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Traditional and molecular diagnosis of *Haemonchus contortus* in sheep in Babylon province, Iraq

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Abstract

Haemonchus contortus one of gastrointestinal helminthes characterized by miner intraspecific variation and a major variation among species which exploited to determine species simultaneously depend on PCR techniques, by amplification of DNA from parasites so goal of study was traditional and molecular detection of this parasite in sheep. Experiment was conducted using adult worms collected from abomasum of sheep obtained from abattoir in Babylon province. All worms divided into two groups, first group for morphological study and second group stored in 70% ethanol for DNA extraction, ITS-2 spacer and 28S gene were amplified using PCR technique sequenced and analysis with a phylogenic tree. According to the available data this study recorded *Haemonchus contortus* in sheep depend on ITS-2 spacer and 28S gene sequences for the first time in Iraq with accession no. LC552170 and LC552171 using molecular data. The phylogeny analysis depended on ITS-2 spacer and 28S gene partial sequences were closely related and high identity 94% with Germany *H. similis* sequence MN708992.1 and 93% identity with New-Zealand and Austria *H. contortus* sequence KC998713.1 and KJ724288.1 respectively, with a low genetic variation among all comparison sequenced isolates.

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Introduction

Gastrointestinal helminthosis infections in ruminants represent the major problems and the major factors responsible for lowered economics of sheep production (1). *Haemonchus ssp*. infection clinical signs include hypoproteinemia, epithelium hyperplasia within nodules affects the full mucosal necropsy thickness, weakness, collapse and death because of unspecific signs (2,3). In addition, it reported that *Haemonchus* was a blood-feeding parasite responsible for important morbidities and mortalities in sheep (4). The major signs of this parasite anemia, pale mucous membranes, submandibular edema, and sudden death (5). acute haemonchosis form occurs with varying rates affected animals appeared weaker depending on intake of infective larvae and blood loss rate, while chronic form characterized by weight loss, poor weight gain, general diagnosis and anemia detected by assessment of conjunctiva membrane color (4). Drug resistance due to excessive and uncontrolled anthelmintic use lead to problems with genetic resistance at high gene flow level to this parasite and enhance resistance (6). Due to the few published studies about genetic diversity that depend on genome-wide data, high polymorphism rate in *Haemonchus* reference sequences make discernable genetic differentiation between *H. contortus* populations in different host species challenge for genome assembly (7). The species identity and comparison of *H. contortus* isolated from sheep and goat indicated that it was more closely related to each other depended on internal transcribed spacer ITS-1, ITS-2 and the 5.8S rRNA regions (8). Many studies depend on ITS sequence indicated that it this region was good genetic marker for distinguishing sympatric *Haemonchus* ssp. (7,8).

Materials and methods

Total 67 sheep samples were checked in study, abomasum was obtained from slaughtered sheep at Babylon province local abattoir, abomasum were placed into a bucket containing physiological saline and transported to Laboratory of Parasitology, Baghdad. Research had been done between June 2019 to May 2020. A total of 86 worms were collected from abomasum continent washed with PBS at pH 7.4 prepared by dissolved 9.86 g of phosphate buffer powder in 1000 ml of distill water according to the manufacture instruction and adjusted PH to 7.2 for use. All worms were divided into two groups, first group loaded into a lactophenol for diagnosis species level of body, cervical papillae, esophagus and vulval distance and morphology to detected morphological characters depend on light microscope (40x) diagnosis (9).

Genomic DNA isolation

The second group of the worms was stored in 70% ethanol; the genomic materials were isolated according to (Qiagen, Germany Kit) directive. DNA estimation for all samples used a Nanodrop (ThermoScientific, USA) reading absorbent at (260 /280 nm); samples were stored at-20ºC (10).

PCR amplification

Depended on Shen *et al.* (11) about ~300 bp fragment encompassing the nuclear ribosomal DNA ITS-2 spacer and 28S gene was PCR amplified by added 2 µl of individual worm genomic DNA as a template, 1 µl of each forward ITS-2F-28S-5'-ACGTCTGGTTCAGGGTTGTT-3' and reverse ITS-2R-28S-5'-TTAGTTTCTTTTCCTCCGCT-3' with 10 (pcoml) to master mix (Maxime PCR PreMix) then complete reaction volume to 20 µl by nuclease-free water. All above were used without DNA template as a negative control; all the tubes were transported to Exispin vortex centrifugation for 1 minute at 2000 rpm, and positioned in PCR Thermo-cycler.

PCR thermocycler conditions and electrophoresis

A PCR thermo-cycler condition was done by utilizing a conventional PCR thermo-cycler (T100 thermal cycler. BioRad USA) (Table 1). To detected PCR products and to verify represented single bands, Agarose gel 1% concentration was prepared according to (12) as 1 gm agarose dissolved in 1X TBE, heated in microwave 1 min, then cool down at 60ºC.

DNA sequence and phylogenic tree analysis

Positive products of PCR were sent by ice bag by DHL to Macrogen Company in Korea for completed DNA sequencing by AB DNA sequencing method. Sequence data were deposited in Gene-Bank and get accession number to identify genetic variation between Iraqi sequences isolates and other sequences that submitted in NCBI. Sequencing of DNA study was directed using utilizing Mega 6.0. The development distances were computed utilizing; Phylogenetic analyses were carried out employing (Phylogny.fr / advanced method).

Table 1: PCR thermo-cycler system conventional

PCR steps	Temp.	Time	Cycles
Initial Denaturation	95ºC	5 minutes	1
Denaturation	95ºC	30 second	
Annealing	52ºC	30 second	35
Extension	72ºC	1 minute	
Final extension	72ºC	5 minutes	1
Hold	4ºC	-	-

Results

Morphological identification

The results showed that 50 adult worms, 36 adult female and 14 male worms were *Haemonchus spp.*, all obtained female samples were detected by their body length, which demonstrated 29.75 mm, cervical papillae recorded 4.55 mm from head (Figure 1), valval flap and uterus morphology was also detected (Figure 2). In male nematodes, the results recorded a morphological attribute that include; body length which demonstrated 18.16 mm as a male average length, 4.9 mm of left and right spicules, barbed tip, and gubernaculum 8.9 mm in length (Figure 3).

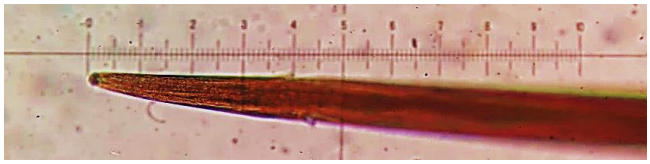

Figure 1: cervical papillae of *Haemonchus spp.* 40X-light microscope.

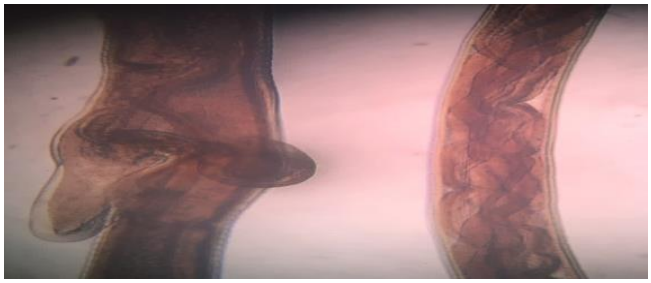

Figure 2: Vulval flap and uterus of *Haemonchus spp.* 40X-light microscope.

Genomic study results

Total genomic DNA were extracted from individual worm's samples (second group of worms) with high concentration and purity measurement 350 ng/µl and 1.74 respectively.

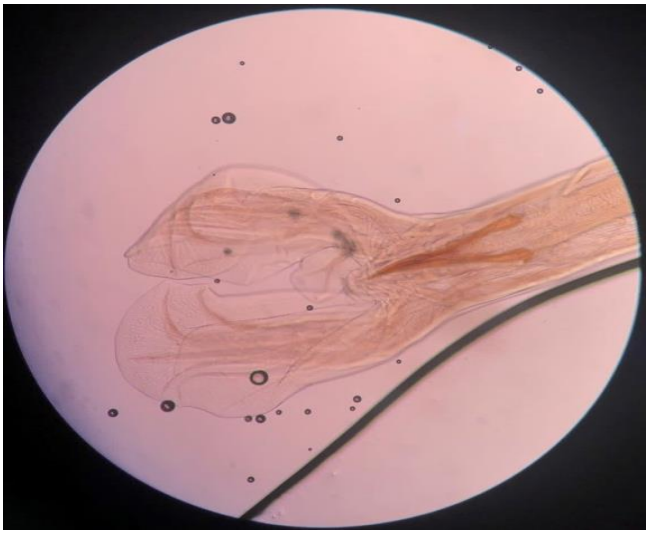

Figure 3: male spicules, barbed tip, and gubernaculum of *Haemonchus spp.* 40X-light microscope.

PCR amplification and sequencing

Specific oligonucleotide primer pairs for ITS-2 spacer and 28S gene we're using with purified worm's DNA as a template in PCR technique were exhibited successfully amplified showing a bands of DNA fragment that indicated the presence of positive results for *Haemonchus*. Representative bands are compared to 100bp ladder in electrophoretic with 1% agarose concentration (Figure 4). Positive PCR product were sequenced and deposed in gene bank NCBI with accession numbers LC552170 and LC552171 at DDBJ and ENA databases for the first time in Iraq.

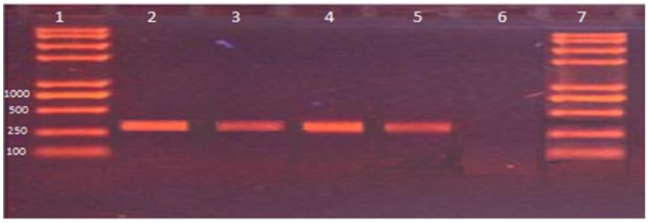

Figure 4: Gel electrophoresis for PCR product of 289bp using (1%) agarose for 90 minutes at 80 volts. Lance (1) molecular marker, Lances (2-5) positive samples for *Haemonchus,* and Lance (6) negative control.

Phylogenic tree analysis

The phylogeny depended on ITS-2 spacer, and 28S gene sequences of neighbor-joining analysis in length sequence was approximately 289 bp editing and alignment using (Phylogny.fr, advanced method). The results indicated that all Iraqi *Haemonchus spp*. isolates in this study were *Haemonchus contortus* and recorded a low genetic variation (0.01) among all comparison isolates (Figure 5).

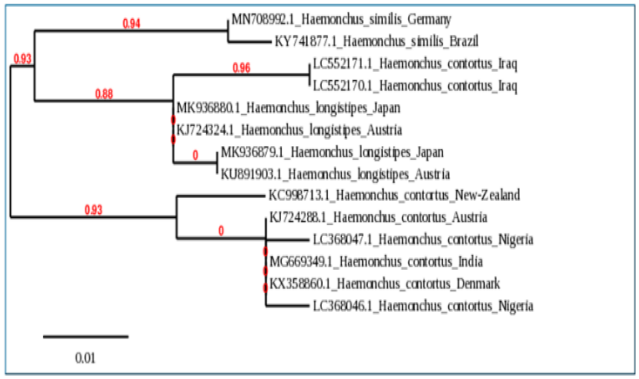

Figure 5: Phylogenetic tree of *Haemonchus contortus*.

Discussion

Many researchers detected specific primers for different genes for *Haemonchus* PCR diagnosis (13). The current study investigated primers that amplify ITS-2 spacer and 28S gene for *Haemonchus* with amplicon size 289 bp as amplifiable targets for PCR that did not match with parasites that infect sheep, which indicated of PCR technique high sensitivity for parasites detection (14,15). Sequence analyses of ITS2 spacer and 28S gene partial sequence were obtained from all worm's specimens that isolated from sheep abomasum and positive amplified by PCR were used to determine taxonomic status of *Haemonchus spp*. for the first time in Iraq, since other studies indicated for many years DNA sequencing was available and good technology to differentiated closely related parasites species (16).

Nucleotide ITS-2 spacer sequencing, BLASTEn alignment, and graphic analysis presented highly similar sequences (megablast) found in NCBI database after sequences blasted at BLASTn program and the study revealed that sequence alignment of ITS-2 spacer and 28S gene lies in *Haemonchus* group with different max score 534 and equal to the total score, E-value were equal to 8e-148 and identity 100% as clarified in NCBI database, and that agree with Jassem *et al.* (17) who indicted that gene sequencing was an excellent target to differentiation isolates by molecular detection. Phylogeny of Iraqi *Haemonchus contortus* isolates were closely related and high identity with Germany and Brazil *H. similis* sequence and showed 93-94% identity respectively, 88% identity with those of Japan, Austria *H. longistipes* sequence. Additionally, there were 93% identities with *H. contortus* sequences of New-zealand, Austria, Nigeria, India, Denmark and Nigeria. The previous studies have shown that the sequencing followed by phylogenic tree analysis was used to detect parasite species (18,19).

Some previous studies have indicated that diagnosis of parasites species based on their morphological characters alone may give imprecise results for accurate identification,

due to the interaction between the many morphological parameters between *Haemonchus spp.* isolates such as parasite length, spicules length, spicules barb length, and many other parameters, as well as the difficulties that the researchers could be facing them in microscopic examination (20). This interaction will lead to measurements overlapping between species and discriminate function in species identification, so other techniques like immunological, histopathological, or molecular methods were required (19-22).

Conclusion

ITS-2 spacer and 28S gene was an excellent molecular marker for detected *Haemonchus spp.*, sequencing followed by phylogeny confirmed the phylogenetic relationship of all species recorded in NCBI database determines genetic affinity percentage among them.

Acknowledgments

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Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this manuscript.

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التشخيص التقليدي والجزيئي للدودة السلكية الملتوية في الأغنام في محافظة بابل، العراق

بلقيس فاضل هادي، سهى طارق البياتي
و حيدر محمد علي الربيعي

فرع الطفيليات، كلية الطب البيطري، جامعة بغداد، بغداد، العراق

الخلاصة

الدودة السلكية الملتوية أحد أنواع الديدان التي تتميز بمستوى منخفض من التباين داخل النوع وتباين مرتفع بين الأنواع لذلك تم الاعتماد على التقنيات الجزيئية لتشخيص الطفيلي اعتمادا على تضخيم المادة الوراثية. الهدف من الدراسة هو الكشف عن الطفيلي في الأغنام باستخدام الطرق التقليدية والجزيئية في التشخيص. أجريت الدراسة باستخدام ديدان بالغة تم جمعها من منفحة الأغنام والتي تم الحصول عليها من مجزرة في محافظة بابل إذ تم نقلها إلى مختبر الطفيليات، كلية الطب البيطري، جامعة بغداد لغرض الدراسة. تم تقسيم جميع الديدان إلى مجموعتين، المجموعة الأولى للدراسة الشكلية التقليدية للطفيلي والمجموعة الثانية المحفوظة في ٧٠ ٪ من الإيثانول لاستخراج المادة الوراثية للطفيلي، إذ تم تضخيم منطقة ITS-2 والجين 28S باستخدام تقنية تفاعل البلمرة المتسلسل وتسجيل متتابعات القواعد النيتروجينية و التحليل الجزيئي للنتائج باستخدام شجرة التطور الوراثي. نتائج الدراسة تؤكد تسجيل الدودة السلكية الملتوية في الأغنام اعتمادا على منطقة ITS-2 وجين 28S لأول مرة في العراق بالأرقام LC552170 و LC552171 إذ استخدام المتتابعات الوراثية للقواعد النيتروجينية ل منطقة ITS-2 و جين 28S للعزلة العراقية التي ارتبطت ارتباطًا وثيقًا وبنسبة عالية ٩٤ ٪ مع طفيلي *H. similis* MN708992.1 للعزلة الألمانية بنسبة ٩٣ ٪ مع تتابعات الدودة السلكية الملتوية النمساوي ونيوزيلندا KC998713.1 و KJ724288.1 على التوالي، مع تسجيل اختلاف وراثي منخفض بين جميع عزلات المقارنة.