Institute of Experimental Morphology, Pathology and Anthropology with Museum
Bulgarian Anatomical Society

Acta morphologica et anthropologica, 26 (3-4)
Sofia ● 2019

Genetic Variability Patterns of Haemonchus Species Affecting Small Ruminants in Egypt and Bulgaria

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The current study was carried out to study the impact of disparate geographical regions on genetic structures variability of *Haemonchus* species populations among small ruminants from Egypt and Bulgaria using mtDNA markers and to diagnose the predominant *Haemonchus* species isolated from the naturally infected sheep and goats through studying the genetic characterization and the phylogenetic relationships of such isolates. This study disclosed that the nucleotide sequences of PCR products belonged to the Cytochrome Oxidase subunit 1 gene of *H. contortus*. The dendrogram elucidated a close relation between Egyptian and Bulgarian goat isolates where they were located in the same sub genotype group. Thus goats' nucleotide sequences from Egypt and Bulgaria showed little variations among all published sequences with one substitution. Contrariwise, the Egyptian and Bulgarian sheep isolates are in two varied sub genotypes where sheep nucleotide sequences from Egypt and Bulgaria demonstrated great variation with others including five substitutions. The data obtained bring out the level of genetic variability among populations of *H. contortus* isolates of small ruminants in Egypt and Bulgaria. Thus, the current results could be a starting point for applying potent diagnosis and control measures and assaying the consequences of altering environment and management conditions.

Key words: Haemonchus, genetic variability, PCR, COI, small ruminants, Egypt, Bulgaria.

Introduction

Haemonchosis represents a serious global parasitic disease that adversely affects livestock production [1]. The blood feeding abomasal nematode *Haemonchuscontortus* (*H. contortus*); the causative agent of the disease is extremely pathogenic and the most economically vandal parasite of sheep and goats all over the world [15]. In Egypt, sheep and goats represent a significant source of the meat, milk and wool/hair industry. Similarly, in Bulgaria, sheep and goats are maintained in small private farms under

extensive conditions and sheep breeding considers one of the most important livestock industries. The pathological, epidemiological and economical impacts of the disease trigger various studies how to control the disease, thus, studying the genetic diversity and genetic relationships of this nematode via phylogenetic trees is indispensable [9]. Also, precise identification and genetic characterization is essential for valid diagnosis and increasing the efficacy of the control programs of parasitic nematodes [5]. Recently, various molecular markers have often been utilized for studying population genetics and phylogenetic analysis in different species [20]. Among the mtDNA, cytochrome oxidase subunit 1 (COI) genes have proved efficacy in various population genetic studies [8]. Furthermore, the rate of the substitution of the mtDNA was higher than the nuclear DNA [3, 6], so it is used to differentiate between firmly related individuals. Thus, the current study was fulfilled to study the comparative genetic diversity of *Hamonchus* species population among small ruminants in Egypt and Bulgaria and diagnose the predominant *Haemonchus* species isolated from the naturally infected Egyptian and Bulgarian sheep and goats through studying the genetic characterization and the phylogenetic relationships of such isolates that provide insight to improve diagnostics, comprehend the relationships between parasites, hosts, and geographic regions participating proactively in determining the potential for disease dissemination.

Material and Methods

Animals

Rapid clinical investigation of the suspected naturally infected sheep and goats was done prior slaughtering process at the abattoirs of Cairo, Giza and Qalubia in Egypt and Sofia in Bulgaria.

Parasite

Haemonchus species worms were harvested from the abomasa of the suspected naturally infected animals. Individual male worms were separated, washed extensively in PBS buffer pH 7.2 (37 °C) identified as stated by [14] and fixed in 70% ethyl alcohol.

DNA extraction

Twenty–two adult male worm specimens (18 samples as 3 of both species from three localities from Egypt and four samples; two from sheep and two from goats from Bulgaria), were prepared. DNA extraction kits (Qiagen) were utilized, as adopted by the manufacturer's protocols. DNA extracts were stored at −20°C.

PCR

The primer set utilized to amplify partially the COI gene of the *H. contortus* genome was, COIF:5′CCTACTATAATTGGTGGGTTTGGTAA–3′,COIR: 5′–TAGCCGCAGTAAAATAAGCACG–3′, as adopted by [12] PCR was done according to [10]. A total volume of 50 µl containing 1 × PCR buffer (20mM Tris–HCl and 50mMKCl at pH 8.4), 1.5mM MgCl2, 0.2mM deoxynucleoside triphosphate mixture (dATP, dCTP, dGTP and dTTP), 100 pmol of each primer, 2.5 units (U) of proof reading Thermusaquaticus (*puf*Taq) polymerase, 0.1 µg of extracted parasite genomic DNA and nuclease–free sterile double–distilled water up to 50.0 µl were used to perform PCR. Thermal profile was utilized through (Biometra thermocycler) as follows: an initial

denaturation was done at 95°C for 120 s; 35 cycles at 95°C for 50 s, 55°C for 45 s and 72°C for 60 s and the final extension at 72°C for 600 s. Agarose gel electrophoresis of 1.5% was used for analysis of the resulting PCR amplicons, as outlined by [16]. To visualize the resulted DNA bands, gel staining using ethidium bromide was used (0.5 µg/ml) against Gene Ruler 100 bp Plus ready–to–use DNA ladder (molecular weight marker) (Fermentas). Gel purification of the PCR products were processed utilizing a DNA gel purification kit (AB gene).

DNA sequencing

The PCR DNA products were sequenced with the previous primers, employing the BigDye Terminator v.3.1 Cycle Sequencing Kit on an automatic sequencer (3500 Genetic Analyzer; Applied Biosystems) [17]. The resulted data of nucleotide sequence of the COI gene of *H. contortus* from local Egyptian sheep and goats were presented to Gen Bank (KT826575, and KT826574) while that of Bulgarian sheep and goats were (KX379142 and KX379143). These sequence data were compared to other related isolates obtained by Gen Bank. The ClustalW (1.82) program of the European Bioinformatics Institute was used to align the nucleotide sequences.

Phylogenetic analysis

The analysis of the obtained nucleotide sequence involved multiple and pair–wise sequence alignments was constructed utilizing the un-weighted pair group method with arithmetic mean (UPGMA), distance method relied on Kimura 2-parameter model [13]. All trees were built utilizing the MEGA 6 program [19] and *H. placei* reference was appended as out groups.

Results and Discussion

In this study, the clinical investigation of the suspected infected animals has cleared that they suffered from weakness, loss of wool/hair, pale anemic mucosal membranes which may be due to infection by haemonchosis [5, 14]. The adult male *haemonchus* worms were selectively utilized for application of PCR to exclude the changeful DNA reinforcement which may have resulted from the eggs existed in the characteristic twisted uterine of the prolific female adult worms [6]. It is found that the mtDNA could define contrasts among closely related individuals due to its high rate of substitution in comparison with nuclear DNA [3]. So the COI gene has been used in determination of population genetic diversity in Brazil [4], in Pakistan [8] and in Egypt [10]. The analysis of PCR products has revealed about 213 bp length of a partial COI gene from the small ruminants under experiment. A particular band to *Haemonchus* species was detected in all reactions using COI specified primers. To explicate the population structure, comparison of the resulted sequences from Egypt and Bulgaria was performed with that of *Haemonchus* isolates from other nations recorded in gene bank. The obtained nucleotide sequences were recorded via MEGA 6.0 program revealing a 213 bp length of target size that coincided to nucleotide position 288 to 547 of *Haemonchus* mitochondrial genome and presented in Gene Bank with accession numbers (KT826575, KT826574, KX379142 and KX379143) from sheep, goats, of Egypt and Bulgaria, respectively. The results disclosed that the nucleotide sequence of PCR products belonged to the *H.contortus* COI gene in small ruminants. This goes in parallel with the finding obtained by [18] who mentioned that small ruminants consider the primary susceptive host that extremely infected by this hematophagus helminthes. The 9 Egyptian sheep isolates showed the

same sequence alignment with no variation so they are considered as one Egyptian sequence for sheep, as well as the 9 Egyptian goat isolates have also recorded similar sequence alignment. On the other side, the two Bulgarian isolates for the sheep were typically the same as well as, the two Bulgarian goats were also similar that may be due to the origin of the ruminants [2].

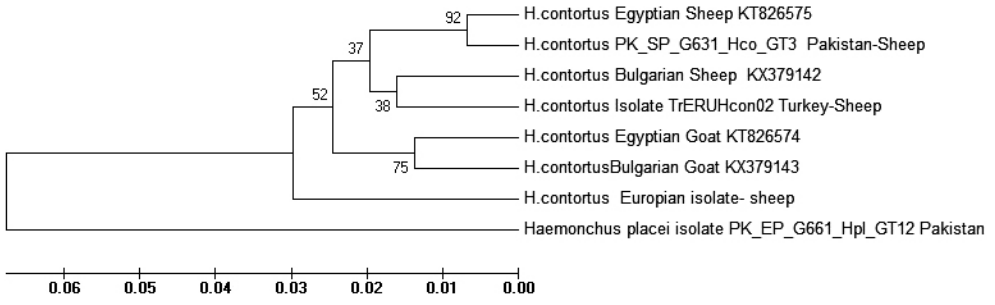

Fig. 1. Rooted Phylogenetic Tree of the Egyptian and Bulgarian *Haemonchus* species isolates with other related *Haemonchus*family and other nations isolates planned from nucleotide sequences encoding for COI gene of the analyzed *Haemonchus* genomes. A weighted pair group method with arithmetic mean dendrogram.

This suggested that the adult worms isolated from each species in each country were identical. The dendrogram showed close relation between Egyptian goat isolates and Bulgarian goat isolates where they are in the same sub genotype group; they have exhibited little diversities among all recorded sequences with one substitution (**Fig. 1**) that may be owing to the animal movement between countries which is an important determinant of population genetic structure in these nematodes [3]. While the Egyptian and Bulgarian sheep isolates are in two different sub genotypes so a major difference was noticed between sheep sequence from both countries with others in 5 substitutions that agree with the result obtained by [8, 12], who recorded variation in COI gene loci with high rate of gene flow among *Haemonchus* species from various hosts worldwide. In other context, [11] outlined that sheep were more susceptible to the heavy infection of helminthes than goats that may be owing to their various feeding behavior. While feeding of sheep and goats together, goats evade the 3rd stage infective nematode larvae as they intake usually woody plants, minimizing the larval swallowing, which usually are still on the grass preferred by sheep [7]. Thus this difference among the sheep nucleotide sequence from Egypt and Bulgaria isolates may be attributed to the repeated exposure of infection to *haemonchus* and other parasites that increase the opportunity of mutation and variation in genetic structure. Concerning more understanding of structural populations, the partial genomic sequences (213bp) of *Haemonchus* COI gene obtained from different Egyptian and Bulgarian sheep and goats was compared with that of four reference genotype sequences recovered from the Gen Bank from other nations and genus (**Fig.1**). The results declared that the Egyptian sheep isolates were more related to Pakistani isolate while Bulgarian sheep isolates were more related to the Turkish isolate. This might be due to several factors including the possible transportation of host via the different geographic areas as explained by [2], beside that both species in the different countries may have originated from the same ancestor during their developmental history [4]. This study has confirmed the *H. contortus* dominance and prevalence in both sheep and goats in Egypt and Bulgaria. The current work revealed

apparition of the homology between goat nucleotide sequences from Egypt and Bulgaria *Haemonchus* isolates among all published sequences while great variation was noticed in sheep sequence from Egypt and Bulgaria *Haemonchus* isolates. Our results declared that the migration of various hosts between the different geographical areas could affect the population genetic structure and facilitate the cross infection between variant hosts. The phylogenetic analysis of *H. contortus* separated from the Egyptian and Bulgarian small ruminants, could be an important aid in proceeding of proper management and control programs.

Acknowledgements: This work was funded from a bilateral project between the Academy of Scientific Research in Egypt and the Bulgarian Academy of Sciences, entitled "Recent methods for diagnosis of haemonchosis".

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