Vol.65: e22210369, 2022
https://doi.org/10.1590/1678-4324-2022210369
ISSN 1678-4324 Online Edition

BABT
BRAZILIAN ARCHIVES OF BIOLOGY AND TECHNOLOGY

Article - Human and Animal Health

First Record of Molecular Confirmation, Phylogeny and Haplotype Diversity of *Haemonchus contortus* from Gaddi (breed) Goats of North India

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Editor-in-Chief: Alexandre Rasi Aoki
Associate Editor: Renata Marino Romano

Received: 05-Jun-2021; Accepted: 21-Oct-2021.

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HIGHLIGHTS

- First report of phylogeny and haplotype diversity of *Haemonchus contortus* isolated from Gaddi (breed) goats of North India.
- The occurrence of different haplotypes evinced presence of indels at distinct places.
- There was probable establishment of heterozygosity in putative hybrid *H. contortus* worms.

Abstract: In the present study, molecular identification and genotypic characterization of *H. contortus* was carried out targeting 28S-18S rRNA intergenic spacer. Faecal samples of Gaddi goats were collected and subjected to qualitative screening. The samples exhibiting the presence of strongyle type eggs were introduced to faecal culturing. The larvae retrieved were molecularly confirmed as of *H. contortus* species and the phylogenetics was performed. For the estimation of evolutionary divergence in between the present study isolates with the GenBank archived sequences, maximum composite likelihood model was employed. Nucleotide and haplotype diversity indices and *Fu's Fs* were also estimated. Approximately 260 bp size amplicons retrieved were confirmatory for the presence of *H. contortus* species. Phylogenetic analysis also accentuated that present parasite isolates were of *H. contortus* only. The nucleotide diversity (π) obtained was 0.06696, whereas, haplotype diversity was 0.92549 [95% CI: 0.77778-1.0000]. In between the isolates, *Fu's Fs* statistic value was positive (1.566), evidencing a deficiency of alleles, which would have happened due to recent population bottleneck. The recovered representative sequences were deposited in GenBank under the accession numbers LC600315-LC600317.To the best of our knowledge, the present study is the first report of phylogeny and haplotype diversity of *H. contortus* isolated from Gaddi goats of North India. The present study would also serve the basis for future detailed molecular epidemiological studies using

discriminative markers for the assessment of genetic diversity in different populations of *H. contortus* in different hosts of the study area.

Keywords: *Haemonchus contortus*; haplotype diversity; Gaddi breed; goats; phylogeny.

INTRODUCTION

Haemonchus contortus, commonly known as 'wire worm' or 'barber pole worm', is one of the most important strongylid infecting millions of sheep and goats globally [1]. As helminthoses pose serious threat to small ruminant production, *H. contortus* is responsible for huge economic losses to the farmers [2]. Most common production losses are associated to decline in wool production, severe weight loss, and eventually mortality [3]. Due to its capability to cause acute, sub chronic and chronic infections in the susceptible hosts, the parasite causes severe gastroenteritis, anaemia, and hence mortality in heavily infected animals of all age groups [2, 4].

In the North-West Himalayan region (especially Himachal Pradesh), Gaddi (breed) goat farming is most commonly practised due to its low investment and good output (wool, milk and meat) [5-6]. The animals are commonly allowed to graze in pastures. The infected pastures are most common source of infection to the susceptible hosts. With the production of around 10,000 eggs/ day, *H. contortus* females are prolific, resulting in heavy parasitic burden in the form of large population size (eggs and larvae) in pastures [7]. The genetic structural differences in *H. contortus* populations are significantly high globally [8], but are generally low within contiguous geographic regions [9]. This could be attributed to the fact that gene flow among nematode subpopulations is being governed by host movements [9-10].

The parasite *H. contortus*, due to its great biological and ecological plasticity possesses a unique survival strategy [2]. Mainly two or sometimes three *Haemonchus* species are sympatric in several regions of the world especially where small and large ruminants share the pastures [2, 7, 11]. Hence, for the successful implementation of sustainable parasitic control programmes, the correct identification of the species becomes extremely important. Also, the small and large ruminants sharing the same pastures result in genetic flux of parasitic populations, eventually leading to intra- and interspecies transmission of resistant genes [12]. Thus, in the present study, we adopted molecular approach to confirm *H. contortus* infection in Gaddi breed goats of North India. We also characterised (including haplotype diversity) the retrieved parasitic isolates targeting 28S-18S rRNA intergenic spacer.

MATERIALS AND METHODS

Sample collection and parasitological examination

Faecal samples were collected from all the Gaddi goats (n=41) reared at Livestock Farm, College of Veterinary and Animal Sciences, Palampur (India). The samples were subjected to standard parasitological examination (floatation and sedimentation techniques) for qualitative assessment of parasitic load [3].

Faecal culture and larvae isolation

The samples found positive for strongyle type eggs were introduced to faecal culture as per Singh [13]. After 7 days of incubation at 27°C, larvae from the culture were harvested using Baermann method [13]. The larvae thus recovered were identified as per van Wyk and Mayhew [14]. Mean and standard deviation values pertaining to the measurements of larvae were assessed using Microsoft Excel. *Haemonchus contortus* larvae were then isolated for further molecular confirmation and phylogenetic analysis.

Genomic DNA extraction, PCR amplification

The larvae (n=10) collected from individual animal were subjected to genomic DNA extraction using DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany). DNA retrieved was stored at -20°C until further use. The polymerase chain reaction (PCR) was performed for amplification of DNA sequences targeting 28S-18S rRNA intergenic spacer. The published primers [15] employed in the present study were; Forward: 5′-TGT CGA ACA CGA AAC TCG TC-3′ and Reverse: 5′-TGT GTC TCT ACC GCC CGA GT-3′. The 25 µl reaction mixture constituted of 12.5 µl Master Mix (Thermo Scientific, USA), 1 µl of each primer (forward and reverse), 1 µl of genomic DNA and 9.5 µl of nuclease free water. The reaction conditions were: initial denaturation (95°C for 5 min.), denaturation (95°C for 30 sec. × 35 cycles), annealing (59°C for 40 sec. × 35 cycles), extension (72°C for 30 sec. × 35 cycles) and final extension (72°C for 5 min.). The amplicons

retrieved were subjected to electrophoretic separation on 1.5% agarose gel and were visualised under gel documentation system for detection of 260 bp amplicon size. The genomic DNA from *Toxocara* species was also run simultaneously (in PCR) as a negative template control to check the specificity of primers.

DNA sequencing and phylogenetic analysis

The amplicons (260 bp) retrieved were subjected to custom sequencing (GeneBio Solutions, Dehradun, India). Manual correction for any misread sequence, analysis and final alignment of resulting gene sequences was performed with BioEdit software [16]. The product sequences were confirmed and their homology was established after comparing them with reference sequences (MW054180, MW054174, MW054176, MW054188, HQ389234, and MW054169) archived in GenBank, by using NCBI BLAST algorithm (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The evolutionary history was inferred after constructing the phylogenetic tree with other isolates (exhibiting similitude with present study isolates), retrieved from GenBank using the Maximum Likelihood method and Tamura-Nei model in MEGA X (Molecular Evolutionary Genetic Analysis) software [17]. The sequence of *Toxocara vitulorum* (MG214153.1) was included in the phylogenetic analysis as out-group species to root the tree. For the estimation of evolutionary divergence in between the present study isolates with the archived GenBank sequences, maximum composite likelihood model was employed [18]. Nucleotide and haplotype diversity indices and *Fu's Fs* were estimated in DnaSP v5 software [19].

RESULTS

Parasitological observations

The faecal samples subjected to faecal culture revealed the presence of third stage larvae of *H. contortus* with characteristic bullet shaped head and a fine sheath tail with a measurement of 71.9 ± 2.8 mm (99% confidence level). The intestine constituted of 16 cells with the end possessing two terminal cells.

Molecular confirmation and phylogenetic observations

Approximately 260 bp size (Figure 1) amplicons retrieved after gel electrophoresis were confirmatory for the presence of *H. contortus* species. Moreover, the nucleic acid (DNA) of *Toxocara* species did not exhibit amplification validating the specificity of the primers. The recovered representative sequences were deposited in GenBank under the accession numbers LC600315, LC600316 and LC600317. The other isolates (archived in the GenBank: MW054180, MW054174, MW054176, MW054188, HQ389234, MW054169) from the definitive hosts showed high similarity (more than 94%) with the isolates retrieved in the present study (Figure 2). Two isolates of the present study (LC600315 and LC600316) represented one haplotype; whereas a single isolate (LC600317) represented another haplotype (Figure 2). The manifestation of these two haplotypes suggested the presence of two distinct genetic variants of *H. contortus*. *Toxocara vitulorum* (MG214153.1, Egypt), included as an outgroup species occupied the basal position in the phylogenetic tree (Figure 2).

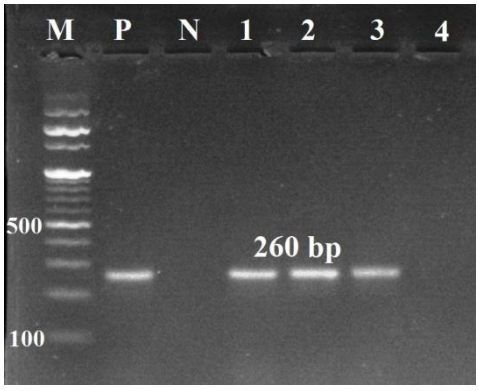

Figure 1. PCR amplification targeting 28S-18S rRNA intergenic spacer. M. 100 bp marker; P. Positive control; N. Negative template (*Toxocara* species) control; 1-3. PCR products of *Haemonchus contortus*; 4. No template control

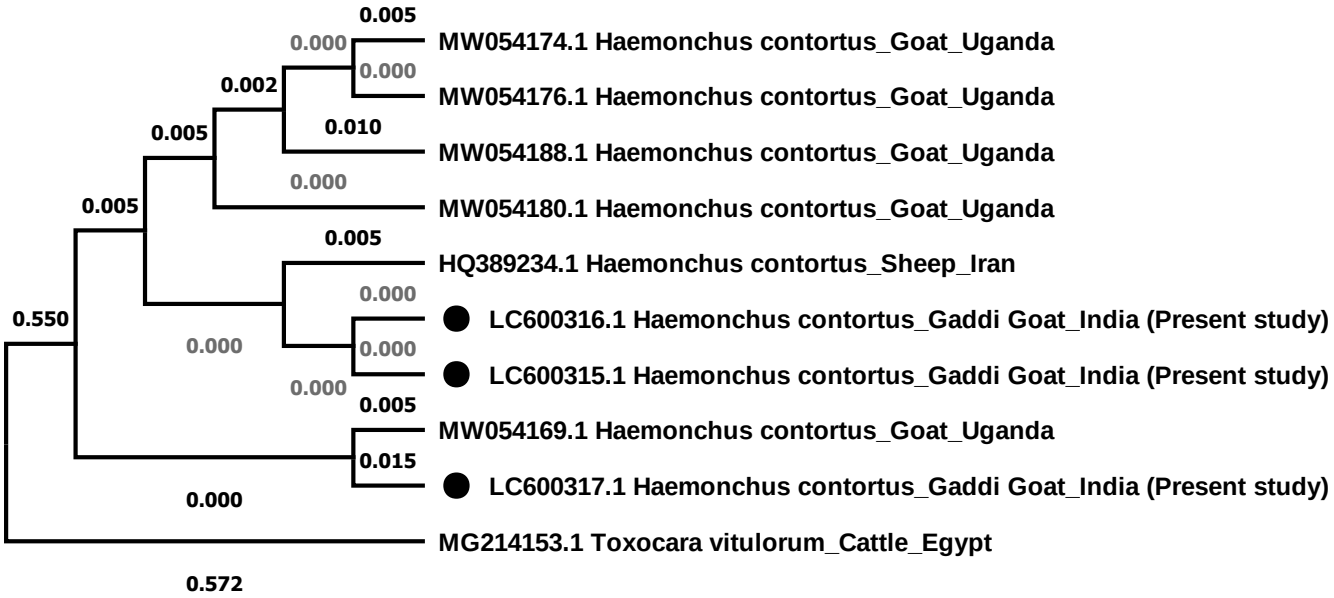

Figure 2. Phenogram of *Haemonchus contortus* isolates based on 28S-18S rRNA intergenic spacer. The phylogenetic tree was constructed by the Maximum Likelihood method and Tamura-Nei model by using MEGA X software. All accession numbers correspond to different isolates followed by their host and country of origin. The new sequences generated in the present study are marked with black solid filled circles.

The nucleotide diversity (π) obtained was 0.06696, whereas, haplotype diversity was 0.92549 [95% CI: 0.77778-1.0000]. In between the isolates, *Fu's Fs* statistic value was positive (1.566). The observations based on maximum likelihood model exhibited low/less differences between the present study and archived isolates, as the values were extremely low (< 0.034) in pair-wise comparison (Table 1). The three isolates retrieved in the present study exhibited nucleotide homology ranging between 92.76-97.30%. Various nucleotide substitutions (at positions 13, 24, 32, 33, 41, 100, 106, 110, 115, 125, 126, 157, 165, 166, 173, 174, 175, 201, 230, 248, 249, 250, 251) and deletions (at positions 1, 2, 3, 25, 35, 36, 52, 81, 92, 103, 111, 112, 116, 117, 127, 128, 129, 130, 131, 159, 160, 185, 186, 236, 237, 250-261) were observed in all the three isolates (Figure 3).

Table 1. Estimates of evolutionary divergence between sequences based on the maximum likelihood model

	1	2	3	4	5	6	7	8	9
MW054188.1									
MW054180.1	0.01931								
MW054176.1	0.01542	0.00382							
MW054174.1	0.01934	0.00767	0.00383						
MW054169.1	0.03546	0.01949	0.01949	0.02348					
LC600317.1	0.03355	0.02375	0.02376	0.02864	0.01901				
LC600316.1	0.01387	0.00458	0.00458	0.00921	0.00921	0.01902			
LC600315.1	0.01413	0.00467	0.00467	0.00938	0.00938	0.01938	0.00000		
HQ389234.1	0.02728	0.01545	0.01157	0.01548	0.02348	0.02370	0.00457	0.00466	

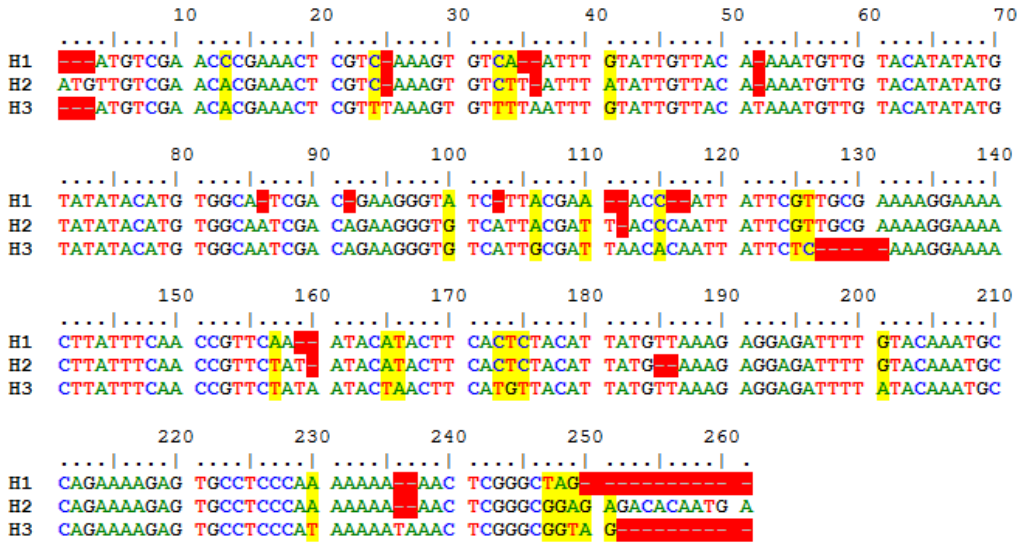

Figure 3. Nucleotide variations in three isolates of *H. contortus* (H1, H2 & H3) based on 28S-18S rRNA intergenic spacer sequences showed substitutions (yellow coloured) and deletions (red coloured)

DISCUSSION

Haemonchus genus comprises of 12 different species with *H. contortus* and *H. placei* are the most widespread [20]. *Haemonchus contortus* is considered as one of the most problematic and successful parasite of domestic and wild artiodactyl hosts in almost all the regions of the world [21]. Its control is becoming difficult day by day due to its remarkably high propensity to develop resistance to anthelmintic drugs [21]. The extremely high levels of genetic diversity of *H. contortus* are responsible for its high adaptive capacity [21]. The detailed understanding of its population structure and genetic diversity is extremely important for many areas of research including epidemiology, control, anthelmintic resistance, drug/ vaccine development, and molecular diagnostics [21].

Various morphological and molecular studies had been carried out in the past to assess the prevalence of different *Haemonchus* species in distinct populations. The epidemiological studies carried out in different parts of the world had even established the presence of genetic hybridization between the two *Haemonchus* species (*H. contortus* and *H. placei*) [2, 22-23]. Genetic hybridization leads to various adverse consequences as it can lead to introgression of genes responsible for pathogenicity, drug resistance, transmission and host specificity [22]. For definitive molecular identification and for the establishment of intra- and inter-species genetic variation most appropriate genetic markers are required [24]. Various molecular markers including species-specific rDNA internal transcribed spacer 2 (ITS-2), mitochondrial NADH dehydrogenase subunit 4 and 28S-18S rRNA intergenic spacer had been employed in the past to adjudge the species of the prevalent parasite in different ruminant populations [22], which is difficult with the application of classical Parasitology (morphology and morphometry) though. It is a constraint in epidemiological studies that *Haemonchus* infection is not diagnosed accurately in the field conditions resulting in unclear picture on true species prevalence and the extent of co-infections [22]. Based on the scarcely available data from different parts of the world, it is quite evident that two species i.e. *H. contortus* and *H. placei* are sympatric and co-infection is common [25-26] including a recent study carried out in South India [22]. Hence, the present study was planned to assess the prevalence of *H. contortus* species in Gaddi breed goats (in North-West Himalayan region) employing both molecular and morphological techniques.

Varying degrees of morphological variations within and between different species of *Haemonchus* were reported in the past, which made accurate species identification challenging as well as time consuming [22]. In case of adults of subfamily Haemonchinae, the most reliable differences are in the patterns of longitudinal cuticular ridges of the synlophe and in chromosomal morphology [22, 27]. The observations pertaining to third stage larvae of *H. contortus* in the present study were in concordance to the findings of van Wyk and coauthors [28]. However, presence of two terminal cells at the end of intestine is a differentiating feature of *Haemonchus* species from *Ostertagia* species, which has only one [28].

An amplicon size of 260 bp retrieved in the present study was in consistency to the findings of Ramos and coauthors [15], justifying the infection of *H. contortus* species in Gaddi goats. The reason for encountering two different haplotypes could be attributed to existence of intra-specific variations in *H. contortus* [29]. Earlier, it was believed that amongst rDNA multigene families, the nucleotide sequence

homogeneity is maintained within individuals/ species by 'concerted evolution' [30]. However, varying degrees of intra-specific variations had been recorded in the nucleotide sequences of ITS-1 and/or ITS-2 [29] and other ribosomal nucleic acid sequences/ genes. The intraspecies variable positions observed in the present study were indicative of probable heterozygosity in putative hybrid worms [22]. The extremely low values obtained in estimation of evolutionary divergence based on maximum likelihood model indicated the absence of diverged lineages.

In between the isolates, ***Fu's Fs*** statistic value was positive (1.566), evidencing a deficiency of alleles, which would have happened due to recent population bottleneck [31]. The present study also exhibited high haplotype diversity (0.92549), which is in concordance with the observations of Shen and coauthors [32]. However, observation of low nucleotide diversity was indicative of an excess of rare polymorphic sites and hence pointed towards purifying selection or recent population expansion [33].

CONCLUSION

It was evident from the morphological and molecular studies that *H. contortus* was the species infecting the Gaddi breed goats. However, presence of two different haplotypes evinced presence of indels at distinct places, which were further suggestive of probable establishment of heterozygosity in putative hybrid worms. Keeping in view the ever rising rates of anthelmintic resistance in *H. contortus* population and the possibility of transmission of resistance genes with intra- and inter-species hybridisation, further detailed study in this aspect is warranted. The present study would also serve the basis for future detailed molecular epidemiological studies using discriminative markers for the assessment of genetic diversity in different populations of *H. contortus* in different hosts.

Acknowledgements: We thank the Dean, College of Veterinary and Animal Science (Hisar) India, for providing the facilities to carry out the research work.
Funding: The present study received no specific grant from any funding agency, commercial or not-for-profit sectors.
Conflict of Interest: The authors declare that there are no conflicts of interest with respect to the research, authorship, and/or publication of this article.
Ethical considerations: No studies involving laboratory animals or invasive techniques were conducted. The faecal samples were collected from goats without any invasive technique.

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