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College of Veterinary Medicine

Polymerase chain reaction-based detection of *Moniezia* in sheep

A Graduation Project Submitted to the Department Council of the Internal and Preventive Medicine-College of Veterinary Medicine/ University of Al-Qadisiyah in a partial fulfillment of the requirements for the Degree of Bachelor of Science in Veterinary Medicine and Surgery.

By
Rabab Hussain Hatem

Supervised by
Dr. Al-fatlawi M. A.

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بِسْمِ ٱللَّهِ ٱلرَّحْمَٰنِ ٱلرَّحِيمِ

فَتَعَٰلَى ٱللَّهُ ٱلْمَلِكُ ٱلْحَقُّ ۗ وَلَا تَعْجَلْ بِٱلْقُرْءَانِ مِن قَبْلِ أَن يُقْضَىٰٓ إِلَيْكَ وَحْيُهُۥ ۖ وَقُل رَّبِّ زِدْنِى عِلْمًا ﴿١١٤﴾

صَدَقَ ٱللَّهُ ٱلْعَظِيمُ

Certify of supervisor

I certify that the project entitled (Polymerase chain reaction-based detection of Moniezia in sheep) was prepared by Rabab Hussain Hatem under my supervision at the College of Veterinary Medicine / University of Al-Qadisiyah.

Supervisor

Al-fatlawi Monyer Abd

Dept. of Veterinary microbiology

Coll.Of Vet .Med. / Univ. of Al-Qadisiyah.

01 / 03/ 2018

Certificate of Department

We certify that Rabab Hussain Hatem
Has finished her Graduation Project entitled (Polymerase chain reaction-based detection of Moniezia in sheep) and candidate it for debating.

Instructor

Dr. Muthanna H. Hussain

01 /03/ 2018

Head of Dept of Int. and Prev. Med.

Dr. Muthanna H. Hussain

01 /03 / 2018

Dedication

To who I waited a long time to dedicate to him my graduation project, Al-Imam Al-mahdi....

To the memory of my great father....

To the source of kindness, the greater my mother....

To the great supporter in my life, my brothers and my sisters ....

To my best friends , Israa Ayad and Israa Jawad ....

To my classmates , Nooraldeen Hakim and Mortada Mohammed....

Rabab Al-zameli

Acknowledgement

I would like to thank merciful God for enabling me to perform this work.

I would like to express my deeply thanks to my supervisor Dr. Monyer Abd Al-Fatlawi for his supervisor, constant help and endless encouragement....

Rabab Al-Zameli

Abstract

The sheep infestation by *Moniezia* spp, cestodes that normally affect intestine of ruminants especially sheep, are wide-spread-parasitic infestation. This study was initiated to investigate the cestodes that infest sheep intestine. From Al-Najaf City-Iraq, 50 sheep intestines were examined seeking for tapeworms. After that searching, the results indicated that 13 sheep were infested with *Moniezia* spp. For studying the morphological features of the tapeworms, 5 cestodes were stained with modified Carmen stain, and the mature segment of these worms showed the presence of genital pore, cirrus sac, vitelline gland, testes, and inter-proglottid gland. For better detection of the tapeworm, 4 samples out of 13 cestodes were subjected to polymerase chain reaction (PCR) test.Using 18S rRNA gene, the results of the PCR have confirmed the identity of these tapeworms as *Moniezia* spp. This study indicates the presence of *Moniezia* spp in the intestine of sheep that belong to

the city of Al-Najaf, Iraq. This issue should alarm the veterinary officials to control and prevent such parasitic infestation.

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1. Introduction

The cestode namely *Moniezia* spp. are wide-spread tapeworms that affect intestines of ruminants especially sheep. The worm infestation allows huge economic losses in different countries of the world (Soulsby, 1982; Mazyad and el-Nemr, 2002). These worms characterized by the presence of the featured scolex, neck and strobila. Moreover,these tapeworms have unique scolex and neck that are small in size with long chain of strobili. Anoplocephalidae is the family of these tapeworms and descent from Cyclophyllidea order. In order to differentiate this genus from other genera, the tapeworms have anterior, posterior, mature and gravid segments. The sexual organs of *Moniezia* spp. are repeated for each proglottid. As this worm needs an intermediate host, mites play as an important source of infestation via grassing and then digesting of these mites to release the active larvae which grow later to the adult stage of these worms, Figure 1,(Denegri, Bernadina, Perez-Serrano, & Rodriguez-Caabeiro, 1998). The disease caused by this worm is called monieziasis. There are few species of *Moniezia* spp. with limited genetic studies regarding 3 out of 7 species. These species are *Moniezia expansa*,

Moniezia benedeni and *Moniezia monardi* ((Ohtori, Aoki, & Itagaki, 2015). The current study was intended to detect and genetically identify *Moniezia* spp. that affect sheep in Al-Najaf City, Iraq.

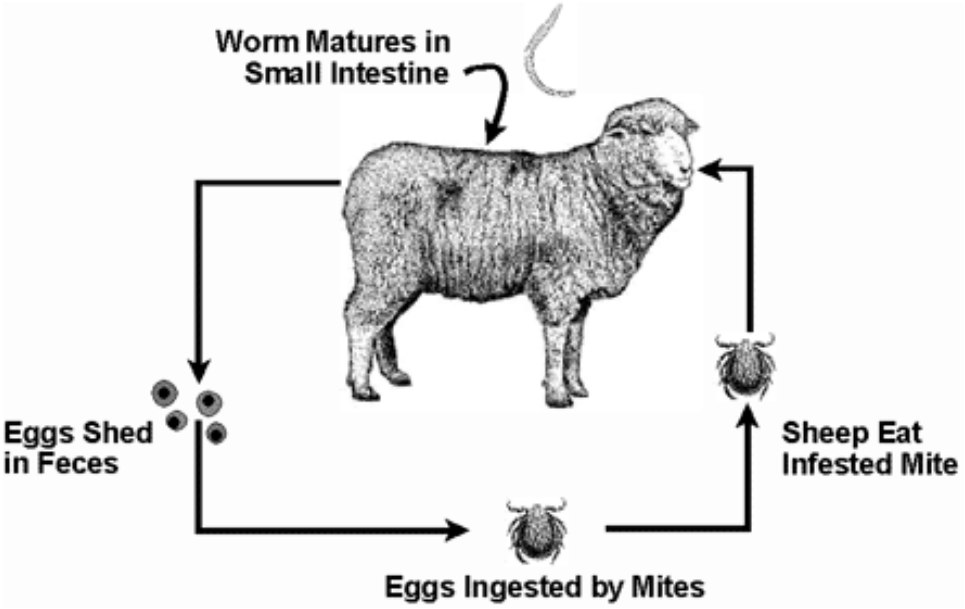


Figure 1: The life cycle of *Moniezia* spp.

(http://www.danekeclublambs.com/Tapeworms.html)

2. Review of literatures

2-1. Morphological Features

Moniezia expansa is a tapeworm that belongs to the *Moniezia* genus one of the widely distributed double-pored ruminant (Gómez-Puerta et al., 2008). In addition to its high incidence in sheep, *Moniezia expansa* was also reported in swine and once unusual case in human (Gómez-Puerta et al., 2008; el-Shazly et al., 2004). It is well known that *Moniezia expansa* mainly infects young animals (Wymann, 2008). *Moniezia expansa* body is segmented into several proglottids and characterized by the presence of an anterior scolex that contains sensory organs, neck which connects the premature segments and the strobilus which represents the longest part of the body. The sensory organs in the scolex are responsible for tactile stimulation, and they are close to the nerves that expand along the parasite body (Brusca and Brusca, 1990). This Cestode size changes through the life of the parasite to be about 8-10 meters in length and 1.5 centimeter in width, and the scolex is about 0.8 millimeter (Chilton, et al., 2007). It does not contain rostellum and rostellar hooks, instead the scolex carrys four suckers that are responsible for penetrating or holding on to the host tissues (Mehlhorn, 2008). This parasite is monecious with both genders sexual organs on each subject. The reproductive organs

locate laterally, and each proglottid is characterized by the presence of cirrus pouches as well as genital pores and abundant testes. Despite the fact that there are no studies about the behavior of the *Moniezia expansa,* it is known that these worms are not able to travel due to the lack of cilia in their bodies (Brusca and Brusca, 1990).

2-2. Digestive System

The body does not involve digestive organs, and it resembles a whole inverted intestine since it is covered with microvilli that increase surface through which absorbing takes place. They get their nutrition such as body fluids by absorption through their tegument, or the external covering (Brusca and Brusca, 1990).

2-3. Natural Environment

There are three natural habitats for *Moniezia expansa* that can be summarized as follw:

1. External habitat: represented by the ground.
2. The gastrointestinal organs of the intermediate host: represented by the oribatid mite.
3. The definitive host: represented by the intestine of sheep, cattle and goat.

2-4. Life Cycle

Life cycle of *Moniezia expansa* needs two hosts; final host that is sheep, cattle or goat and intermediate host that is the oribatid mites (Elliott, 1986). In details, eggs go out of angulates intestine with the feces to be eaten within one day by mites that are highly available in soil.Then, the eggs reach the intestine of the mites where they develop and hatch into oncospheres (larvae with 6 hooks). As their intermediate host, the eggs stay in the mites'intestines for 1-3 months. After that, the oncospheres pass through the haemocoel to grow into the cysticercoid stage. Finally, ruminants graze on the mites infected grass and the cysticercoids get released into the digestive system to be developed to full grown up parasite within 5-6 weeks in the large intestine (Barriga, 1994). For the life cycle to go on and on, the proglottids that involve the *Moniezia expansa* mature eggs are discarded with the animals' feces to be consumed and broken down by the mouth of the intermediate host (Barriga, 1994). Interestingly, there are no studies about the life span of this tapeworm yet.

2-5. Reproduction

Moniezia expansa adult worm has both male and female reproductive system as mentioned above. The mature proglottids can mate internally since they have both sexes. They also can couple with other tapeworm to

reproduce. Reproduction produces gravid proglottids that pass out with angulates feces (Melhorn, 2001). Dry conditions destroy the eggs unless otherwise they were eaten by the soil mites. These tapeworms can communicate with each other chemically in addition to the tactile and chemical ways for perception communication.

2-6. Pathogenesis

No recorded signs of the *Moniezia expansa* infection in sheep and ruminants generally. It is worth to notice that in intensive infection;the disease might cause intestinal blockage, diarrhea and weight loss (Bauer, 1990).

2-7. Diagnosis

Since *Moniezia expansa* is asymptomatic, it is accidently detected on parasitological postmortem fecal examination. Importantly, examiners sometimes mix between *Moniezia benedeni* and *Moniezia expansa* due to their similar interproglottidal glands. Accordingly and in order to have very accurate results, examiners must be able to differentiate those glands in the *Moniezia expansa* and *Moniezia benedeni*. Those glands have rosette shape in the posterior part of the *Moniezia expansa*, but they make striate line in the other species (Taylor, 1928).

Moniezia expansa infection can be diagnosed by either of the next ways:

1. Macroscopic examining of the anal area and fecal samples to grossly detect the gravid proglottids since they are big enough to be seen easily by naked eye.
2. Microscopic examining of fecal samples in laboratory to detect the eggs of *Moniezia expansa*.
3. Enzyme Linked Immunosorbent Assay (ELIZA) (Jiménez et al., 2007).
4. Polymerase Chain Reaction(PCR) (Khan et al., 2010).

2-8. Treatment

The following antiparasites are often prescribed to treat the *Moniezia expansa* infection:

1. Niclosamide
2. Praziquantel (Bauer et al., 2007)
3. Albendazole (Bauer et al., 2007)
4. The compination of Praziquantel + Levamisole(Southworth et al., 1996).

2-9. *Moniezia expansa* Impact on the Community

Moniezia expansa can impact the community by affecting the definitive hosts such as sheep, cattle and goats. As mentioned above, those parasites can cause blockage to the intestine under severe conditions. Moreover,

the infection can also cause diarrhea and weight loss especially in young ruminants which ends up with economical stock breeding loss (Gomez-Puerta and Denegre, 2008).

3. Materials and methods

3-1. Intestinal exploring and sample collection

This study was initiated to investigate the cestodes that infest sheep intestine. From Al-Najaf City-Iraq, 50 sheep intestines were examined seeking for tapeworms. After that searching, the results indicated that 13 sheep were infested with *Moniezia* spp.

3-2. Morphological examination

For studying the morphological features of the tapeworms, 5 cestodes were stained with modified Carmen stain.

3-3. Polymerase chain reaction

To confirm the tapeworm identity, 4 samples out of 13 cestodes were subjected to polymerase chain reaction (PCR) test using 18S rRNA gene.

3-4. The Equipment, instruments, and kits

Table 1 mentions the equipment and instrument that were used in this study. Table 2 refers to the kits that were used in the present study.

Table 1: mentions the equipment and instrument that were used in this study.

No.	Equipment & instrument	Company
1	High Speed Cold centrifuge	Eppendorf /Germany
2	Incubator	Mammert/Germany
3	Sensitive Balance	Sartorius/Germany
4	Water Bath	Mammert/Germany
5	Vortex	CYAN/ Belgium
6	Micropipettes 5-50, 0.5-10, 100-1000µl	CYAN/ Belgium
7	Refrigerator	Concord /Lebanon
8	Thermocycler PCR	MJ-Mini BioRad/ USA
9	Exispin centrifuge	Bioneer/ Korea
10	Eppendorf tubes	Bioneer/ Korea
11	Disposable syringe 10 ml, 5ml and 3ml	Sterile EO. / China
12	Sterile test tube	Superestar/ India
13	UV Transilluminator	ATTA/ Korea
14	Gel electrophoresis	Shandod Scientific/ UK
15	Digital camera	Samsung/ china

Table 2: refers to the kits that were used in the present study.

No.	Kit	Company	Country
1	**gSYAN DNA Extraction Kit**	**Geneaid**	**USA**
	GST buffer		
	GSB buffer W1 buffer		
	Wash buffer		
	Elution buffer GD column		
	Collection tube 2ml		
2	**AccuPower™ PCR PreMix**	**Bioneer**	**Korea**
	Taq DNA polymerase dNTPs (dATP, dCTP, dGTP, dTTP)		
	Tris-HCl pH 9.0		
	KCl $MgCl_2$ Stabilizer and Tracking dye		

3-5. Primers of the PCR

The primers were designed in the current study and were purchased from (Bioneer Company, South Korea). The primers were deposited in the ribosomal database under the number AY752651.1. Table 3 shows these primers.

Table 3: shows the primers used for the PCR of the *Moniezia* spp.

Amplicon	**Sequence**		**Primer**
18S ribosomal RNA gene	F	**TGCTACCCGCATGATGTTGT**	1124bp
	R	**ACACAGTTGGCTGCACTCTT**	

3-6. Chemicals used for the PCR

Table 4 reveals the chemical substances that were used in the current study.

Table 4: reveals the chemical substances that were used in the current study.

No.	**Chemical**	**Company and Origin**
1	Absolute Ethanol	BDH (England)

2	Agarose	BioBasic (Canada)
3	TBE buffer	BioBasic (Canada)
5	Ehidium Bromide	BioBasic (Canada)
6	Proteinase k	BioBasic (Canada)
9	Free nuclease water	Biolab/ USA
10	PCR water	Bioneer (Korea)

3-7. Worm DNA Extraction

Using gSYAN DNA mini kit-based extraction kit (Geneaid, USA), the genomic DNA was extracted from the tapeworm tissues and following the manufacturer's protocol as shown in the following steps:

1. From the tapeworm tissues, 200mg was placed in a 1.5ml tube, and 200ul of the GST buffer was added. The mix was homogenized using micropestle.
2. Next, 30µl proteinase K was added and mixed by vortexing. Then, the mix was incubated at 60℃ for 1 hour.
3. Later, 200µl of GSB was placed and vortexed vigorously, which then were incubated at 70℃ for 15 minutes and inverted every 3 minutes until done.

4. After that, 200µl ethanol was added and immediately mixed by shaking vigorously.

5. Then, DNA filter column was inserted into a 2 ml collection tube. Next, the mixture containing the precipitant was transferred to the column. Then, it was centrifuged at 10000 rpm for 5 minutes. The 2ml-collection tube was discarded and a new tube was used with the column.

6. Following that, the filter column received 400µl of W1 buffer and then was centrifuged at 10000 rpm for 30 seconds. The 2ml-collection tube was disposed and a new one was used.

7. Later, the column received 600µl of Wash Buffer (ethanol). Then was centrifuged at 10000rpm for 30 seconds. The flow-through solution was disposed and the tube was used again.

8. Then, the tubes were centrifuged at 10000rpm for 3 minutes for drying out purposes.

9. The filter column containing the DNA was transferred to a clean 1.5ml-centrifuge tube. These tubes received 50µl-pre-heated elution buffer.

10. Finally, 5-minute standing for the tubes was given to increase the absorption of the elution buffer by the matrix. Then, these tubes were centrifuged at 10000 rpm for 30 seconds for the DNA to be eluted.

3-8. Nanodrop for extracted DNA

The resulted DNA was checked for quality and quantity using NanoDrop spectrophotometer (Thermo, USA), and the measured

quantity was resulted using the unit;ng/µL. The measured quality was counted using the unit as purity by absorbance measurement at (260 /280 nm) as following steps:

When run the NanoDrop software, the suitable process was 1. selected, Nucleic acid, DNA process.

The measurement pedestals were cleaned using dry wipes several 2. times. Then,2µl of free-nuclease water was placed on the lower measurement pedestal for blanking the reading.

After that, the moving arm was lowered,and the "Ok" button was 3. hit to run the process of the blank reading.Finally and to read the DNA quality and quantity, the pedestals was cleaned using wipes, and 1µl DNA was placed to run the process of measurement.

3-9. PCR master mix preparation

AccuPower PCR PreMix Kit was used to generate the PCR mastermix following the manufacturer's protocol, table 5.

Table 5: the PCR mastermix components

PCR Master mix	Volume
DNA template	5µl
Forward primer (10pmol)	1.5µl

Reveres primer (10pmol)	1.5µl
PCR water	12µl
Total volume	20µl

The components appeared in the table 5 were placed in a standard tube that belonged to theAccuPower PCR PreMix Kit. This tube contained all other ingredients which were required to perform the PCR reaction such as Taq DNA polymerase, dNTPs, Tris-HCl pH: 9.0, KCl, MgCl2,stabilizer, and tracking dye). The PCR tubes were then centrifuged at 3000rpm for 3 minutes. Finally, these tubes were inserted into the PCR Thermocycler (MJ-Mini BioRad, USA).

3-10. PCR Thermocycler Conditions

The thermocycler conditions used in this study are shown in table 6.

Table 6: the thermocycler conditions

PCR step	Temp.	Time	repeat
Initial Denaturation	95C	5min	1
Denaturation	95C	30sec.	30cycle
Annealing	58C	30sec	
Extension	72C	1 min	
Final extension	72C	5min	1
Hold	4C	Forever	-

3-11. PCR product analysis

Electrophoresis of agarose gel (1.5%) was used to analyze the PCR products as appeared in the following steps:

1- Agarose(1.5%) powder was dissolved in 1X TBE using water bath at 100 °C for 15 minutes and later was left to cool down until 50°C.

2- After that, 3µl of ethidium bromide was added into the solution of the agarose solution.

3- After fixing the comb in the tray, the solution of the agarose was poured and was allowed to solidify for 15 minutes at room temperature.

After solidification of the solution, the comb was removed gently, and 10µl of the PCR products were pipetted into the pre-selected wells.Ladder of 100bp size was pipetted at 5µl.

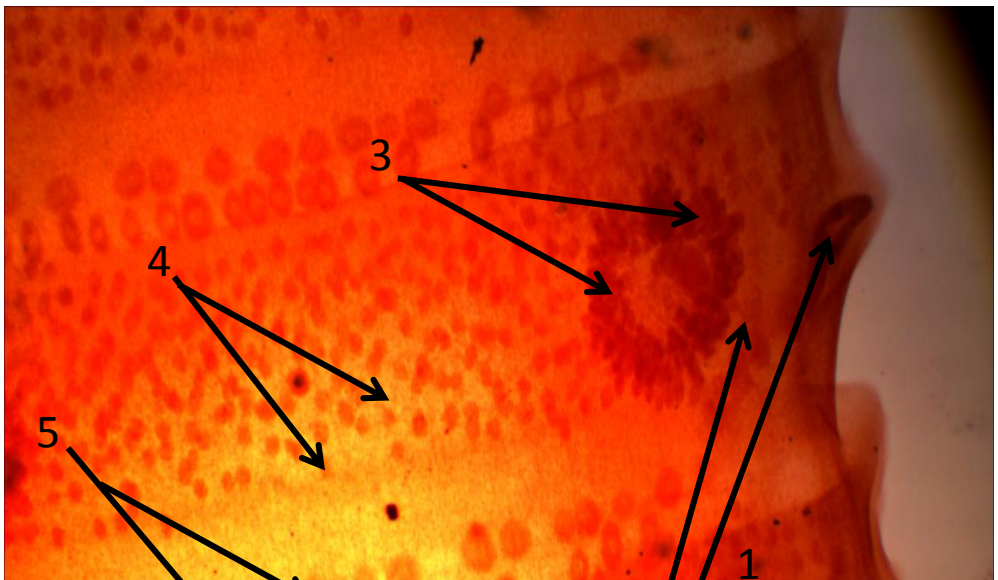
4- The gel was then covered by 1X TBE buffer. Electric current was setup at 100 volt and 80A for 1hour.

5- Finally, the gel was visualized under UV Transilluminator.

4. Results

From Al-Najaf City-Iraq, 50 sheep intestines were examined searching for tapeworms. After that searching, the results indicated that 13 sheep were infested with *Moniezia* spp. For studying the morphological features of the tapeworms, 5 cestodes were stained with

modified Carmen stain, and the mature segment of these worms showed the presence of genital pore, cirrus sac, vitelline gland, testes, and inter-proglottid gland, figure 2. For better detection of the tapeworm, 4 samples out of 13 cestodes were subjected to polymerase chain reaction (PCR) test. Using 18S rRNA gene, the results of the PCR have confirmed the identity of these tapeworms as *Moniezia* spp., table 3.



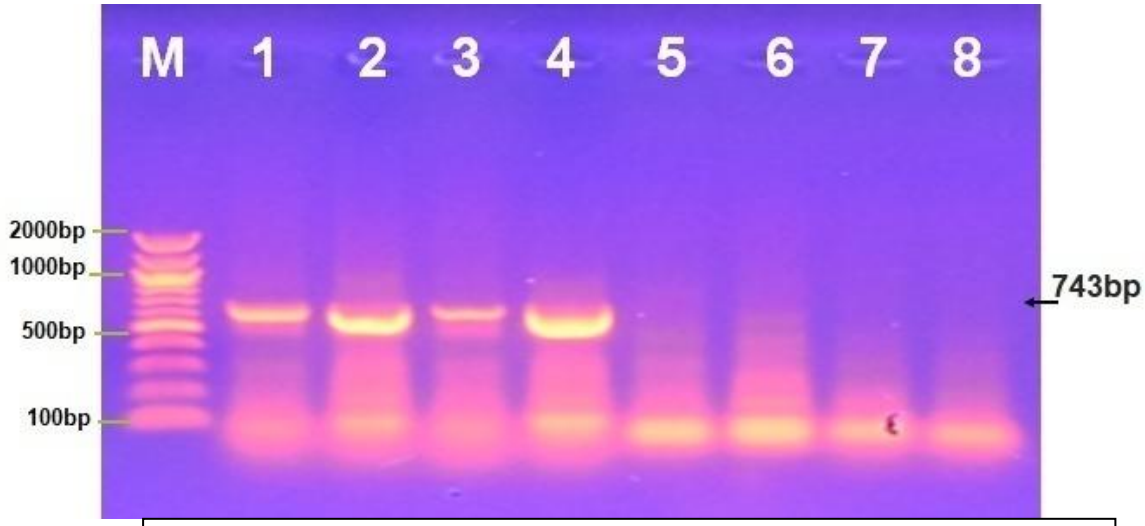


Figure 3: Image of agarose gel electrophoresis that demonstrates the amplification of the 18S rRNA gene for *Moniezia* sp. M is the Ladder (2000-100bp). Lanes 1 to 4are the amplification of this gene the *Moniezia* spp. of sheep. Lanes 5 to 8 are the amplification of this gene for *Moniezia* sp. of cattle. The product size is at 743bp.

5. Discussion

The result of the current study has detected the presence of

Moniezia spp. in the intestine of sheep, and this is considered to the wide-

spread of this tapeworm in small ruminants. The finding that 13 sheep were infested with *Moniezia* spp. indicates a major problem of how big is the economic loss that might this worm introduce to the livestock industry in south of Iraq(Diop et al., 2015). This worm has previously been identified in camels in Al-Najaf and Al-Diwaniyah cities (Anisimova, 2012), who found that the morbidity rates were 32.35% and 15.38% respectively. The current study result agrees with (Fadl et al., 2011) that found that the prevalence of *Moniezia* spp. in sheep in Baghdad regions was 0.9%. Increasing the occurrence rate of the infestation is happened during summers; however; this increase could start in spring when there are much numbers of mites that carry the intermediate stage of this worm on grass (Fadl et al., 2011).

For studying the morphological features of the tapeworms, 5 cestodes were stained with modified Carmen stain, and the mature segment of these worms showed the presence of genital pore, cirrus sac, vitelline gland, testes, and inter-proglottid gland, figure 2.The morphological feature results agree with the following literatures. *Moniezia expansa* adult worm has both male and female reproductive system as mentioned above. The mature proglottids can mate internally since they have both sexes. They also can couple with other tapeworm to reproduce. Reproduction produces gravid proglottids that pass out with

angulates feces (Melhorn, 2001).*Moniezia expansa* is a tapeworm that belongs to the *Moniezia* genus one of the widely distributed double-pored ruminant tapeworm that infects sheep, cattle, and goats (Denegre, 2008, Gómez-Puerta et al., 2008). In addition to its high incidence in sheep, *Moniezia expansa* was also reported in swine and once unusual case in human (Gómez-Puerta et al., 2008; el-Shazly et al., 2004). It is well known that *Moniezia expansa* mainly infects young animals (Wymann, 2008). *Moniezia expansa* body is segmented into several proglottids and characterized by the presence of an anterior scolex that contains sensory organs, neck which connects the premature segments, and the strobilus which represents the longest part of the body. The sensory organs in the scolex are responsible for tactile stimulation, and they are close to the nerves that expand along the parasite body (Brusca and Brusca, 1990). This Cestode size changes through the life of the parasite to be about 8-10 meters in length and 1.5 centimeter in width, and the scolex is about 0.8 millimeter (Chilton, et al., 2007). It does not contain rostellum and rostellar hooks, instead the scolex carrys four suckers that are responsible for penetrating or holding on to the host tissues (Mehlhorn, 2008). This parasite is monecious with both genders sexual organs on each subject. The reproductive organs locate laterally, and each proglottid is characterized by the presence of cirrus pouches as well as genital pores and abundant testes. Despite the fact that there are no studies about the

behavior of the *Moniezia expansa,* it is known that these worms are not able to travel due to the lack of cilia in their bodies (Brusca and Brusca, 1990).

For better detection of the tapeworm, 4 samples out of 13 cestodes were subjected to polymerase chain reaction (PCR) test. Using 18S rRNA gene, the results of the PCR have confirmed the identity of these tapeworms as *Moniezia* spp., table 3. It has been shown that PCR could be utilized as a suitable molecular tool to identify the presence of *Moniezia* spp. in sheep and goat (Nguyen et al., 2012). These current study results allow researchers to use PCR as a trusted method to detect this tapeworm in sheep. The results also alarm the veterinary officials to place control and prevention procedures against the infestation by this parasite.

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