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Haemonchus contortus: Conventional and molecular identification and characterization via sequencing-based phylogenetic analysis in sheep

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Abstract The purpose of this study, conducted from 01-October-2023 to 28-February-2024, was to determine the prevalence and genetic diversity of *Haemonchus contortus* Infection from sheep in Al-Diwaniyah slaughterhouse, it was carried out to investigate the level of infection at two levels stages; adult's animal, and human, and also helped us to evaluate the genetic similarities between local isolates from Iraq in comparison with other strains of *H. contortus* from all over the world. In the slaughterhouse in Al-Diwaniyah City, 250 carcasses were sampled. The samples were divided into two tubes, one of them used for microscopic examination by lacto phenol-solution and the other used for molecular tests by using 70% ethanol. Targeting the β-tubulin region by PCR was done, which was followed with the positive PCR-based sequencing. The findings of this study revealed high prevalence of *H. contortus* infection rate in sheep which leads to parasite transmission among the animals. In addition, a high level of genetic similarities was seen between local isolates from Iraq and strains from Sweden, Canada, Switzerland, the USA, and the Netherland. Overall, this study concludes that the level of infections by *H. contortus*. High genetic similarities, with world strains, indicating high diversity and common ancestor.

Keywords: Enteral parasites, *Haemonchus contortus*, roundworms

Introduction *Haemonchus contortus*, one of the most important gastrointestinal nematode parasites in the abomasum of small ruminants, primarily infects at the wetland map bordering the northern tropical and subtropical zones. The traditional control method using chemical anthelmintics is developing drug resistance (1-3).

Haemonchus contortus is a hematophagous parasitic nematode of the abomasum, being one of the most harmful due to the mass production of eggs that facilitates its transmission and the economic losses by non-productivity that it causes to livestock producers (3). Its control is not only anthropocentric; it is also influenced by the presence of other gastrointestinal nematodes, such as Trichostrongylus, which also affect the quality of life and productivity of the domestic ruminants. The reproductive strategy of this nematode counts on a very short egg-to-egg generation time, the ability to survive for a long time without feeding, and a reduced number of cell division cycles, making it difficult to detect them and apply the appropriate management alternative. The lack of genetic and physiological knowledge of this parasitic nematode has delayed the development of effective treatments, but the interest of the scientific community has allowed advances to be made in understanding which areas of their biology this nematode might exploit to survive and replicate (5-7). Sheep are the animals most affected by these parasites, which cause anemia and even death if infection levels reach high values. However, other susceptible hosts include goats, wild ruminants, camelids, and domestic cattle; in the latter, the presence of these parasites directly compromises the economic losses associated with milk production and reproduction (5, 8, 9).

H. contortus is an intestinal nematode with a direct life cycle and proportional dependence on the environment for transmission of its infective stages. The life cycle depends on the release of eggs containing larvae (L1) by adult females in the host digestive system and environmental factors like temperature, humidity, and oxygen. The suitable conditions favor the development of cuticular larvae (L2) and directly expose infective third-stage larvae (L3) to the external environment. In moderate climates, they favor the survival and transmission of the infective larvae to new hosts throughout the year.

The ability of the L3 to cope with oral ingestion by the ruminant allows them to remain free on the pasture for long periods (10, 11).

The genus Haemonchus can cause haemonchosis, severe damage, and increased susceptibility to abomasal gastritis. This condition can be fatal in sheep and goats. According to previous reports, this nematode caused the highest economic damage to small ruminant production. Since the 70s of the last century, parasitic nematodes of the Haemonchus species have developed resistance to anthelmintic drugs practically used in control. According to the FAO, parasitic nematode resistance to anthelmintic agents is a widespread problem worldwide; some countries have reported resistance to all families of anthelmintic drugs. Widespread use of one or more drug groups can cause a high level of resistance and multiple drug resistance in some parasitic nematodes (12-13).

The aim of this study was to determine the prevalence & genetic diversity of *H. contortus* Infection from sheep in Al-Diwaniyah slaughterhouse, it was carried out to investigate the level of infection at three levels stages; adults, animal, and human, and also helped us to evaluate the genetic similarities between local isolates from Iraq in comparison with other strains of *H. contortus* from all over the world.

Material and Methods

Ethical approval

The current study was approved by the Ethical Committee in the College of Veterinary Medicine, Universityu of Al-Qadisiyah, Al-Diwaniyah City, Iraq.

Samples

These samples were randomly collected from the sheep abomasum in three different months from October 2023 to February 2024 during the slaughtering sheep at the abattoir of Al-Diwaniyah slaughterhouse. Each month, 50 carcasses were examined and were randomly selected from male and female sheep, 25 of each sex. The samples obtained were submitted for macroscopic examination where each sample was cut into half, we put one part in one disposable cup which contained lactophenol solution which is used in the microscopic examination and the other part of each sample was put in containers which contains 70% ethanol which used in the molecular tests.

Microscopic examination

For microscopic studies, the worms were taken directly onto glass slides and examined under a universal light microscope with 10X and 40X magnifications to confirm their identity. Additionally, the worm was examined for its morphology. Preserving the worm in lactophenol solution helps in its clear visualization of structures and is essential for identifying and differentiating it from other parasitic species (14).

Molecular techniques

The DNA was extracted using DNA extraction kit (AddBio, Korea) and following the kit protocol. The β-tubulin gene region of this extracted DNA was amplified using Polymerase Chain Reaction (PCR). The primers were designed for the β-tubulin region with the following sequences and amplified the 785 bp: β-tubulin-F 5’ GGAACAATGGACTCTGTTCG 3’ β-tubulin-R 5’ GAATCGAAGGCAGGTCGT 3’ (Accession number KF269107). Designed by Zongze et al (2018) the PCR compound was as followed: 10 ml of master mix (AddBIO)2 ml of forward primer 0.5 pmol/20 µl 2 ml of reverse primer 0.5 pmol/20 µl 4 ml of PCR water 2 µl of DNA template The thermocycling conditions were as followed: Initial denaturation 95°C 5 min (1 cycle) Denaturation 95°C 35 s (40 cycles) Annealing 55°C 30 s (40 cycles) Extension at 72°C 30 s (40 cycles) Final extension 72°C 5 min (1 cycle).

PCR products were loaded on a 1.5% agarose gel for electrophoresis to determine the appearance and size of the amplicons. Here, 5 µl of PCR products was loaded into each gel well and conducted electrophoresis at 100 volts and 80 amperes for 1 hour. The gel was then placed in the gel documentation system and detect the DNA bands.

Sequencing

First, 10 positive PCR samples were chosen for DNA sequencing and phylogenetic analysis. The products were purified, and then forwarded samples for the Sanger sequencing system (PCR products were sent to Macrogen Company in Korea). The nucleotide sequences from the Sanger sequencing system were trimmed for noise signals and sent to the NCBI-GenBank to obtain accession numbers. The species then were determined using DNA sequencing and phylogenetic tree analyses by the MEGA X software with multiple sequence alignments based on Clustal W alignment analysis. The comparison of the local isolates with other global sequences from the NCBI-Blast database included species typing and evolutionary relationships were investigated.

Results

The number of infected sheep and the relative percentages for each month were 8/50 (16%), 11/50 (22%), 14/50 (28%), 17/50 (34%), and 25/50 (50%) for October, November, December, January, and February, respectively.

In summation, 75 out of the 250 sheep analyzed tested positive for the infection, which gives an overall infection figure of 30%. We then calculated how the infection rates changed month by month and found it to be highly significant (p-value = 0.003).

Figure (1) shows a steady rise in the infection rate. Macroscopic and microscopic examinations showed that the sheep were infested with *Haemonchus contortus* worms

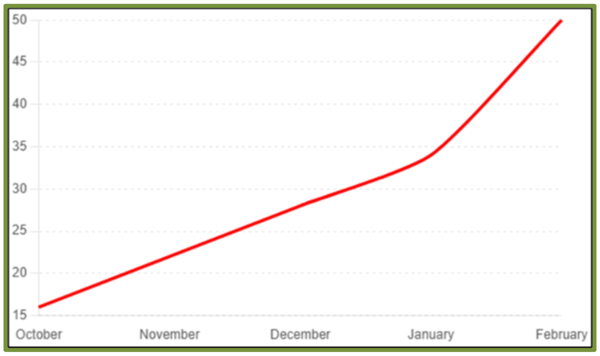

Figure 1: Trend percentage in the infection rates by *Haemonchus contortus* in sheep.

The postmortem and microscopic appearance of the presence of the *H. contortus* is shown in Figure 2.

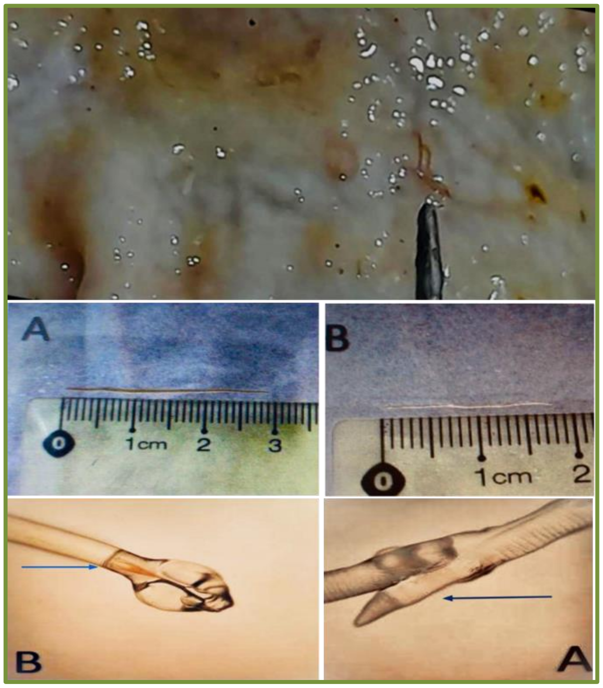

Figure 2: Macroscopic and microscopic appearance of *Haemonchus contortus* from sheep. Male and female.

The PCR amplification revealed positive PCR products, which are shown in Figure 3.

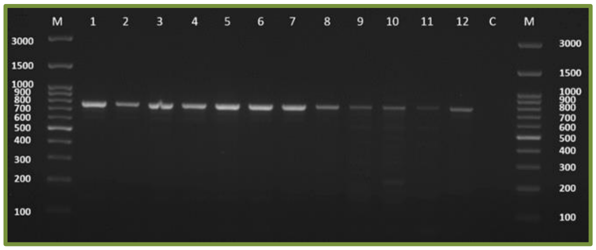

Figure 3: Agarose gel electrophoresis of PCR products for β-tubulin gene region of *Haemonchus contortus* from sheep. Product size: 785 bp. Ladder: (100-3000 bp).

A high level of genetic similarities was seen between local isolates from Iraq and strains from Sweden, Canada, Switzerland, the USA, and The Netherland (Table 1 and Figure 4).

Table 1: Homology Sequence identity (%) between local isolates of *Haemonchus contortus* from sheep and world isolates.

Accession number (Current study)	Accession number (World isolates)	Country	Identity (%)
PP393534	MK382798	Sweden	100
PP393535	MK382800	Sweden	95.75
PP393536	MK382797	Sweden	96.08
PP393537	MK382761	Sweden	95.92
PP393538	KF483614	Canada	95.92
PP393539	DQ469245	Switzerland	95.59
PP393540	MK382772	Sweden	95.42
PP393541	MK382788	Sweden	95.26
PP393542	KX246652	USA	94.60
PP393543	X80046	Netherlands	94.60

Discussion

The number of infected sheep slaughtered at the Al-Diwaniyah slaughterhouse increased during the study period, rising from 16.0% (October) to 50.0% (February). The trend suggests that the burden of the parasite *H. contortus* is increasing in the sampled population, with an overall infection rate of 30%. These data are in agreement with *H. contortus* infections reported from multiple locations around the world, though specific prevalence rates can vary by region and environmental conditions and based on management practices.

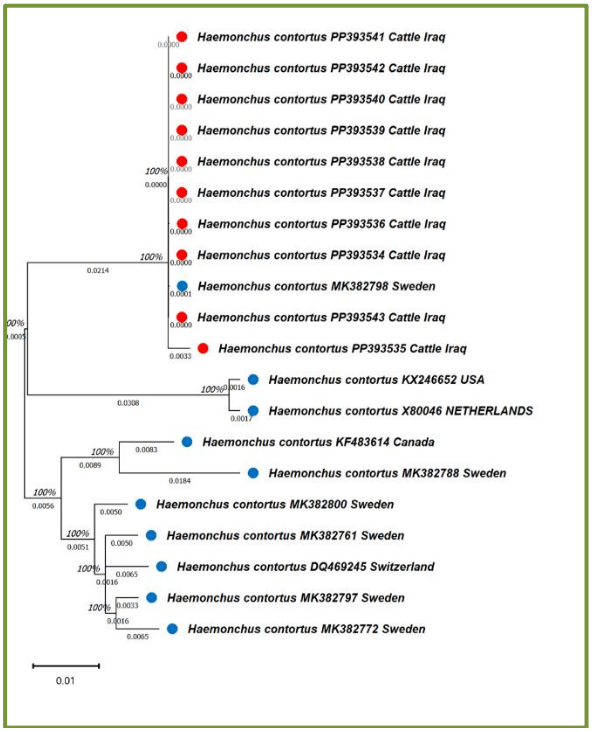

Figure 4: Phylogenetic tree of PCR products for β-tubulin gene region of *Haemonchus contortus* from sheep.

Other studies conducted in other parts of Iraq report different prevalence of *H. contortus* infection, a study in Baghdad report the prevalence among sheep to b 25.8 percent and a study in Basrah report 21.4 percent prevalence (15, 16). However, the higher prevalence in the current study %30 may be due to differences in the sampling period, climatic conditions, animal management and their associated environmental factors. Another important factor is seasonal variation and its effects on *H. contortus* prevalence on livestock, temperature of days and months were higher during the summer season, and humidity also increased, which influences the survival and transmission of the parasite. The trend, during the cooler months, was rising and may be associated with the most favorable of the time for *H. contortus* development and transmission.

Meanwhile, in the broader Middle Eastern region, previous studies have estimated the prevalence of *H. contortus* infections in the sheep at 28.3% (17), 33.7% (18) and 7.6% (19). Again, these results are similar to this current study, which indicates that there is a consistent appearance of *H. contortus* across the Middle East region and similar climates contribute to its persistence. Furthermore, we expect that movement of livestock between and within the neighboring countries is the main reason behind the consistent appearance of *H. contortus* and similar prevalence rates across the region.

Internationally, wide variation in the prevalence of *H. contortus* infections have been reported. In countries practicing intensive livestock industry with routine worming of animals, researchers have reported low prevalence rates. Regassa et al (20) record a 12.5% prevalence which is important due to the stringent control measures and regular use of anthelmintics. In tropical and subtropical forests favored by climatic conditions for the survival of larvae of *H. contortus*, such high figures are prevalent; Mannan et al (21) reports 45.3% prevalence. In regions where intensive control measures are a rarity and climatic conditions are favorable for larval development, the parasite affects a significant percentage of the sheep population.

The detection and molecular analysis of the parasites present revealed high genetic identity of the isolates with various global strains of *H. contortus*, ranging from 94.60% to 100% sequence identities to strains from Sweden, Canada, Switzerland, USA and the Netherlands. These results revealed the genetic conservation of *H. contortus* across geographical regions, and also suggested close evolutionary relationships between local and international strains, indicative within sources and modes of transmission.

Similar patterns have been observed using comparative studies. Laing et al. (22) described high levels of genetic similarity between *H. contortus* isolates from different parts of the world. These studies supported the hypothesis of a single origin of strains and global gene flow, leading to the potential dissemination of resistant alleles in association with high levels of gene flow. In conclusion, the high genetic similarities suggest that integrated and coordinated control of *H. contortus* infections is important because resistance genes can rapidly disseminate if not monitored (23-25).

Conclusion

In conclusion, the study revealed a very high prevalence of *H. contortus* infections in sheep in the Al-Diwaniyah region (33.8 to 50%) with an increase in rates of infections over the study period. The findings of the study on the molecular diagnostics and phylogenetic analyses showed a high degree of genetic similarities between local strains and strains circulating internationally, which urges for a close monitoring of the local and the spread of *H. contortus* strains to minimize their economic impacts on livestock production. Finally, a comparison of the results of the present study with some studies from Iraq, the Middle East and worldwide shows the presence of *H. contortus* in sheep with persistent prevalence and genetic richness, which suggests the importance of integrated and region-specific HA control strategies.

Conflict of interest

There is no conflict of interest in this study as stated by the authors.

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