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Molecular Identification of *Mecistocirrus digitatus* in Cattle from Mymensingh Region of Bangladesh

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ABSTRACT

Gastrointestinal nematode infections of livestock are ranked in the top twenty diseases affecting small-holder farmers' livestock. *Mecistocirrus digitatus* is one of the most prevalent parasitic nematode among the trichostrongylids causing severe health hazards leading to production losses in cattle worldwide. This study was conducted to explore the existence and genetic diversity of *M. digitatus* parasite populations from cattle characterizing second internal transcribed spacer (ITS-2) gene of nuclear ribosomal DNA (rDNA). A total of 23 adult *Mecistocirrus* parasites were collected from abomasa of slaughtered cattle from Mymensingh district of Bangladesh. After the extraction of DNA from adult parasites, ITS-2 of nuclear rDNA gene was amplified and sequenced. The edited and aligned sequences were employed for analysis to determine sequence variation and genetic diversity. All the sequences were found to have high identical ratio with *M. digitatus* of a published sequence and sequence identities ranged from 97.9% to 100%. Genetic analysis revealed 3 distinct ITS-2 genotypes among the *M. digitatus* isolates. The nucleotide and genotype diversities were 0.00089 and 0.170, respectively for ITS-2 sequences. Phylogenetic analysis (neighbour joining, maximum likelihood and maximum parsimony) of ITS-2 sequences indicated the existence of a single cluster within *M. digitatus* population in the study area. In conclusion, our study could confirm *M. digitatus* in the analyzed parasite isolates by amplifying and sequencing ITS-2 gene. Most of the isolates from our present study presented identical genotypes indicating that low genetically diversified parasites are circulating in Mymensingh region of Bangladesh. The findings of our study creates a basis for further molecular epidemiological surveys applying more *M. digitatus* parasite isolates from different regions of Bangladesh.

Introduction

Gastrointestinal nematodes (GINs) of domestic ruminants are of major veterinary importance due to their high prevalence, ubiquitous distribution, pathological consequences and the associated economic production losses. GINs are one of the major threats to the cattle industry due to their detrimental effects on weight gain and milk production of cattle (Perri *et al*., 2011; Sutherland and Leathwick, 2011; van der Voort *et al*., 2013). Infections with blood-feeding GINs, such as *Haemonchus contortus* and *Mecistocirrus digitatus*, significantly hamper animal welfare and cause production losses globally (Molento, 2009). *M. digitatus* is an important blood-sucking nematode of cattle in Asia and Central America (Soulsby, 1982). GINs of livestock are ranked in the top twenty diseases of livestock affecting the farmers' ability to maintain food security and contribute to economic growth in Asia and Africa, where resource-poor small holder farming is usually common (Dicker 2014). *M. digitatus* causes severe micro- and macroscopic lesions such as mucosal inflammation, hemorrhage, ulcers, and necrosis in the abomasum of infected ruminants (Gaur and Dutt, 1973). The high pathogenicity of this nematode causes significant losses in the cattle industry (Van Aken *et al*., 1997). Several studies have reported the presence of *M. digitatus* infection in cattle and buffaloes in different parts of India and Bangladesh (Mondal *et al*., 2000; Sreedhar *et al*., 2009; Laha, 2013). In Bangladesh, *M. digitatus* has been found as one of the most prevalent GINs in cow calves (Mondal *et al*., 2000). Islam *et al.* (1992) reported 15.2-25.5% of *M. digitatus* and *H. contortus* in water buffaloes in Bangladesh. Moreover, this GIN has been found in humans as a consequence of accidental infections, thereby representing a public health risk (Tantalea´n and Sa´ nchez, 2007).

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From the morphological standpoint, nematodes are rather uniform which makes topical elaboration of the molecular methods especially for their identification. In the applied aspect, the accuracy in identification of species and intraspecific groups is of paramount importance for the nematodes parasitizing domestic animals (Aksenov and Spiridonov, 2013). Variation of the DNA domains is being used as markers at the levels of species, population, and even individuals; genetic identity of morphologically distinct forms; and the presence of genetically independent species within a morphological homogeneous sample of parasites (Anderson, 1995). The problem of species and genotype identification may be resolved by the morphology supported by molecular characterization of the marker sequences. Molecular characterization is mainly used as an essential tool for the validation of nematode species and phylogenetic analysis. Knowledge about the genetic variation of *M. digitatus* within and among populations can help to understand the transmission patterns, spread of drug resistance alleles, and eventually supports the formulation of an effective control strategy (Gasser *et al*., 2008).

PCR-based methods using specific genetic markers in the internal transcribed spacer (ITS)-2 of nuclear ribosomal DNA (rDNA) have provided enhanced epidemiological tools. ITS-2 region of nuclear rDNA is designated as an important marker because of its ease of amplification, availability of conserved regions, sufficient number of rRNA clusters, fast evolution of variable nuclear loci and adequate amount of variation to distinguish closely related species (Avramenko *et al*., 2015; Cerutti *et al*., 2010). To the best of our knowledge, no research has been conducted in Bangladesh to explore the genetic variation of this blood feeding GIN, *M. digitatus* of cattle. The present work was therefore carried out with the purpose of molecular identification and characterization of *M. digitatus* based on the ITS-2 sequences and provides some imperative information/data on genetic diversity of this GIN isolates in the study area.

Materials and Methods

Parasite materials and isolation of DNA

Recovery of adult worms was carried out according to standard procedures as described by MAFF (1986). Briefly, after slaughtering of cattle, the abomasum was separated from the other stomach parts and ligated at both ends. The abomasum was then taken directly to the laboratory and the contents were poured into a glass beaker. Both the abomasum and its contents were carefully examined and individual adult male worms were collected. The collected parasites were then washed extensively in physiological saline. The male parasites were identified based on the morphological features such as the bursa having a small, symmetrical dorsal lobe, shorter ventroventral ray than lateroventral and anteolateral rays with 3.8-7 mm long slender spicules united together for almost their whole length (Soulsby, 1986). Only adult male worms were utilized for DNA amplification by PCR in order to avoid the danger of temperamental DNA enhancement from the eggs of female worms (Gharamah *et al*., 2012). The parasites were preserved in absolute alcohol, until DNA was extracted. Total genomic DNA was isolated from 24 individual worms using QIAamp DNA Mini Kit (Qiagen, Germany) according to manufacturer's instructions. Briefly, one adult parasite was taken into an eppendorf tube, tissues were disintegrated, lysed and proteins were digested by proteinase K and washed extensively. Trapped DNA was eluted by elution buffer. Extracted DNA was measured by a Nanodrop spectrophotometer and stored at −20 ^{o}C until further use.

PCR amplification and sequencing

Molecular characterization of *M. digitatus* using ITS-2 gene was performed. ITS-2 region (~320 bp) was amplified using previously published primers; forward 5'-ACGTCTGGTTCAGGGTTGTT-3' and reverse-5'-TTAGTTTCTTTTCCTCGCCT-3' (Stevenson *et al*., 1995). PCR was performed in a 50 µl reaction for 30 cycles (Initial denaturation of 95 °C for 5 min followed by denaturation at 95 °C for 1 min, primer annealing at 55 °C for 60 s and extension at 72 °C for 60 s), followed by a final elongation of the PCR product for 5 min. at 72 °C. A negative and positive control containing distilled water and DNA of *Haemonchus contortus*, respectively, were included during PCR to ensure reliability, validity and to check for possible contaminations of the amplification reactions**.** PCR products (5µl) were visualized on agarose gel (1.5%) with EZ-Vision® IN-Gel (Amresco, USA) (Figure 1).

Sequencing of PCR products of ITS-2 gene

All PCR products were sequenced directly using appropriate forward and reverse primers. The PCR products were column purified (Wizard PCR-Preps, Promega,) and then subjected to sequencing directly (BigDye Terminator v.3.1 cycle sequencing kit, Applied Biosystems in an automated sequencer (PRISM3730, ABI using respective forward and reverse primers (in separate reactions). Forward and reverse sequences were aligned and edited using the BioEdit software (Hall, 1999). The sequences were aligned using MEGA v.10.1.8 software (Tamura *et al.*, 2013) and deposited in GenBank under the accession numbers: LC594625-LC594627 .

Molecular data analysis

The sequences of ITS-2 were aligned using the program Clustal W within MEGA v.10.1.8 (Tamura *et al.*, 2013).

Pairwise comparisons were performed with previously published sequences, and identities (%) were calculated using the program BioEdit (Hall, 1999). Phylogenetic analysis was performed using neighbour joining (NJ), maximum likelihood (ML) and maximum parsimony (MP) methods based on the Tamura-Nei model (Tamura *et al.*, 2013). Confidence limits were assessed using the bootstrap procedure (1000 replicates) for constructing NJ, MP and ML trees, and other settings were obtained using the default values in MEGA v.10.1.8 (Tamura *et al.*, 2013). A 50% cut-off value was implemented for the consensus tree. A hierarchical analysis of molecular variance (AMOVA) was performed to estimate the genetic diversity within and among populations (isolates) using the Arlequin 3.1 package (Excoffier *et al.*, 2005). In addition, sequences retrieved from GenBank were used for comparisons.

Results

Species identification and genotyping

ITS-2 gene was characterized for identification of the *M. digitatus* parasite. ITS-2 gene fragment was PCR amplified from the genomic DNA for confirmation of the *Mecistocirrus* species . PCR amplified (~350 bp) products (Figure 1) were sequenced to determine the species of *Mecisticirrus* of cattle from Mymensingh region in Bangladesh. After editing and alinging of the resulted sequence, 290 bp consensus length was obtained for all the samples. ITS-2 PCR products of 23 worms were randomly sequenced for confirmation of the species that showed that all the specimens were belonged to *M. digitatus*. The analysis of the 23 ITS-2 sequences revealed 3 distinct genotypes. Identical ITS-2 genotype profiles were found for 21 isolates, other two isolates showed unique profile. The sequence identities ranged from 98.6% to 99.6% when compared with each other and 98.6 % to 100.0 % while compared with ITS-2 reference sequence of *M. digitatus* from GenBank (accession no. AJ577468.1) (Table 1). These sequences of *M. digitatus* were also compared with one publicly available sequence of *H. Contortus* (accession no. X78803.1) that revealed the identities of 62.0% to 62.5%. The overall nucleotide diversity and genotype diversity were 0.00089 and 0.170, respectively, among the ITS-2 sequences of M. *digitatus* from Mymensingh region of Bangladesh. The results indicated Identification of almost genetically identical isolates of *M. digitatus* in Mymensingh region of Bangladesh.

Phylogenetic analysis

The NJ phylogenetic tree was constructed using 23 ITS-2 sequences of *M. digitatus* from Mymensingh, Bangladesh. To build this tree, the NJ method in MEGA v.10.1.8 was used and the evolutionary distances were computed using the Tamura-Nei model. For comparison, 6 ITS-2 sequences of *M. digitatus* and one reference sequence of *H. contortus* (accession no. X78803.1) as out-group from different countries were retrieved from GenBank databases. The analyses showed that the NJ phylogram falls into two main clusters. One cluster containing 23 isolates from Mymensingh (Bangladesh), 2 isolates from India (accession nos. MG679517.1 and KJ192195.1), and one from France (accession no. AJ577468.1). Another cluster containing 2 isolates from Japan (accession nos. AB222059.1 and AB114420.1) and one from Philippines (accession no. AB222060.1). The cluster including 23 *M. digitatus* isolates from Mymensingh, Bangladesh has 72% bootstrap support. Our results demonstrated the existence of a single cluster within *M. digitatus* population in Mymensdingh, Bangladesh.

Table 1. Pairwise identities (%) among 3 ITS-2 genotypes of *M. digitatus* representing 23 samples from Bangladesh using selected sequences of *M. digitatus* and *H. contortus* from GenBank

Sample ID	1	2	3	4	5	6
BDMYMD26	–					
BDMYMD22	99.6	–				
BDMYMD21	98.6	98.9	–			
M. digitatus (MG679517.1)	100	99.6	98.6	–		
M. digitatus (AJ577468.1)	100	99.6	98.6	100	–	
H. contortus (X78803.1)	62.5	62.2	62	62.5	62.5	–

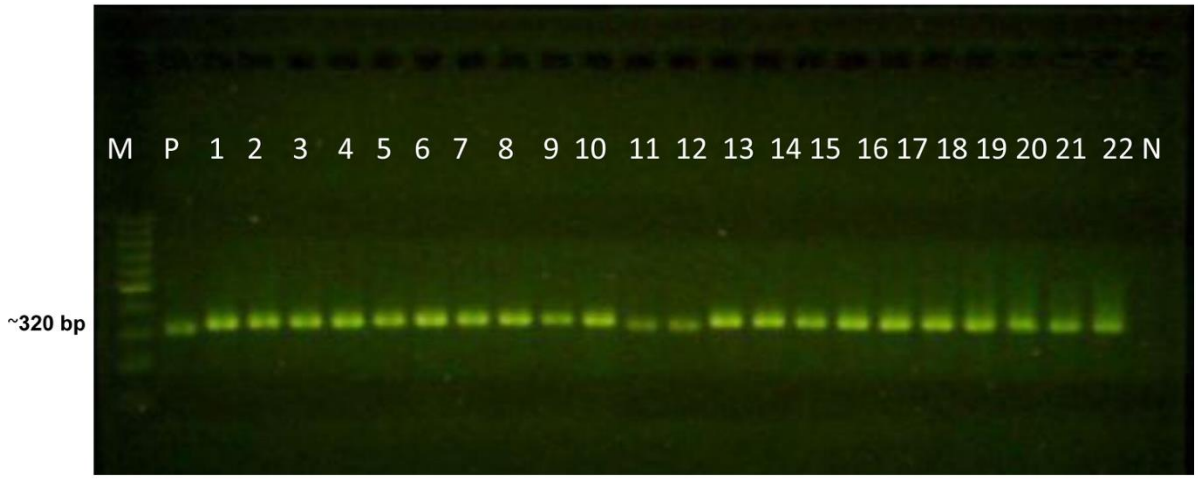

Figure 1. PCR amplification products of *Mecistocirrus* isolates. Amplification of ~350 bp ITS-2 gene for *Mecistocirrus* sp. represented on 1.5% agarose gel; lane M: 100 bp marker; Lane 1-22: ITS-2 PCR products; P: Positive control; N: Negative control.

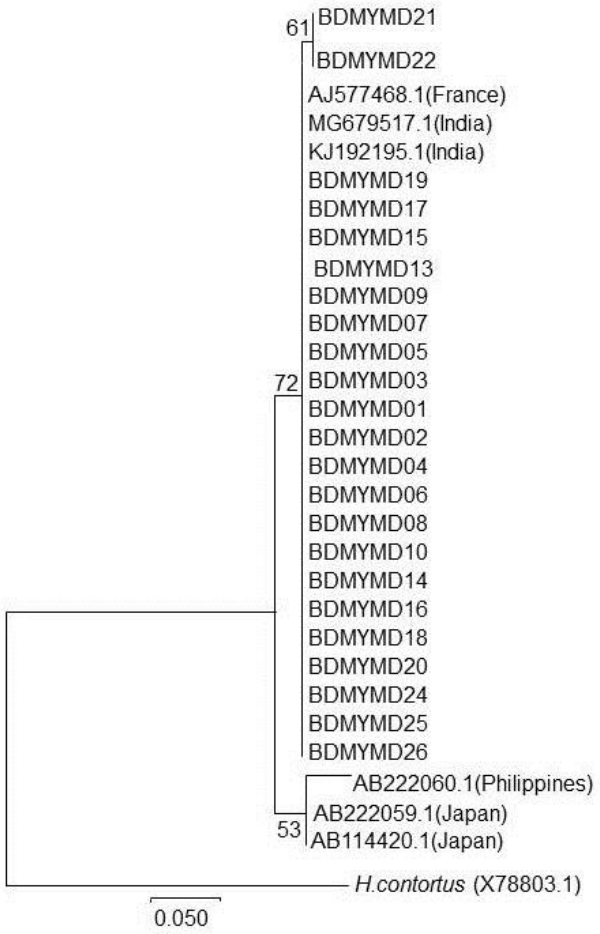

Figure 2. Neighbour-joining tree constructed using 23 *M. digitatus* isoaltes and. Six sequences of *M. digitatus* and one reference sequence of *H. contortus* ((X78803.1) as outgroup from different countries were retrieved from GenBank databases and used for comparison. The bootstrap value of >50% was displayed in the tree.

Discussion

M. digitatus is a hematophagous abomasal nematode which causes significant blood and production losses in cattle. Molecular techniques recommend some advantages over morphology-based detection in that it is more objective, scalable, easier to implement, and permit rapid characterization of nematode parasites to address the shortcoming of the usual diagnosis of parasitic gastroenteritis (Kandil *et al*., 2018). The rDNA is present as numerous repeated sequences in the animal nuclear genome. Although a considerable part of such sequences is rather conserved; they contain several variable regions, such as, ITSs and some segments within the rDNA large subunit. From an evolutionary standpoint, both the variation of ribosomal sequences and their certain intraspecific conservation are interesting phenomena.

For identification of the parasite species, PCR-sequencing is a reliable molecular tool. In the present study, *M. digitatus* was differentiated from *H. contortus* on amplification and sequencing of ITS-2 gene. Twenty three worms were randomly sequenced for ITS-2 gene for confirmation of the species and identified all the specimens as *M. digitatus.* Based on the sequence data of ITS-2 gene, the worms collected from the cattle in Mymensingh region were identified as *M. digitatus*. ITS-2 region has been used as markers earlier by several workers for differentiating strongyle worms including *H. contortus* (Silvestre and Humbert, 2000).

This study analyzes sequence variation in the ribosomal ITS-2 in isolates of the *M. digitatus* from cattle of Mymensingh origin. *M. digitatus* had the lowest degree of ITS-2 sequence polymorphism when compared with very closely related parasite, *H. contortus* (Dey *et al.*, 2019). Among the ITS-2 sequences of *M. digitatus*, the overall nucleotide diversity and genotype diversity were 0.00089 and 0.170, respectively, indicating that low genetically diversified parasites are circulating in Mymensingh region of Bangladesh. Most of the isolates from our present study presented identical genotypes. The identification of genetically almost identical isolates of *M. digitatus* in this study a might be due to the origin of our analyzed samples confined only to the study area. Additionally, we could not retrieve a considerable number of *M. digitatus* ITS-2 sequences from the GenBank to make the comparison. In the present study, *M. digitatus* parasite isolates were closely related to the isolates from India and France. The explanation behind such findings is that the introduction of parasite population due to random access to hosts within continent or among the neighboring countries for trading purpose (Dey *et al*., 2019).

The findings of our study provide a preliminary data on genetic diversity of *M. digitatus* isolates in Mymensingh region of Bangladesh. These results will be useful for further molecular epidemiological survey applying more parasite isolates from different regions of Bangladesh and following up on epidemiological changes, as well as for designing the control strategies against this GIN infection.

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Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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