Molecular characterization of gastrointestinal nematode parasites (*Haemonchus contortus* and *Trichuris ovis*) of goat (*Capra hircus*)

Savita[1], Naveen Kumar[2], Suman Mishra[3], and Kamal Jaiswal[4]

[1,3,4] Department of Zoology, Babasaheb Bhimrao Ambedkar University, Vidya Vihar, Raebareli Road, Lucknow - 226 025, India.

[2] Department of Ecology and Environmental Sciences, Pondicherry University-605014

Email Id: savitagm98@gmail.com

ABSTRACT: Nematodes (Roundworms) are also known as strongylids parasites belonging to the order Strongylida, superfamily Trichostrongyloidea. *Haemonchus contortus* and *Trichuris ovis* species are most important nematode parasites, which are found in the gastrointestinal tract in the goat (*Capra hircus*). Molecular techniques were used to the identification of parasites and amplification is done with the help of ITS2 (Internal Transcribed Spacer 2) fragment of the ribosomal DNA. The present study revealed that the amplified the product band size of gastrointestinal nematode parasites were *Haemonchus* (325bp-MW595998.1), and *Trichuris* species (205bp-MW667589.1). The ITS2 sequencing and phylogenetic tree (Neighbour joining method by MEGA7.0 software) revealed 99 and 100% (*Haemonchus contortus* and *Trichuris ovis*) homology.

Keywords- *Nematode, goat (Capra hircus), gastrointestinal tract, molecular characterization.*

INTRODUCTION

Parasitic infestation contributes towards major loss of production and reduced income of farmers in the world (Hoste *et al*., 1995). Therefore, effective prevention and control of gastrointestinal parasitic diseases are important for the host. The identification of parasitic species is based on morphological and molecular characterization method. Morphology identification used for some specific characters using molecular methods like genomic, species specific study. Bioinformatics method was a unique method used for better understanding of relationship between two species and host & parasitic interaction (Ahmad, 2011).

ISSN 2515-8260 Volume 10, Issue 01, 2023

Molecular characterization is used as a diagnostic tool since 1990s for the identification, quantification and discrimination of the nematode parasites. Data about genetic variation in genomic and species is determined through molecular method (Karp *et al*., 1996). These methods of nematode identification also provide accurate and alternative diagnostic approaches (Punja *et al*., 2008). Based on this method genomic, character of genomic DNA of different species of parasites, PCR and sequencing are identified (Gasser *et al.*, 1993). These techniques are extremely sensitive for accurate identification for parasites up to genomic and species level (Tan *et. al.,* 2014). Molecular techniques have been played a major role in biological studies leading to the understanding of the genetic variation in the parasites in the host. Logically, it has been also very useful diagnosis tool for the identification of gastrointestinal nematode parasites. The molecular tools such as PCR (Polymerase Chain Reaction) and DNA sequencing are advanced techniques, most sensitive and highly accurate for the identification of nematodes at the genomic level. These techniques are used for detecting specific based on methods have also been genotype organisms. The detection sensitivity of PCR is higher than that of light microscopy; therefore, this technique is useful for detecting a low number of parasites in stool samples. The PCR technique has also been used to investigate non-intestinal parasites (Piarroux *et al.,* 1994 and Duraisingh *et al.,* 1998). Gasser and Newton (2000) reported that genetic variation of the ITS2 (Internal Transcribed Spacer 2) sequences in relatively many parasites. The ITS2 (Internal Transcribed Spacer2) sequences can be used as a tool for species differentiation such as in gastrointestinal nematodes (Stevenson *et al.,* 1995).

The Internal Transcribed Spacer (ITS) region has been confirmed to be a very functional for DNA from which universal species-specific primers are used in PCR reactions (Powers *et al*., 1997) and also have very reliable genetic marker for nematode parasitic identification (Gasser *et al*., 1993 and Gharamah *et al*., 2012). Sequence data of the ITS2 is extensively used for the high-resolution marker for several parasitic nematodes (Heise *et al*., 1999; Dallas *et al*., 2000 and Abramatov *et al.,* 2013). The nematode parasitic species can be genetically characterized by rDNA sequences (Chilton *et al.,* 1995). The desirable gene have been encoding with rRNA and conserved with small subunit gene (Highly variable the intergenic spacer regions) (Dorri *et al*., 1999).

ISSN 2515-8260 Volume 10, Issue 01, 2023

Sequences of the ITS2 (Internal Transcribed Spacer 2) of rDNA have been widely used as genetic markers for the differentiation of many nematodes and identification (Gasser and Newton, 2000; Lin *et al*., 2012 and Liu *et al*., 2014). The sequence variation in rDNA within a species is significantly less than the levels of sequence differences among species. That trait allows the specific identification of nematodes, including species of *Haemonchus, Trichuris, Cooperia, Nematodirus, Bunostomum, Oesophagostomum* and *Chabertia,* (reviewed by Gasser, 2006 and Gasser *et al.*, 2008).

Sequence of parasites is analyzed and defined homology by the phylogenetic tree, which is shown in the relationship between different species of parasites taxa (sequences) (Felsenstein 2004 and Hall, 2011). The method is described by some steps to build up the phylogeny tree from the molecular data. NCBI, BLAST, CLASTAL-W and MEGA-6 version software are used for identifying the molecular evolutionary genetic analysis (Tamura *et al*., 2011).

MATERIAL AND METHODS

Collection and preservation of parasites

Gastrointestinal tracts of goat (*Capra hircus*) were collected from different slaughtered house of Lucknow and bring to the laboratory at BBA Central University, Lucknow (Uttar Pradesh, India). Dissected the gastrointestinal tract and collected and examined the presence of parasites. Collected parasites were preserved in 70% alcohol for the further processing.

DNA extraction

DNA extraction was done by P:C:I method (Phenol/Chloroform/Isoamyl alcohol) Which is composed by Harris *et al*., 1999 and Cornman *et al*., 2009. The protocol of DNA extraction-firstly sample crushed with ddH2O (Double-Distilled Water), prepared the homogenate and homogenate collected in 1.5 ml eppendorf tube and centrifuge (3000 rpm for 15 mins.). Pellets was collected added 700 µl Lysis buffer and 10 µl RNaseA and then add 10 µl protenase k. The solution was left overnight Further solutions after lysis treatment were vortex and centrifuge at 14000 rpm for 2 mins. Supernatant transfer to new tube and add equal ratio of Phenol/chloroform/isoamyl alcohol (P:C:I) in the ratio of (25:24:1). Centrifuge at 13000 rpm for 10 mts. Transfer aqueous phase into a new tube (Repeat- (Add -Phenol/ Chloroform/ Isoamyle-

ISSN 2515-8260 Volume 10, Issue 01, 2023

alcohol) into aqueous phase centrifuge –collect aqueous phase) add Chloroforms – 700 µl (Centrifuge- 1300 rpm. for 10 mins) add 60 µl of 3M NaoAc (Sodium acetate) and add 2 volumes of 100% ETOH (Ethanol alcohol). The DNA start to precipitate after mixing at -40°C for 2 hr. and again centrifuge at 13000 rpm for 15 mins. Washed 2 times with 70% ETOH (Ethanol alcohol) Centrifuge at 13000 rpm for 10 mins. DNA pellate was dried by air (in room temp). Dissolve DNA pellet in appropriate volume of TE buffer and mixed by thawing. DNA pellet preserved in -20°C in deep freezer for further studies.

Quality analysis and DNA quantification

This method is used for the analysis of quality and quantification of DNA which is based on the Etbr (Ethidium Bromide Fluorescent) staining of DNA. The quantity of DNA can be predictable by fluorescent yield of the samples and provide good quality of the DNA. Quantification of Genomic DNA were Isolated from desired sample done by spectrophotometer (Biophotometer D-30). Quantification of nucleic acids is an accurate and simple view of the concentration of DNA sample. The ratio of OD260/OD280 should be used checking the purity and quantity of DNA.

PCR programme

PCR (Polymerase Chain Reaction) product was amplifying with the help of Internal Transcribed Spacer 2 (ITS2) marker for the identification of three nematode parasitic genome. PCR was setup for preparation of Genomic DNA to need the volume of 25 µl of sample mixture. The final 25 µl reaction volume was set up using 10 µl TaqPCR, Mastermix, 2.0 µl of forward primer, 2.0 µl reverse primer, 8.0 µl DNA, 3.0. PCR reagents were used for the purposes of optimization. The PCR reaction was performed according to the PCR condition. PCR set up conclude the reaction was conducted based on denaturation, optimal temperature of annealing and extension. Reaction conditions depend on the denaturation temperature necessary to activate the enzyme in each master mix. The amplification concerned an initial denaturation of 94°C -95°C for maximum of 1 mins. Annealing temperatures ranging from 54°C-62°C were tested and sample was optimized with and a final extension at 72°C for 10 mins.

ISSN 2515-8260 Volume 10, Issue 01, 2023

Primers

Three sets (Forward and Reverse) of primers of nematode parasites species were considered for PCR amplification. Reported Primer by Ahmad *et al*., (2015) (ITS2 region) of *Haemonchus sp.* primer set F- ACGTCTGGTTCAGGGTT, R-TTAGTTTCTTTTCCTCCG. *Trichuris sp.* parasites primer was self designed by self with help of the NCBI-Fasta format – BLAST – Clustral-W, Primer 3 plus and Oligo-analyzer from different Accession no. –LN813018.1, AJ238220.1, FR87027741.1 etc. amplified primers set (F-GTCGTCCTAAGCAGGAGTCG R-TCATTGCCGTAAACCAACAA)

Methods for PCR amplification

Electrophoresis of amplified DNA fragment was conducted in 2% agrose gel in 1x TBE buffer and stained with ethidium bromide (Etbr) to verify the bands. These bands compared to the 1 kb DNA ladder. PCR product band size was checked under UV transilluminator and taken image from the gel doc. The PCR product has been purified by the PCR purification kit. The pure PCR product was sent for sequencing (Progen Biolab and Scientific Traders).

Phylogenetic tree

Sequences generated after sequencing BLAST (Basic Local Alignment Search Tool, NCBI) was performed and aligned for knowing homology and edited using MEGA software version 6 (Tamura *et al*., 2011) in both directions (forward and reverse sequences). Clastal-W is aligned and sequenced. The sequence identity computed using the BioEdit (Hall, 1999) and compared with the reference sequences of each nematode parasites species with accession number. Phylogenetic analysis was conducted using Neighbour Joining (NJ) method The phylogenetic tree analysis was conducted using the (UPGMA), distance method based on Neighbour joining method by MEGA7.0 software (Tamura *et al*., 2011).

RESULTS

This investigation revealed that the nematode parasites species (*Haemonchus contortus,* and *Trichuri ovis*) by molecular technique (PCR, DNA sequencing and bioinformatics). The amplified PCR product range of approximately 325 bp and 205 bp (base pair). The generated

sequence was blasted (BLAST) in nucleotide and compared to different accession number which showed homology (99% and 100%), is identified species of all nematodes after using bioinformatics methods (BLAST, Clastal W, Oligo analyzer and Mega 7.0 software) generating sequence. The phylogenetic tree analysis showed relationship between various species of parasites and represented by phylogeny tree.

DNA extraction and PCR amplification analysis

Genomic DNA of gastrointestinal nematode parasites of goat was elaborated by molecular techniques. The molecular characterization techniques are utilized for the characterization and identification of specific Genomic studying of nematode parasites. These techniques have become widely used in the world (Dahlgren *et. al*., 2010). The study was conducted to extract the genomic DNA from nematode parasites with the standard method of PCI (Phenol: chloroform: Isoamyl alcohol) (Mabe, L. T., 2012). Extracted DNA was quantified by Spectrophotometer and Gel electrophoresis Unit and observed the high intensity of band. The PCR product was amplified from the genomic DNA of *Haemonchus sp*. and *Trichuris sp*. PCR amplification products have yielded 325 bp and 205 bp fragment in size. Three sets of primer targeting the Internal Transcribed Spacer 2 (ITS2) for genomic-specific were successfully amplificafied and sequenced.

Sequencing

The amplified product was purified by the purification kit and PCR product was send for sequencing. The PCR amplified product sizes of approximately 325 bp to 205 bp (*Haemonchus* sp., and *Trichuris* sp.) were confirmed by the genome of parasitic species by checking sequences size.

Phylogeny tree analysis

DNA sequences were edited using MEGA 7.0 software (Kumar *et al*., 2016). The generated sequences of *Haemonchus* and *Trichuries* sp. were submitted to GenBank with an accession number. Multiple sequence alignments data were analyzed by Clustal W and GenBank database in the BioEdit program (Hall, 1999). The phylogenetic tree was constructed with the help of

UPGMA method in the MEGA 7.0 programs. The evolutionary distances were computed using the Kimura 2-parameter method.

The sequences were aligned and compared with the ITS2 sequences of the *Haemonchus* sp. from the GenBank database, using NCBI and BLAST tool are used for the similarity determination. Alignment of sequences revealed 99.07% similarity with generated accession no. MW595998.1 of *Haemonchus contortus* (Accession no. -MF398451.1, MF398452.1, MF398446.1, MF398443.1, MF398439.1, MF398442.1 and MF398434.1). The second generated sequence of amplified ITS-2 sequences number MW667589.1 of *Trichuris* sp. revealed that 100% similarity with *Trichuris ovis* (Accession no.- HS586910.1, HF586911.1, KR025525.1 and JN252307.1).

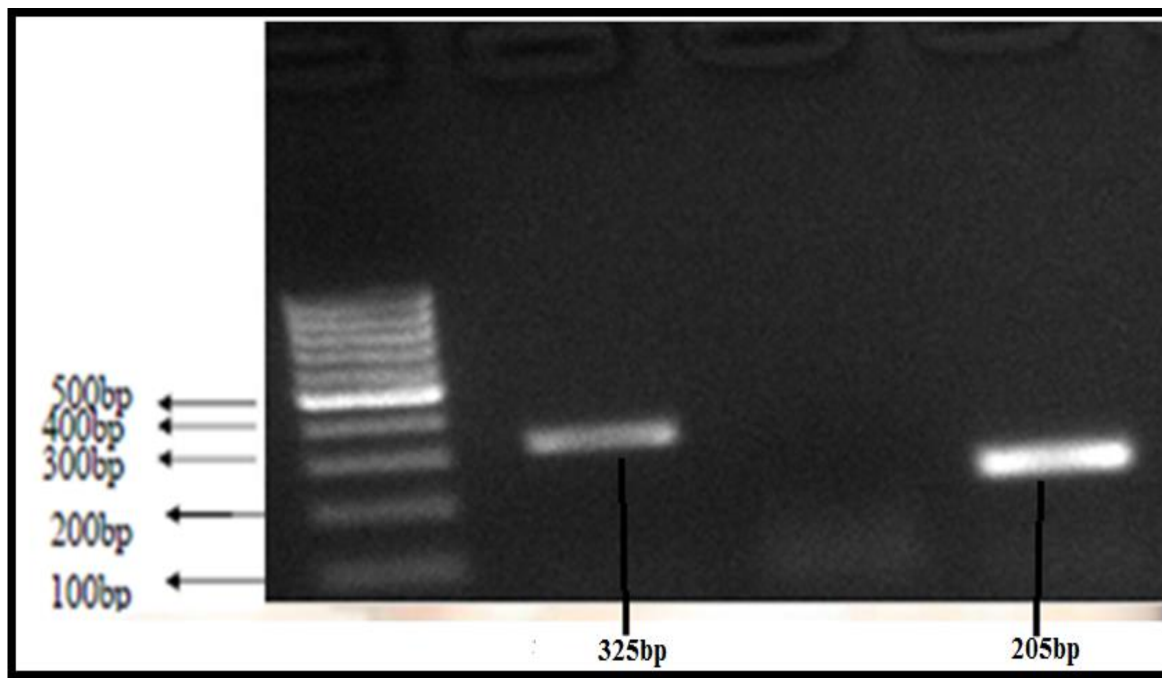

Figure 1- PCR purified product size 325 bp and 205 bp, loaded with 1 kb DNA ladder. 2 % agarose gel showing amplified DNA of *Haemonchus and Trichuris* species.

1.-

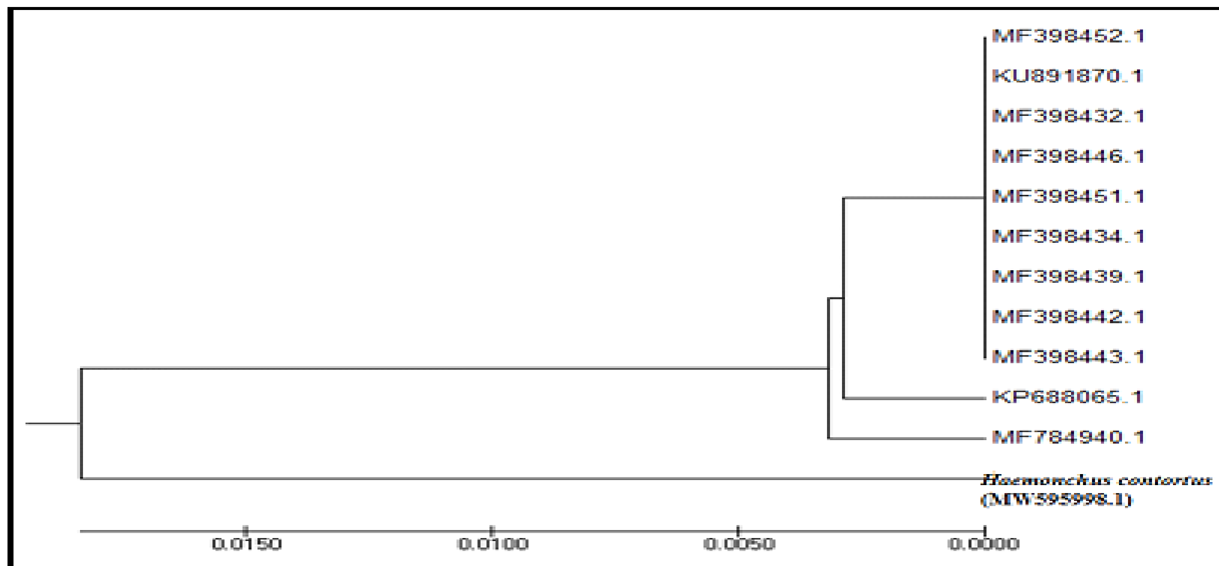

Figure 2-Phylogeny tree analysis was constructed by using UPGMA method. Accession no. for each sequence is given on the tree. The tree has been represented homology and evolutionary distances with *Haemonchus contortus* (MW595998.1).

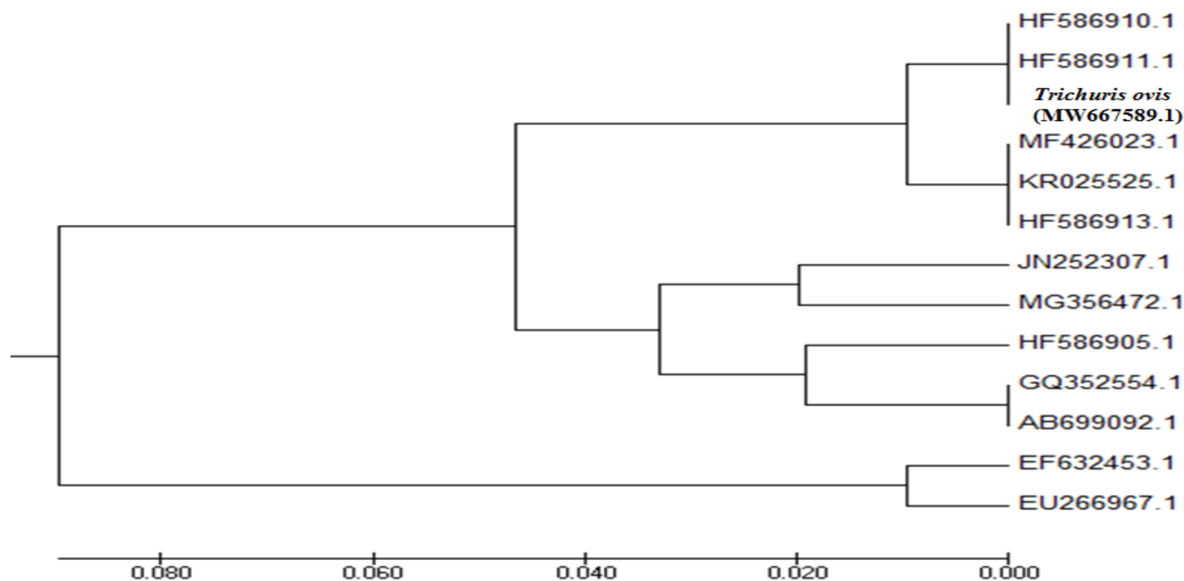

Figure 3- Phylogeny tree analysis was constructed by using UPGMA method. Accession no. for each sequence is given on the tree. The tree has been represented homology and evolutionary distances with the *Trichuris ovis* (MW667589.1).

DISCUSSION

Molecular is very advanced technique, which is highly sensitive, enabled the accurate, widely used for identification and diagnosis of gastrointestinal parasitic infection (Dahlgren *et al.*, 2010; Rosenthal *et al*., 2008 and Dangoudoubiyam *et. al.,* 2009). PCR (Polymerase Chain Reaction), DNA sequencing and Bioinformatics are the most important tools in my study (Gasser *et al*., 1993 and Sweeny *et al.*, 2011). Ghai *et al.*, (2014) have reported that PCR technique is most sensitive than microscopy for analysis of parasitic species. Abbaszadegan *et al.,* 2007, reported that PCR is used as analytical methods and observed the genetic variability between the parasites at genomic and species-specific level. rDNA sequences are most important molecular markers used for differentiation of parasites (Rosenthal *et al.*, 2008 and Xiang *et al.*, 2009). Internal Transcribed Spacer 2 (ITS2) of nuclear rDNA (Ribosomal Deoxyribonucleic acid) sequences is most important reliable genetic marker used for species identification (Campbell *et al*., 1995; Stevenson *et al*., 1995; Gasser, 2006; Gasser *et al*., 1993; Gharamah *et al*., 2012 and Xiang *et al.*, 2009) due to the high inter-specific sequence difference and intra-specific sequence correlation. It is very useful tool for species specific identification of the parasites (Heise *et al*., 1999; Luton *et al*., 1992, Hoste *et al*., 1995 and Bott *et al*. 2009) ITS2 DNA region are species specific primers of *H. contortus* and *Trichostrongylus* spp. in a livestock (Nei and Kumar 2000) .

However, the genetic variability of Internal transcriber spacer 2 (ITS2) within depending on the parasite population studied (Gasser and Newton, 2000 and Gasser, 2006). Sequence analyses of the multicopy of ribosomal DNA (rDNA) encoding of genes and more conservative primers has binding to the rDNA of many nematode parasites (Heise *et al.,* 1999) and phylogenetic tree analysis (Cerutti *et al*., 2010). Phylogenetic tree analysis of the ITS2 sequences was most reliable genetic marker for the identification of nematode parasites (*Haemonchus contortus,* and *Trichuri ovis*) they were constructed by UPGMA method in the MEGA7 program (Kimura, 1980, Saitou, 1987; Chilton *et al*, 1995; Tamura *et al.*, 2007; Lin *et al.*, 2012; Callejón *et al.,* 2013 and Kumar *et al.*, 2018).

Molecular characterization is one of the most common and crucial for identification of species, which have a broad range of utilization (Criscione, 2005 and Gasser, 2006). This study provided

ISSN 2515-8260 Volume 10, Issue 01, 2023

the first data of the ITS2 (Internal Transcribe spacer 2) region derived from 325 bp amplifying sequence of *Haemonchus contortus* from gastrointestinal parasites of goat in different areas of Lucknow (Uttar Pradesh). This data was based on findings of Ahmad *et al.,* (2015), Hassan *et al.,* (2017) and Stevenson *et al*. (1995). ITS2 region for identification of *Haemonchus contortus*, which is widely used by many authors (Blouin, 199, Bensch *et al*., 2004, Chilton *et al*., 2001, Brasil *et al*., 2012) in analyzing sample from many parts of the world. Similarly, a high diversity of ITS2 sequences was observed with 98 to 100 homologies in *Haemonchus contortus* sequences from the GenBank database in goats, sheep, and cattle. (Akkari *et al*., 2013 and Mangkit, *et al*., 2014). Fakae and Chiejina (1993) also reported significant higher variation and 90% to 100%, homology depicted in species of *Haemonchus contortus* in gastrointestinal tract of goats (Tiong *et. al.,* 2014). Ahmed *et al*., 2015 suggested that alignment of ITS2 sequences at 187,196 and 208 nucleotide and Phylogenetic tree analysis was showed using Neighbour-Joining method, 99 and 100% homology. Abramatov *et al*., 2013 have also reported similar study.

The genus of *Trichuris* was recognized by Roederer 1761. This is very common nematode parasites which are found in the gastrointestinal tract in the goat. In this study, it was found that 205 bp binding sized of *Trichuris ovis* and phylogeny tree analysis homology showed that 100% similar studies were found in the paper of Bandyopadhyay *et al.,* (2009), Abildgaard, 1795 and Misal, *et al.,* 2015. Many research articles related to other species, *Trichuris Skarjabini*, *Trichuris discolor* and *Trichuris leporis* (Baskakaw, 1924, Linstow, 1906 and Froelich, 1789). Liu *et al*., 2014 and Cutillas *et al*., 2009 have also suggested the genomic study of *T. ovis* and *T. discolor*, and detected a substantial difference.

Conclusion

The study was based on the molecular characterization of nematode parasites. This study highlights the integrated study of detection of nematode parasites and allowing a more complete understanding of the genetic variation, bioinformatics, and long evolutionary process by sequences and phylogeny tree analysis. The findings of this study revealed that the data was amplified the product band size of gastrointestinal nematode parasites (*Haemonchus* (325 bp-MW595998.1), and *Trichuris* species (205 bp-MW667589.1). ITS2 region was using rDNA interspecific difference and showed genetic relationship. The online blasting (BLAST) revealed

that the nucleotide sequence has finding significant homology with nematode parasites species and higher score homology which showed 100% were obtained with *Trichuris ovis* species following to another species of parasites, *Haemonchus contortus* (99.07%). The study provided the molecular evidence of parasitic infection in Lucknow region. Molecular method having rapid changes in progress of research field with in significant genetic diversity. This observation might have implications for further study epidemiology, diagnosis of parasitic infection and control program.

ACKNOWLEDGEMENT

The authors would like to acknowledge the supervisor and other authorities of the University for providing necessary infrastructure facilities to carry out the research work.

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