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RESEARCH ARTICLE

Molecular Confirmation and Genetic characterization of *Haemonchus contortus* Isolates at the Nuclear Ribosomal ITS2 Region: First Update from Jhang Region of Pakistan

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A B S T R A C T

Haemonchus (*H.*) *contortus* is a blood-feeding parasite causing substantial losses to sheep and goats sector across the globe. Pakistan's livestock industry comprises of more than 100 million head of small ruminants contributing to meet the protein requirement of people. To date, there is no report on genetic characterization of *H. contortus* from Jhang District of Pakistan. With the aim to narrow this research gap, the current study was planned and molecular confirmation and genetic diversity of *H. contortus* was determined. Species-specific primers were used to amplify partial ITS-2 region. Sequencing and subsequently identity of every isolate was verified through NCBI BLAST program. Molecular analysis revealed a total of 14 haplotypes out of 30 isolates with 23 mutations. High haplotype diversity (Hd = 0.846) was observed with statistically significant Tajima's *D* (-2.00336*) value suggesting population expansion. The phylogenetic analysis showed existence of unique haplotypes of *H. contortus* at Jhang. Further, significantly negative Tajima's *D* (-2.16660*) and non-significant Fu's Fs (-4.218) also indicated population expansion as obtained in the median-joining network consisting of a dataset of 39 sequences. The study has reflected high genetic variation and existence of unique haplotypes of *H. contortus* in Jhang, Pakistan. The results of this study will serve as an important database for future studies *H. contortus* in the country.

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INTRODUCTION

Parasitic diseases lead to heavy economic setback to owners of small ruminants as a result of reduced growth and mortality (Alvi *et al*., 2020). Haemonchosis, caused by *Haemonchus contortus* (Phylum Nematoda; Family Trichostrongylidae), is a parasitic disease affecting small ruminants (sheep and goats) in tropics and subtropics across the globe (Vongnady *et al*., 2020). *H. contortus* is commonly known as barber's pole worm having fecund rate up to 10000 eggs per day (Abutarbush, 2010) and resides in the abomasum. Hematophagous behavior of the worm leads to anemia resulting in edema and death in heavily infected animals (Taylor *et al*., 2007; Besier *et al*., 2016). Chronically infected animals show reduced feed intake and poor weight gain (Mushonga *et al*., 2018) leading to decreased production and increased production cost causing substantial economic losses throughout the world (Wang *et al*., 2017; Chinchilla-Carmona *et al*., 2020). It is estimated that this infection causes huge annual economic losses amounting up to $26 million in South Africa, $103 million in India and $46 million in Kenya (Dey *et al*., 2018).

Molecular characterization is an essential tool for the validation and phylogenetic analysis of nematodes. Understanding about molecular epidemiology and genetic variation of *H. contortus* helps to determine the patterns of transmission and dissemination of alleles responsible for drug resistance and ultimately paves the way for designing the effective control strategies (Gasser *et al*., 2008).

Pakistan is an agricultural country with livestock comprising up to 11.70% in the national gross domestic product and livelihood of at least 80 million people is directly or indirectly associated with this sector. Pakistan

consists of 31.20 million head of sheep and 78.20 million head of goats producing 41,000 tons and 65,000 tons of milk, respectively whereas sheep and goats collectively contribute up to 74,8000 tons of meat available for human consumption (Economic Survey of Pakistan, 2019-20).

As far as the authors know, there is no study on molecular characterization and population structural analysis based on *ITS*-2 gene of *H. contortus* from Jhang, Pakistan prior to inception of this study. Keeping in view, heavy global economic losses due to *H. contortus*, high population of sheep and goats in Pakistan and advantages of molecular characterization for effective control measures, this study was designed to provide baseline molecular epidemiological data of *H. contortus* isolates recovered from small ruminants slaughtered at abattoir of Jhang district, Pakistan.

MATERIALS AND METHODS

Study district: Jhang district spreads over 8809 sq. km with population density up to 321.8 per sq. km and the majority (76.61%) belongs to the rural areas (Pakistan Bureau of Statistics, 2021). The district consists of four sub-districts namely Jhang city, Athara Hazari, Shorkot and Ahmad Pur Sial. The district is located in the central section of the Punjab province of Pakistan at the east bank of the Chenab River. The coordinates of the city are 31.2781N and 72.3317E (Pakistan Meteorological Department, 2021). The temperature in summer is harsh reaching up to 45ºC while in winter mercury level goes down up to 5ºC. The land of Jhang is purely feudalistic agriculture with it 85% land have irrigation supplies. Huge population relies on agriculture and rearing of livestock to earn their livelihoods (Jhang Chamber of Commerce and Industry).

Parasite material: The central abattoir of the city was selected for the collection of *H. contortus* worms from sheep and goat. The slaughterhouse was visited during 2019 and a total of 30 worms were recovered from the abomasa of slaughtered small ruminants. The worms were transported to the Parasitology Laboratory of Department of Pathobiology, College of Veterinary and Animal Sciences, Jhang. Worms were washed with phosphate-buffered saline to remove the abomasal contents.

DNA extraction and PCR: Using Qaigen DNA extraction kit, DNA was extorted strictly according to the instruction of the company. A total of 25 µl reaction volume consisted of 12.5 µl PCR premix, 1 µl of forward and reverse primers each, 0.5 µl DNA, and 10 µl nuclease free water. Negative control was also run in each PCR. RNAse / DNAse free water was added in the negative control instead of template. A portion of ITS-2 gene was amplified using a pair of primers as previously described by Bott *et al*. (2009). Initial denaturation was carried out at 95ºC for 5 min. Thirty-five cycles of denaturation (95ºC; 30 sec) annealing (55ºC; 30 sec) and extension (72ºC; 30 sec) were followed by final extension (72ºC; 7 min). The amplicons (5 ul) were run on 2% agarose gel to visualize the bands and the remaining 20 µl of the positive samples was sent to LabGenetix, Lahore, Pakistan for Sanger sequencing.

Molecular and population genetics analysis: Misread nucleotides in DNA sequences were manually corrected and multiple sequence alignment was done using UGENE software. Identity of each sequence was confirmed through NCBI BLAST program. Population diversity indices (haplotypes and nucleotide diversities, and haplotypes number) and Tajima's D and Fu's Fs (neutrality indices) were calculated using DnaSP software (Rozas *et al*., 2017) while the geographic relationship of isolates from this study and different parts of the world was assessed by building median joining network (Bandelt *et al*., 1999).

Phylogenetic analysis: A dataset comprising of the *ITS*-2 (192 bp) region of the haplotypes found in this study and those recovered from GenBank was used to draw the phylogenetic tree. MrBayes software was used to construct the tree while TreeView software was employed to present the tree. Posterior distribution of the parameters was judged through Markov Chain Monte Carlo (MCMC) sampling. *Haemonchus placei* was used as an out-group.

RESULTS

Nucleotide polymorphism and population indices: Followed by DNA extraction and amplification of partial *ITS*-2 gene along with part of 28s rRNA, a PCR product of approximately 265 bp was found. After sequence alignment and DNA polymorphism analysis of the sequences, 12 parsimony informative sites and 23 mutation sites were observed that resulted in 14 haplotypes (Table 1).

Representative haplotype sequences of *ITS-2* genes are available online in the GenBank database (Accession numbers: MW376203 to MW376209 and MW381017 to MW381023).

Low nucleotide diversity (π=0.02142) and high haplotypes diversity (Hd=0.846) were observed for *H. contortus* (Table 2). The value of Tajima's *D* was negative and statistically significant (-2.00336*) while Fu's Fs value was also negative (-3.487) and statistically insignificant (P>0.05). The pairwise distances among the observed haplotypes of *H. contortus* found in this study is shown in Table 3.

To determine the geographic relationship between the isolates from the current study and those obtained from GenBank (different parts of the world), a set of 39 *ITS*2 sequences (180 base pairs) was used to depict the median joining network (Fig. 1). The analysis revealed 14 haplotypes, with high haplotype diversity (Hd=0.792), and low nucleotide diversity (π =0.01627). Significant Tajima's *D* (-2.16660) and statistically non-significant Fu's Fs (-4.218) were found. Haplotype 1 was found to be the central one and consisted of isolates from Pakistan, Bangladesh, Austria, Iran, India, Kenya, Poland and South Africa. Haplotype 1 occupied a central position with a maximum of 25 mutational differences from other encountered haplotypes.

Phylogenetic analysis: A total of two clades and three clusters were formed (Fig. 2). Cluster-1 consisted of only Pakistani haplotypes (PAK-Jhang 8, -Jhang 9, and –Jhang 10). Cluster-2 and -3 were constituted of sequences from Iran and Thailand, and Bangladesh, respectively. In clade-2, sequences were randomly distributed without any relation geographical origin of isolates.

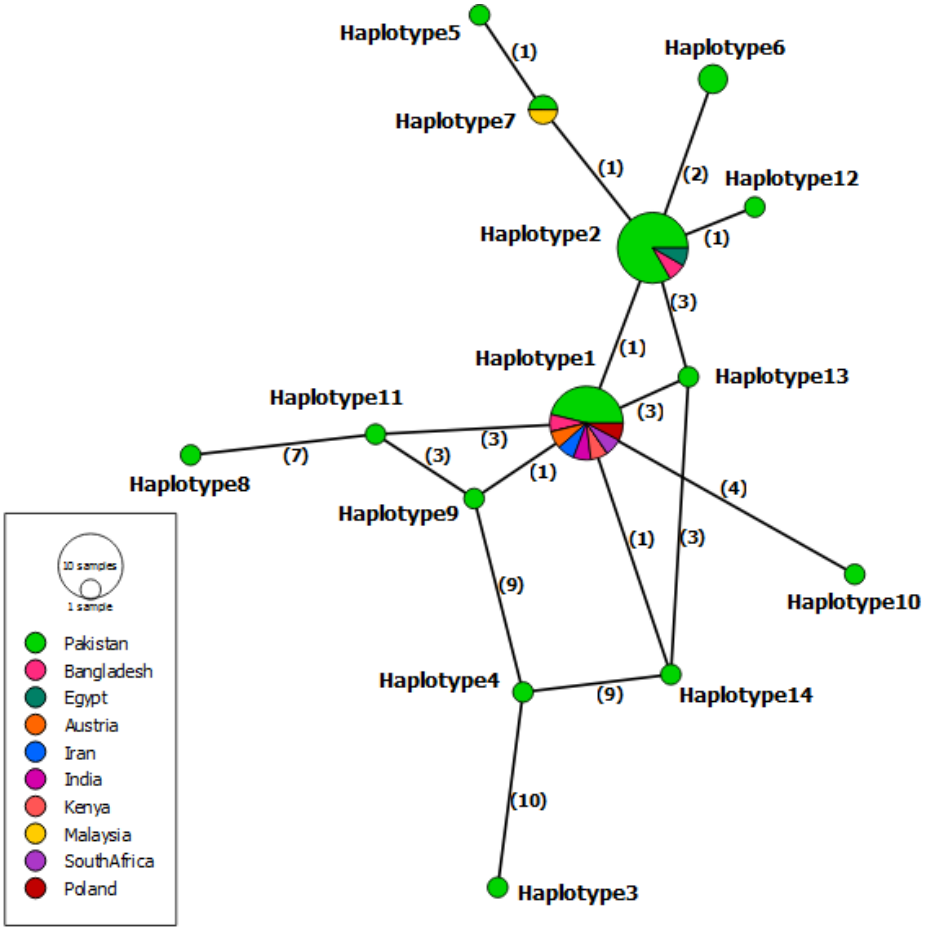

Fig. 1: Median joining network of Pakistani isolates of *Haemonchus contortus* and different countries (obtained from GenBank) based on *ITS-2* (192 bp) gene sequence. Sphere sizes are relative to the frequencies of the haplotypes while number of mutations is written in parentheses.

Table 1: *Haemonchus contortus* internal transcribed spacer-2 gene nucleotide sequence polymorphism

Haplotype	*ITS-2* DNA mutation sites																						
	1	2	3	9	10	13	14	15	16	17	18	19	20	21	22	23	24	25	26	29	86	95	107
PAK- Jhang1	G	A	T	G	A	T	T	G	T	A	A	C	A	T	T	C	T	T	G	T	G	T	G
PAK- Jhang2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	A	.	G	.	.	.
PAK- Jhang3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C		.	.	.	.	.	.	.
PAK- Jhang4	A	T	G	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.
PAK- Jhang5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.
PAK- Jhang6	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	T	.	.	.	.	.	A	.
PAK- Jhang7	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	A	A	A
PAK- Jhang8	.	.	.	C	.	C	A	A	.	T	C	T	T	C	.	T	T	C	.	.	.	.	.
PAK- Jhang9	.	.	.	.	.	.	.	.	A	.	C	.	T	.	A	T	A	A	A	.	.	.	.
PAK- Jhang10	.	.	.	.	G	.	C	C	A	T	T	T	C	.	.	T	T	.	.	.	.	.	.
PAK-Jhang11	.	.	.	.	.	.	.	.	.	.	.	.		.	.	C	.	.	.	.	.	.	.
PAK-Jhang12	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.
PAK-Jhang13	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.
PAK-Jhang14	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.

Table 2: Diversity and neutrality indices for *Haemonchus contortus* populations from Jhang, Pakistan.

Indices	*ITS2* (192 bp)
No. of isolates	30
No. of mutations	36
Parsimony informative sites	12
No. of haplotypes	14
Haplotype diversity (Hd)	0.846
Nucleotide diversity (π)	0.02142
Tajima's *D*	-2.00336*
Fu's Fs	-3.487

DISCUSSION

Haemonchus contortus is an important hemato-phagous nematode prevalent throughout the world affecting small ruminant population (Tsotetsi and Mbati, 2003; Choubisa and Jaroli, 2013). The parasite possesses high biotic potential along with heavy infections in sheep and goats. Additionally, direct life-cycle of *H. contortus* results in marked genetic variations in the population (Blouin *et al*., 1998; Prichard, 2001; Brasil *et al*., 2012). The two species of *Haemonchus viz. H. contortus* and *H. placei* are closely related to each other and confirmation of the species is essential to be carried out through investigation of intra-specific variations between these two species (Jacquiet *et al*., 1995).

The internal transcribed spacer (ITS) 2 region of nuclear ribosomal DNA is considered as one of the most important potential candidates to investigate genetic variations. It contains the conserved regions to design universal primers, easy amplification, and plenty of variability to differentiate closely related species (Yao *et al*., 2010).

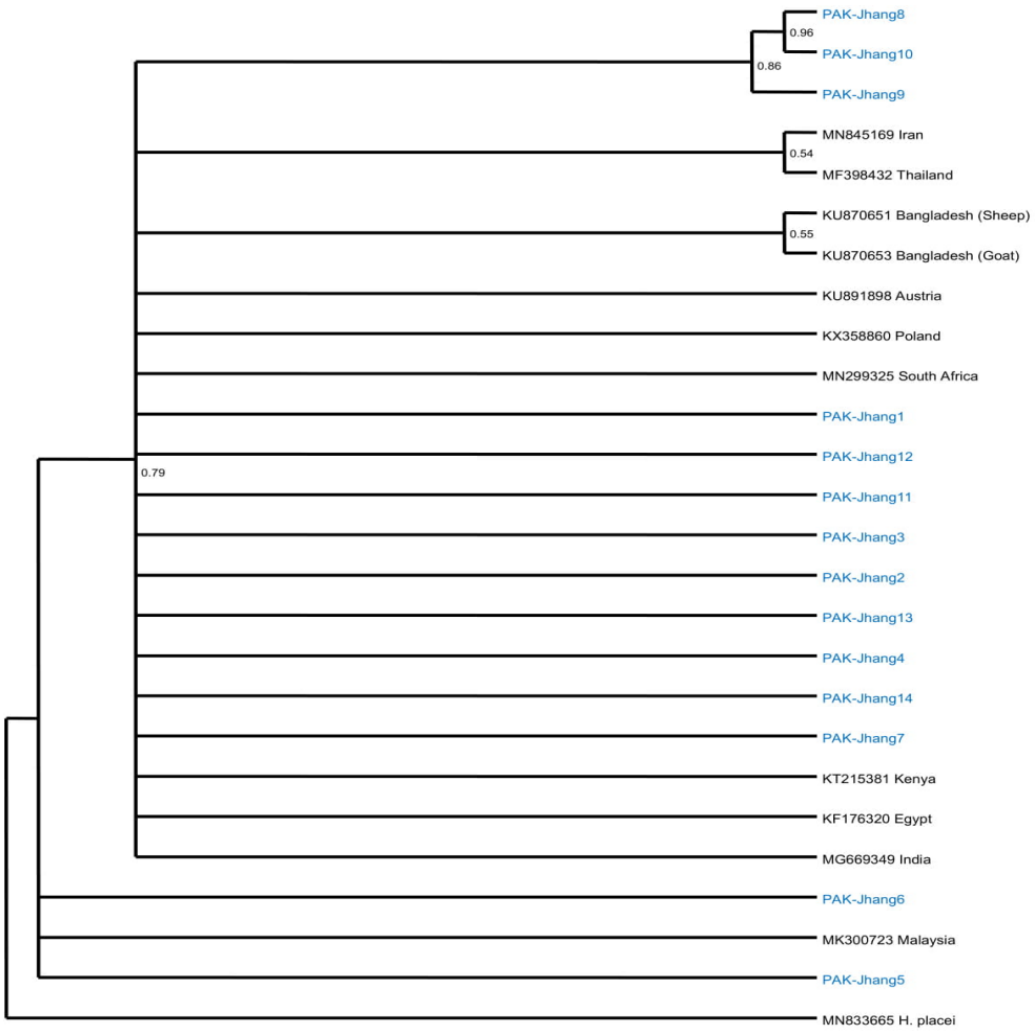

Fig. 2: Phylogenetic tree of *Haemonchus contortus* isolates from Pakistan deduced from the *ITS-2* gene. *Haemonchus placei* was used as an outgroup. Blue color represents the haplotypes *Haemonchus contortus* obtained in this study.

Table 3: Pairwise distances of partial ITS-2 gene between the genotypes of *H. contortus* found in this study

	PAK Jhang1	PAK Jhang2	PAK Jhang3	PAK Jhang4	PAK Jhang5	PAK Jhang6	PAK Jhang7	PAK Jhang8	PAK Jhang9	PAK Jhang10	PAK Jhang11	PAK Jhang12	PAK Jhang13
PAKJhang1													
PAKJahng2	0.016												
PAKJhang3	0.005	0.021											
PAKJhang4	0.021	0.037	0.026										
PAKJhang5	0.010	0.026	0.005	0.032									
PAKJhang6	0.016	0.032	0.010	0.026	0.005								
PAKJhang7	0.016	0.032	0.010	0.037	0.016	0.021							
PAKJhang8	0.065	0.070	0.070	0.076	0.076	0.070	0.081						
PAKJhang9	0.043	0.037	0.043	0.053	0.048	0.043	0.053	0.065					
PAKJhang10	0.053	0.065	0.059	0.065	0.065	0.059	0.070	0.053	0.059				
PAKJhang11	0.005	0.021	0.000	0.026	0.005	0.010	0.010	0.070	0.043	0.059			
PAKJhang12	0.005	0.021	0.010	0.026	0.016	0.021	0.021	0.065	0.043	0.048	0.010		
PAKJhang13	0.005	0.021	0.000	0.026	0.005	0.010	0.010	0.070	0.043	0.059	0.000	0.010	
PAKJhang14	0.005	0.016	0.010	0.026	0.016	0.021	0.021	0.059	0.043	0.048	0.010	0.010	0.010

In this study, we molecularly confirmed the prevalence of *H. contortus* in small ruminants slaughtered at Jhang abattoir, Pakistan using a partial segment ITS-2 gene and described genetic variations. We found a total of 14 haplotypes out of 30 isolates recovered from small ruminants diverged from previous reports of Yin *et al*. (2013) and Gharamah *et al*. (2012) who reported 18 and 6 haplotypes from China, Malaysia and Yemen, respectively.

Haplotype diversity (Hd = 0.846) found in this study corresponds to the findings of Dey *et al*. (2018) who also reported high haplotype diversity (Hd = 0.8695) in Bangladeshi isolates of *H. contortus*. However, the value of nucleotide diversity (π = 0.02142) calculated in this study differed from reports of Dey *et al*. (2018) and Shen *et al*. (2017) who found nucleotide diversity of 0.007 and 0.0098 in Bangladesh and China, respectively. Sequence analysis conducted by Laosutthipong *et al*. (2019) revealed 9 haplotypes of ITS-2 gene among 20 isolates of *H. contortus* in Thailand with low nucleotide (π = 0.017) and high haplotype diversity (Hd = 0.832) which is in tandem with our results. In another Chinese study, nucleotide and haplotype diversities in domestic sheep

were observed to be 0.004 and 0.719, respectively whereas the values of these diversity indices were 0.008 and 0.787, respectively in wild blue sheep (Shen *et al*., 2017). Our results are coherent with that of the Chinese reports in term of values of haplotype diversity while the results of nucleotide diversity differed greatly.

The phylogenetic analysis showed that there is existence of unique haplotype of *H. contortus* recovered from abomasa of sheep and goats slaughtered at Jhang abattoir, Pakistan that were clearly at distant places from *H. placei* in the phylogenetic tree which is the most closely related species and was used as an outgroup.

Further, negative and statistically significant Tajima's *D* (-2.00336*) and Fu's Fs (-3.487) indicated population expansion. Global median-joining network consisting of a dataset of 39 *ITS*2 sequences (180 bp) revealed 14 haplotypes with a high haplotype diversity (0.792), and nucleotide diversity (0.01627). Further, significant and negative Tajima's *D* (-2.16660*) and non-significant Fu's Fs (-4.218) also indicated population expansion. These diversity and neutrality indices can be attributed to the substantial genetic differentiation between the isolates from different continent (Troell *et al*., 2006).

Conclusions: To the best of authors' knowledge, it is the first attempt to molecularly characterize *H. contortus* isolates recovered from slaughtered sheep and goats in Jhang, Pakistan. The results will serve as a foundation for future molecular epidemiology studies employing hypervariable microsatellite markers as well as for designing of control strategies.

Authors contribution: WQ and MAZ conceived and designed the study. MAZ and RZA developed the methodology while WQ performed the experiments. WQ wrote the original draft. Project supervision and funding was acquired by MAZ. MF, IH, KA interpreted the data. MFK, HMI and FAA critically revised the script for its contents. Final version was endorsed by all the authors.

Supplementary materials: Representative haplotype sequences of *ITS-2* genes are available online in the GenBank database (Accession numbers: MW376203 to MW376209 and MW381017 to MW381023).

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