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Co-infection of *Haemonchus contortus* and *Trichostrongylus* spp. among livestock in Malaysia as revealed by amplification and sequencing of the internal transcribed spacer II DNA region

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Abstract

Background: *Haemonchus contortus* and *Trichostrongylus* spp. are reported to be the most prevalent and highly pathogenic parasites in livestock, particularly in small ruminants. However, the routine conventional tool used in Malaysia could not differentiate the species accurately and therefore limiting the understanding of the co-infections between these two genera among livestock in Malaysia. This study is the first attempt to identify the strongylids of veterinary importance in Malaysia (i.e., *H. contortus* and *Trichostrongylus* spp.) by amplification and sequencing of the Internal Transcribed Spacer II DNA region.

Results: Overall, 118 (cattle: 11 of 98 or 11.2%; deer: 4 of 70 or 5.7%; goats: 99 of 157 or 63.1%; swine: 4 of 91 or 4.4%) out of the 416 collected fecal samples were microscopy positive with strongylid infection. The PCR and sequencing results demonstrated that 93 samples (1 or 25.0% of deer; 92 or 92.9% of goats) contained *H. contortus*. In addition, *Trichostrongylus colubriformis* was observed in 75 (75.8% of 99) of strongylid infected goats and *Trichostrongylus axei* in 4 (4.0%) of 99 goats and 2 (50.0%) of 4 deer. Based on the molecular results, co-infection of *H. contortus* and *Trichostrongylus* spp. (*H. contortus* + *T. colubriformis* denoted as HTC; *H. contortus* + *T. axei* denoted as HTA) were only found in goats. Specifically, HTC co-infections have higher rate (71 or 45.2% of 157) compared to HTA co-infections (3 or 1.9% of 157).

Conclusions: The present study is the first molecular identification of strongylid species among livestock in Malaysia which is essential towards a better knowledge of the epidemiology of gastro-intestinal parasitic infection among livestock in the country. Furthermore, a more comprehensive or nationwide molecular-based study on gastro-intestinal parasites in livestock should be carried out in the future, given that molecular tools could assist in improving diagnosis of veterinary parasitology in Malaysia due to its high sensitivity and accuracy.

Keywords: Strongylid, *Haemonchus contortus*, *Trichostrongylus*, Infection rate, Livestock, Co-infection, Second internal transcribed spacer (ITS2) of ribosomal DNA

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Background

Nematode parasites commonly known as strongylids belonging to the order Strongylida and superfamily Trichostrongyloidea significantly affect the health of livestock [1]. Among these strongylid species, *Haemonchus contortus* and *Trichostrongylus* spp. are reported to be the most prevalent and highly pathogenic in livestock, particularly in small ruminants. It is indisputable that *H. contortus* is the most notorious parasite in livestock (i.e., ruminants) due to its biotic potential and blood sucking ability [2]. *Haemonchus contortus* infection (i.e., haemonchosis) may exhibit clinical signs such as anemia, followed by lack of appetite, lethargy, loss of weight, dehydration, oedema and death as a consequence of the disease [2-5]. As compared to *H. contortus*, *Trichostrongylus* infection may show milder clinical signs, which may result in inappetence, weight loss, poor body condition, emaciation, diarrhea, hypoproteinaemia and death in the case of heavy infection, particularly in malnourished animals [5,6].

In animal treatment management, species identification of strongylid is often deemed unnecessary; given that drug treatment is usually similar for the different species. Nonetheless, strongylid species identification is crucial in obtaining a greater understanding of the epidemiology, population biology and anthelmintic treatment efficacy, all of which are essential factors for formulating effective parasite control strategies. It is important to emphasize that this information is rarely obtained from conventional diagnostic technique. Strongylid species can only be successfully identified via advanced tools such as molecular techniques. It is important then to know this fact as it is possible that an individual animal could be susceptible to more than one strongylid species when several species are circulating in a farm pasture [7,8]. The occurrence of mixed infections may pose a serious problem as they could aggravate the health consequences of the infected animal.

In Malaysia, detection of ova is routinely performed by a floatation principle and observation under a light microscope in veterinary diagnostic laboratories, namely, universities and government agencies (i.e., Department of Veterinary Services or DVS, Malaysia). Although this technique enables a wide range of parasite detection, information of genus and species cannot be easily deciphered. Given that each genus of strongylid has a certain range of egg sizes, the overlapping sizes make it more challenging to pinpoint its genus especially for inexperienced staff. Although fecal culture is another technique for strongylid identification by defining the specific genus characteristics at larval stage, this method is unfortunately time-consuming and requires technical expertise. Furthermore, the accuracy of identification may be questionable and it is impossible to identify the strongylid up to species level.

The utilization of molecular tools such as PCR and DNA sequencing has enabled the accurate identification of parasite species [9]. These advanced techniques are highly sensitive, providing highly accurate identification of strongylids up to species level. Starting from 1990, the Internal Transcribed Spacer (ITS) of nuclear ribosomal DNA (i.e., Second Internal Transcribed Spacer or ITS2) has been developed as a reliable genetic marker in strongylid species identification [9-13] due to its high interspecific sequence divergence and intraspecific sequence homogeneity [14,15]. Among these studies, Bott et al. [12] developed a real time-PCR coupled with melting curve analysis based on the ITS2 of ribosomal DNA for the improvement in veterinary parasitology diagnosis on seven common strongylid parasites, namely *H. contortus*, *Trichostrongylus* spp., *Teladorsagia circumcincta*, *Cooperia oncophora*, *Chabertia ovina*, *Oesophagostomum columbianum* and *Oesophagostomum venolosum* in small ruminants.

In the present study, species specific primers from Bott et al. [12] were applied to amplify ITS2 DNA region of *H. contortus* and *Trichostrongylus* spp. from microscopy positive fecal samples of Malaysian livestock. This study is the first attempt to accurately identify the Strongyles of veterinary importance in Malaysia (i.e., *H. contortus* and *Trichostrongylus* spp.) by molecular methods. The application of advanced molecular tools in determining the specific identity of strongylid species will provide complementary evidence to the microscopy detection of eggs and larvae.

Results

A total of 416 rectal fecal samples from four types of livestock (i.e., 98 cattle; 70 deer, 157 goats and 91 swine) were examined (Table 1). Among the examined samples, 118 (11 or 11.2% of cattle; 4 or 5.7% of deer; 99 or 63.1% of goats; 4 or 4.4% of swine) were microscopically positive for strongylid parasites and these parasites were subsequently subjected to molecular identification of *H.contortus* and *Trichostrongylus* spp.

ITS2 DNA region of *H. contortus* was amplified in 94 (79.7% of 118) individuals, consisting of 93 isolates from goats (93 of 99 or 93.9%) and one from deer (1 of 4 or 25%) (Table 1). Of these, 92 amplicons were successfully sequenced and represented by two distinct sequence types [GenBank accession numbers KF204571 and KF204572]. Neighbour-Joining analysis revealed that both sequences were clustered with *H. contortus* sequences available from GenBank (99-100% similarity) and apparently differed from its closely related species *H. placei*. As for *Trichostrongylus* spp. detection, a total 81 amplicons were amplified, comprising 79 goats (79.8% of 99) and two deer (50.0% of 4). Of these, all amplicons were successfully sequenced revealing five sequence types [GenBank accession numbers KF204573 to KF204577]. Neighbor-Joining