Original article

MOLECULAR IDENTIFICATION OF ABOMASAL NEMATODES
(HAEMONCHUS SPP. AND OSTERTAGIA OSTERTAGI) IN
NATURALLY INFECTED CALVES

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Summary


Clear variation in the pathogenicity and epidemiology of gastrointestinal nematodes and sensitivity to anthelmintic drugs require precise epidemiological information for such worms. Our work is the first effort to detect the abomasal nematodes (Haemonchus spp, Ostertagia ostertagi) in calves in Mosul, Iraq through amplification of rDNA ITS1 and ITS 2 regions. Four hundred and eighty (480) faecal samples were collected from 35 private calf herds between October 2021 and April 2022. Ages, origins of the herds, size of the herds, and gender were recorded. Microscopic examination and larval culture were conducted. Specified primers were used for molecular identification. The study showed a 50.6% total prevalence of gastrointestinal nematodes in calves. Haemonchus spp. and Ostertagia ostertagi rates were 62.13%, and 60.9%, respectively. The percentages of L3 of Haemonchus spp. and Ostertagia spp. were 32% and 14% respectively. Polymerase chain reaction (PCR) showed reaction products of 321 bp, and 257 bp for studied parasites, respectively. In conclusion, classical laboratory tools may be unable to identify precisely the species and subsequently, minimise the comprehension of parasitic species that circulate in animal populations. Molecular detection is a rapid, highly sensitive, specific and cost-effective procedure that accurately identifies parasite species.

Key words: Haemonchus, Ostertagia, PCR, prevalence

INTRODUCTION

Gastrointestinal parasites affect health, productivity, and profitability of livestock around the world, moreover pose a threat of deworming and drug resistance. Monitoring and control of these parasites is a priority of control programmes, predominantly in tropical and subtropical zones (Maurizio et al., 2021). Infestation with numerous gastrointestinal nematode (GIN) species is widespread in cattle worldwide, with diverse assembly species between countries (Avramenko et al., 2017). The majority of digestive worms parasitise their hosts by ingestion of infective larvae or eggs that are already excreted in the faeces from previously affected
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animals into the environment that become a source of transmission (Sato et al., 2014; Puspitasari et al., 2016; Craig, 2018; Income et al., 2021).

Although some GIN like Haemonchus spp. and Ostertagia spp. are able to induce fatal outcomes, most GIN Infections are subclinical or result in mild disease in the affected animals. Moreover, the severity of parasitic infections may also be assigned to the integrated adverse impact of diverse genera and species and the number of worms, termed parasitic gastrointestinal (Uriarte & Valderrábano, 1989). In addition, the acuteness of infection relies on the immunity and nutritional status of the animal. Usually, invasion of abomasal submucosa by grown worms and penetrating the capillaries using buccal tooth leads to haemorrhage, blood loss, and reduced clotting of blood. About 0.05 mL of blood/day can be lost through ingestion or infiltration from occupied tissue for each single worm, which eventually leads to anaemia and bottle jaw as serum proteins and packed cell volume are decreased (Hildreth & McKenzie, 2020). Substantial losses in the affected herds are related to feeble growth, reduced weight gain, low-grade wool, and mortality of the acutely affected animals (Amarante, 1997; Emery et al., 2016). *Trichostrongylus* sp. infection may clinically display signs such as gain loss, emaciation, inappetence, diarrhoea and even death in intense infection (Getachew et al., 2007).

The genus *Haemonchus* contains about 12 species or more with two major prevailing species: *H. contortus* and *H. placei* (Akkari et al., 2013; Hussain et al., 2014). Both *H. placei* and *H. contortus* have been documented in large and small ruminants in the field and experimentally proposing the presence of cross-infection and broad range of host species (Hogg et al., 2010; Akkari et al., 2013; Chaudhry et al., 2015). In cattle, *Ostertagia ostertagi* is the commonest pathogenic nematode species (Kim et al., 2015). *Ostertagia* spp. produce serious pathological damage in the abomasum which may clinically result in poor weight, diarrhoea, milk drop and anthelmintic resistance in the affected animals (Craig, 2009; Charlier et al., 2012; Höglund et al., 2013; Peña-Espinoza et al., 2016; Ramos et al., 2016).

While conventional procedures are commonly utilised for the diagnosis of gastrointestinal nematodes and anthelmintic resistance in animals, researchers are currently looking for new techniques such as molecular evaluation which are faster, less labour-intensive, and accurate alternatives. Examination of faecal samples is predominantly categorised as either quantitative or qualitative. The quantitative tests identify a specific parasite depending on the characteristic morphology of the parasite’s eggs, oocytes, cysts, or larvae (Charlier et al., 2010). However, several limitations are encountered in microscopic examination, like time-consuming and labourous protocols, experience and skills needed for distinction of eggs of analogous appearance and size. Moreover, there are clear gaps between the scientific literature on the length property of diverse nematode parasites and considerable disparity inside the species. Meanwhile, morphometric distinguishing of the larvae varies based on environmental influence including climate (Roebert et al. 2013; Roebert & Kahn, 2014; Roebert et al., 2017; Borkowski et al., 2020).

Finally, due to the scarcity of data on the helminths associated with calves in the region, this study was aimed at molecular assessment of distribution of abomasal nematodes of veterinary importance among cattle in Mosul, Iraq.
MATERIALS AND METHODS

Ethical approval

This research was confirmed by the Institutional Animal Care and Use Committee of the Faculty of Veterinary Medicine, the University of Mosul on the 6th of September 2021 (Approval code UM.VET. 2021.20).

Animals and location of the study

Forty hundred eighty (480) calves from thirty-five (35) private herds were included in this study through a cross-sectional survey of different areas in Mosul from October 2021 till the end of April 2022. The animals were: ≤1 and ≥1 year old, from both sexes, local and imported or ins. According to the herd size, they were from small herds (≤40) and large herds (≥40 calves). The epidemiological information mentioned above and the case history were recorded from the breeders, and the most important clinical signs were recorded in a pre-prepared clinical card during farm visiting and sampling process.

Collection and handling of faecal samples

A total of 480 samples of faeces were collected from animals in sterile rubber gloves for each sample, placed in clean, dry, leak-proof, transparent plastic containers, and transported to the laboratory for examination in the clinical pathology laboratory of the Veterinary Medicine College, University of Mosul. Samples that were not examined on the same day were kept cooled at 4 °C to be examined on the following day.

Laboratory analysis

Faecal egg counts. For each faecal sample, the egg counting was done by using Mini-FLOTAC technique. In our work this method and according to our knowledge was used for the first time as a new and alternative method to McMaster method. Briefly, two grams of the faecal sample were added to 38 mL of saturated salt solution and homogenised. Then, the two counting chambers in the disc were filled with filtered slurry by the filter on the top cover. After that the disc was left for 10 minutes horizontally. It was transferred to the microscope for examination with a magnification power of 100× (Cringoli et al., 2017; Amadesi et al., 2020). Eggs per gram were calculated using the following equation:

EPG = \frac{\text{(total eggs of two chambers)}}{10}

Larval culture (LC). Thirty grams of faeces were laid in a jar and mixed with a little fine charcoal, and drops of water were added to the faeces to maintain the appropriate humidity. The cultures were kept at 25 °C for a period of 1–2 weeks for the purpose of obtaining infective larvae (L3) in order to distinguish them (Roberts & O'Sullivan, 1950; Zajac, 2012; Van Wyk & Mayhew, 2013). Then the larvae were harvested using Baermann technique (Van Wyk & Mayhew, 2013). Moreover, the L3 were collected for identification and additional examinations (Zhou et al., 2019).

DNA extraction. The DNA of Oster tagia spp. and Haemonchus spp. worms was extracted according to the instructions of the manufacturer (FAVORGEN Biotech Corporation Taiwan). Measurement of the extracted DNA was confirmed by agarose gel electrophoresis by mixing 5 μL of the extracted DNA with 1 μL loading dye and placing it in the gel well with the addition of the DNA ladder indicator. The gel was prepared and transported as mentioned later.

Polymerase chain reaction procedure. In this study, a 321 bp fragment from the rDNA ITS-2 region was amplified for
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**Haemonchus spp.** using the universal primers NC1 and NC2 proposed by Gasser et al. (1993): NC1F-ACGTCTGGTGTTAGGTTGTTGGTCTTCTTCTCCTCGCT. The global initiator forward (NC1-F) is located in the S5.8 subunit gene and the universal initiator reverse (NC2-R) in the S28 subunit gene. It is known that the S5.8 gene is located between the 18S and S28 genes and is separated from each of them by ITS1 and ITS2 respectively. The specific primers for the genus Ostertagia ostertagi were HSSF-TAAAAGTCGTAACAAGGTATCTGTAGGT and HSSR-GTCTCAACCATATCCACACCATG, 257 bp size at the ITS1 gene (Zarlenga et al., 2001).

The master mix was prepared with final volume 20 µL. Master mix (10 µL) was mixed with 1 µL (10 pmol/µL) from each of forward and reverse primers, 2 µL of DNA template and 6 µL of PCR grade water. After preparing the PCR tubes, the DNA was amplified in a Thermocycler T100 TM Thermal Cycler, (Bio-Rad, USA). Thermo-cycling parameters included one cycle of primary denaturation at a temperature of 94 °C for 10 min, 35 cycles (denaturation) at 94 °C for 45 s, annealing at 60 °C for 45 s for Ostertagia ostertagi and 55 °C for Haemonchus spp., extension at 72 °C for 1 min, and final extension at 94 °C for 45 s. At 72 °C for 10 min, the tubes were removed from the device and amplification process was detected on 1.5% agarose gel, power supply 80 V and 300 mA for 60 min. The gel was extracted and placed in a special imaging device Gel Doc EZ Gel Documentation System, (BioRad, USA), then the images were saved for later analysis.

**Statistical analysis**

Total prevalence of gastrointestinal nematodes in calves was calculated in the SPSS program for Windows (v. 21, IBM SPSS, USA).

**RESULTS**

Out of 480 faecal samples examined, the proportion of gastrointestinal nematodes in calves was 243/480 (50.6%) depending on the faecal examinations using Mini-FLOTAC methods (Table 1).

In the current study, the prevalence rates of abomasal nematodes Haemonchus spp., and Ostertagia spp. were 62.13% and 60.9% respectively (Table 2). The results of larval culture indicated that the percentages of L3 of Haemonchus spp. and Ostertagia ostertagi were 32% and 14% respectively (Table 3).

| Table 1. The proportion of gastrointestinal nematodes in calves (Mini-FLOTAC method) |
|-----------------------------------|----------------|----------------|
| Total number tested              | Positive number (%) | Negative number (%) |
| 480                               | 243 (50.6%) | 237 (49.4%) |

| Table 2. The proportion of Haemonchus spp., and Ostertagia spp. among positive animals |
|---------------------------------------------|----------------|----------------|
| Gastrointestinal nematodes species          | Positive animals number (%) |
| Haemonchus spp                             | 151 (62.13%) |
| Ostertagia ostertagi                       | 148 (60.90%) |

| Table 3. L3 percentage of abomasal nematodes after larval culture |
|-----------------------|----------------|
| Type of nematodes     | Percentage (%) |
| Haemonchus spp        | 32% |
| Ostertagia ostertagi  | 14% |
The results from the PCR showed for the first time the possibility of diagnosing *Haemonchus* spp. in DNA samples in Nineveh Governorate, and to our knowledge in Iraq. They were confirmed on agarose gel using Internal transcribe spacer 2 (*ITS*-2) gene, with reaction product of 321 bp (Fig. 1). The results also showed the possibility of diagnosing *Ostertagia ostertagi* using the specific primer in the form of bundles indicated through the presence of its complement sites at internal transcribe spacer-1 (*ITS*-1 region), with a reaction product of 257 bp (Fig. 2).

**DISCUSSION**

A high prevalence of gastrointestinal nematodes in calves in Mosul, Iraq, was detected: 50.6%. This result might concur or differ from earlier reports from Iraq.
Molecular identification of abomasal nematodes (Haemonchus spp. and Ostertagia ostertagi) in ... and different countries worldwide. Abdulhameed et al. (2011) revealed 60.99% total prevalence rate, represented by Ostertagia spp., with the highest percentage of 61.62% and Haemonchus spp with 40.69%, respectively. A recent study showed 18.60% prevalence of GIT nematodes in Sulaymaniyah (Aram, 2020). The data for Iran showed 81.25% GIT nematodes (Kordi et al., 2019), and in Germany – 41.1% (Gillandt et al., 2018). The reasons for different prevalence rate between the current study and the studies conducted locally and in some countries may be the laboratory tools, management systems, region, climate, sample size, breed and resistance to repellents. The results of this study are in line with those of researchers in different countries worldwide (Rupa & Portugaliza, 2016; Income et al., 2020). It is well known that traditional methods of diagnosing nematodes necessitate experience. At the same time, molecular techniques provide the possibility of additionally effective and authoritative distinction between different types of parasites. It has been pointed out that molecular biological assays are applied to identify and analyse phylogenetically different types of nematodes. This result matches published data (Tan et al., 2014; Sharidini et al., 2017). It is necessary to point out that the GIT nematodes can be precisely identified through modern methods like molecular techniques because even a single animal could be vulnerable to one or several worm types which can lead to significant impact on health and productivity of the affected animal (Agyei, 1997; Tan et al., 2014).

In summary, in Mosul, classical flotation procedures and microscopic inspection are commonly used in the diagnostic laboratories for the detection of parasite eggs. Though these methods can discriminate parasites, knowledge of genus and species is difficult to be obtained precisely, given the need for additional efforts for inexpert staff. Moreover, the larval culture is also a facility for distinguishing the specific genus properties at larval stage, but unluckily needs practical skills of staff and is time-consuming. Furthermore, differentiation up to species level might be questionable (Vlassoff, 1973; Agyei, 1997).

CONCLUSION

According to what was mentioned above, the study demonstrated a high prevalence rate of GIT nematodes in calves in the study region with Haemonchus spp. and Ostertagia ostertagi being the predominant species. The PCR approaches can be utilised as precise complementary tools for identification of worm species to avert the limitations related to morphology-based methods.

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