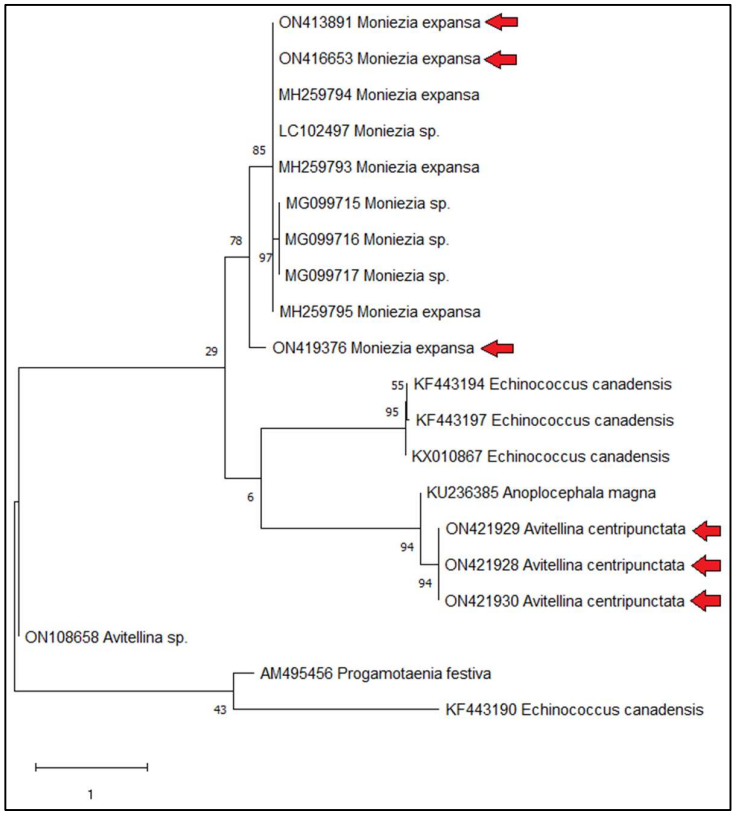

Fig 2: Phylogenetic tree based on the *cox1* gene partial sequencing of *Moniezia* spp. Isolated from sheep (red arrows). Maximum Likehood tree was aligned by using Muscle method associated with nucleotide substitution models (HKY:Hasegawa-Kishino-Yano) Tree was made by using MEGAX VERSION 10 with bootstrap value of 1000 replicates. Numbers on the nodes are bootstrap values.

However, the highest percentage of positive cases were seen among local sheep with 32.3% rate of infection compared to 8.1 % in imported sheep with high statistical significance (Table 2).

Molecular tools-Polymerase chain reaction (PCR) was applied and confirmed the detection of *Moniezia* species with product amplification at 743bp for the ITS 1 gene and 364 bp for *cox1* gene. Different species of Tapeworm were initially determined by The BLAST program on NCBI based on the (ITS1) and (*cox1*) sequence of the type strains. The sequence analysis by using ITS 1 gene detected 20 *Moniezia expanse* and 12 *Moniezia* spp. while sequence analysis via *cox1* gene indicated 25 *Moniezia expanse* and 7 *Avitellina centripunctata*.

Table 1: The relationship between the infection with *Moniezia* species and sex of animals

Sex	No. of examined	No. positive	% infection
Male	195	32	16.4
Female	5	0	0
Total	200	32	16

$X^2 = 0.977$: *p* value = 0.323

Table 2: The relationship between the infection with *Moniezia* species and source of animals.

Types of source	No. of examined	No. positive	% infection
Local	65	21	32.3
Imported	135	11	8.1
Total	200	32	16

$X^2 = 19.054$: *p* value <0.05

All sequenced isolates were recorded in GenBank under the following accession number: ON421928-ON421930 for *Avitellina centripunctata,* ON419376, ON416653 and ON413891 for *Moniezia expanse* by using *cox1* gene and ON508851- ON508853 for *Moniezia expanse* by using ITS 1 gene as targeting gene (Fig. 1 and 2). All isolated strains collected from sheep in Duhok city and their sequence were compared with different *Moniezia* spp. in different countries. Phylogenetic analysis based on the ITS 1 gene showed that the tapeworms from sheep are genetically distinct, two isolates ON508852 and ON508853 are sister taxa and form a separate clade, while the isolate ON508851 form a well-supported clade with other isolates from Iraq (MZ724156, MZ724158, ON456485, ON456487, ON454637-8), China (KX377890), Japan (AB367793) with bootstrap 93% as shown in Fig. 1.

Phylogenetic analysis using *cox1* gene discovered that the tapeworms isolated from sheep are genetically distinct, two isolates ON413891 and ON416653 are sister taxa and form a clade with other isolates from Iraq (MH259793 and MH259794), China (LC102497) and Finland (MG099715, MG099716, MG099717) with bootstrap 85%. The isolate ON419376 joins the other isolates in a well-supported clade. All isolates of *Avitellina centripunctata* (ON421929-30) are sister taxa in 94% of the bootstrap resampling and form a clade with isolate of *Anoplocephala magna* from China (KU236385) as shown in Fig. 2.

DISCUSSION

One of the most common cestodes tapeworms of domestic animals especially sheep and goats belong to the genus *Moniezia* species. High tapeworm burden in the animal's intestine can develop some problems to the health of animals, besides that lead to financial losses by increasing the cost of veterinary treatment and service (Diop *et al*., 2015; Kouam *et al*., 2019). In our investigation, a high prevalence of *Moniezia* spp. were detected in male, with no significant difference and that may be because male sheep were slaughtered more frequently than female in Zakho abattoirs. Our finding was in disagreement with other previous studies (Nurling and Admasu, 2014; Ghanim *et al*., 2022). Hence, among infected sheep, sexual dimorphism does not affect the incidence of *Moniezia* spp. Regarding the source of animals, local sheep showed the highest rate of infection than imported sheep breed, which may be due to low levels of awareness of these infections among owners of domestic animals and hence less treatment of infected domestic local animals against tapeworms (Ijaz *et al*., 2018). Among species of *Moniezia*, *M. expanse* and *M. benedeni* are well known for their effective infection in sheep and goat. Although several international studies have confirmed the presence of *Moniezia* by using PCR methods; but few have been conducted in Iraq (Ali *et al*., 2018; Ghanim *et al.,* 2022). The sequencing analysis of *cox1* gene detected 25 *Moniezia expanse* and seven *Avitellina centripunctata,* while sequence analysis by using ITS 1 gene revealed 20 *Moniezia expanse* and 12 *Moniezia* spp. Hence sequence analysis using *cox1* gene is proved to be reliable in identifying tapeworms since it is useful in detecting more species than that in the other method that utilized ITS1 as a target gene.

All isolates form close matching with global isolates on the phylogenetic tree. However, some isolates form branches out from the other global isolates due to distinct mutations that occur in the past and may be due to their different ancestors (Ohtori *et al*., 2015; Diop *et al.,* 2015; Guo, 2017). The phylogenic tree conducted for isolated tapeworms is different from others in China, Japan and Finland and this may be due to the geographical differences, as well as in Iraq, the disease control systems may not be as good as the ones in those places which lead to development of new strains of tapeworms due to modification in the nucleotide sequences.

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