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RESEARCH ARTICLE

First Genetic Evidence of *Trypanosoma theileri* in Indigenous Cattle in Southern Punjab Province of Pakistan

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A B S T R A C T

Trypanosomes species, including *Trypanosoma evansi* (*T. evansi*) and *Trypanosoma theileri* (*T. theileri*) have been reported in a wide range of mammalian species worldwide. We report the first genetic evidence of *T. theileri* in indigenous cattle originating from the Southern Punjab province of Pakistan. The archived blood samples (n=30) were taken from cattle clinically suggestive of Trypanosomiasis, screened through microscopic examination, and processed for amplifying hypervariable region in the 18S rDNA and sequence analysis. Only *T. evansi* and *T. theileri* infections were identified, where the presence of *T. evansi* (22/30, 73.33%) was higher than that of *T. theileri* (8/30, 26.6%). Single nucleotide polymorphism (SNPs) suggested considerable interspecies specific variations between *T. theileri* and *T. evansi* haplotypes than intraspecies where a few variations within *T. theileri* imply circulation of closely related haplotype in the cattle. The genetic variations among haplotypes of *T. theileri* and *T. evansi* coupled with inferred monophyletic clustering (bootstrap CI>90) suggest both as two distinct species. Future studies are required to screen a large population of susceptible hosts further and elucidate population dynamics of trypanosome species that can infect hosts either alone or as a co-infection.

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INTRODUCTION

Animal Trypanosomiasis is a severe threat to animals and presents a significant constraint to the productivity losses and international trade in developing countries such as Pakistan (Shahzad *et al.,* 2010; Tehseen *et al.,* 2015; Tehseen *et al.,* 2017). The disease infects a wide range of domestic and wild animals, such as cattle, buffalo, camels, equines, dogs, pigs, deer, antelopes, duikers, sheep, and goats (Davila and Silva, 2000; Desquesnes *et al.,* 2013). The said infection can be acute (high mortality) or chronic (subclinical infection) and characterized by recurrent episodes of parasitemia-associated pyrexia, progressive anaemia, generalized loss of body condition, infertility, spontaneous abortions, neuropathy, and eventually death (Desquesnes *et al.,* 2013).

Trypanosomiasis is caused by numerous species divided into two major sections. Salivarian includes *T.evansi*, *T. brucei*, *T. vivax,* and *T. congelense.* Stercorarian includes *T. theileri*, *T. melophagium,* and *T. cruzi* (Sudan *et al.,* 2018). *T. evansi*, *T. brucei*, *T. vivax,* and *T. congelense* are well-thought-out to be highly pathogenic species. *T. theileri* is potentially pathogenic for animals under stress, such as physical and nutritional stress, including pregnant and newborn animals (Verloo *et al.,* 2000; Villa *et al.,* 2008).

Previous studies reported trypanosomiasis infection in buffalo, sheep, goats, camel, and equines but limited to detection of *T. evansi* using traditional diagnostic approaches, including microscopic screening and PCR assay in Pakistan (Shah *et al.,* 2004; Bhutto *et al.,* 2010; Shahzad *et al.,* 2010; Tehseen *et al*., 2015; Tehseen *et al*., 2017; Sabir *et al*., 2018; Sobia *et al*., 2018). However, information on the prevalence of other *Trypanosoma* species in Pakistan is rare. Although previous studies reported some cases of *T. theileri* infections in Europe (Verloo *et al.,* 2000; Villa *et al*., 2008), American (Jaimes-Duenez *et al*., 2018; Pacheco *et al*., 2018), African (Ngomtcho *et al*., 2017), Asian countries (Lee *et al*., 2010; Garcia *et al*., 2011a; Yokoyama *et al*., 2015) and its susceptible population. However, the pathogenicity of *T. theileri* and its economic importance in domestic

ruminants is still not fully understood. Hence, the present study is designed to investigate the molecular characterization of *T. theileri* infecting cattle populations in Southern Punjab, Pakistan.

MATERIALS AND METHODS

Collection of Trypanosoma positive blood samples: A total of 30 *Trypanosoma* positive blood samples originating from indigenous cattle were collected and archived from March 2020 to October 2020. Samples were collected by puncturing the ear vein from cattle showing a clinical representation of parasitic infestation, including pyrexia, anaemia, loss in productivity, poor body condition, and history of abortion. Detailed discussions with administrative and community leaders were held to raise awareness of the study. Furthermore, farmers were also encouraged to participate in the current study. Samples were collected with the help of veterinary professionals and sent to the University of Veterinary and Animal Sciences (UVAS) parasitology laboratory for further processing and analysis. The ethical committee of the Institutional Review Board of UVAS, Punjab, Pakistan, approved the research work (UVAS-793-1).

Trypanosoma positive blood samples were used to make thin and thick blood smears on the glass slide following the previously described methodology (Shah *et al*., 2004). After drying, the smears were fixed in methyl alcohol for 3 minutes. The slides were immersed in 10% Giemsa's stain for 30 min and were examined under an optical microscope (at 100x oil immersion objective) to identify the *Trypanosoma* at the genus level (Sadek *et al.,* 2021). The examination revealed 30 blood samples positive for *Trypanosoma* in cattle.

Genomic DNA isolation: WizPrep™ gDNA Mini kit was used to extract the genomic DNA (Wizbiosolutions, Seongnam, South Korea). To assess the quantity and quality of extracted DNA, the NanoDrop (NanoDrop Technologies Inc., Wilmington, DE, USA) was used to determine the quality and quantity of genome spectrophotometrically at λ=260/280 nm and λ=260/230 nm. The extracted DNA samples were stored at -20ºC till further use.

PCR amplification and sequencing of Trypanosoma 18S rDNA: For the molecular identification, Polymerase chain reaction (PCR) amplification using WizPrep™ PCR 2X Master reaction (Wizbiosolutions, Seongnam, South Korea) with a pair of 23-mer forward (5'-ACGTTCGCAAGAGTGAAACTTAA-3') and a 23-mer reverse (5'- GCATCACAGACCTGCTGTTGCCC-3') primers targeting V7-V8 region of 18S small subunit ribosomal gene (SSU rDNA) to target 370-bp fragment. WizPrep™ Gel/PCR Purification Mini Kit was used to purify the PCR amplicons (Wizbiosolutions, Seongnam, South Korea). The targeted fragment was sequenced in both directions using the same primers utilizing a conventional BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) and an automated DNA sequencer, as per the manufacturer's instructions (ABI PRISM Genetic Analyzer 3130x1).

Bioinformatic analysis of Trypanosoma 18S rDNA: The ChromasPro software (Version1.7.4) was used to evaluate each sequence's chromatograms, then compared to the GenBank database through nucleotide sequence homology using the National Center for Biotechnology information BLAST Tools (NCBI). Similar sequences of *Trypanosoma* species were retrieved from the public database, and all sequences were aligned using Clustal W methods in BioEdit® version 7.2.5 (Sunantaraporn *et al.,* 2021). A phylogenetic tree was constructed using the MEGA-X software and a maximum likelihood statistical method with a bootstrap value of 1000 to investigate the taxonomic position of under-studied sequences (Kumar *et al*., 2018; Stecher *et al*., 2020).

RESULTS

Prevalence of trypanosome species in the Field Samples: The sequencing was performed on 30 *Trypanosoma*-positive blood samples to detect *Trypanosoma* species. Only *T. evansi* and *T. theileri* infections were identified. The results revealed that the presence of *T. evansi* was higher than that of *T. theileri* in cattle. A 22/30 (73.33%) samples were *T. evansi* positive, while 8/30 (26.6%) samples were *T. theileri* positive. The sampling site and distribution of Trypanosoma species identified in this study are shown in Fig. 1. The FASTA sequence files have been made freely available through the Mendeley database at DOI: 10.17632/d66sjjrwyn.1.

Assessment of Trypanosoma 18S rDNA genetic variations: A 96 filtered sequence data sets of *T. theileri*, *T. congolense*, *T. vivax*, *T. simiae*, *T. evansi*, and *T. brucei* were derived from NCBI GenBank. The 22 samples of *T. evansi* and eight *T. theileri* in the present study were aligned using Clustal W methods in BioEdit® version 7.2.5 and then imported into the FaBox v1.5 online software to calculate the number of "haplotype" generated from each species. In total, 48 haplotypes of 18S rDNA of six species were generated for the reference database, where study sequences corresponded to 5 haplotypes of *T. theileri* and four haplotypes of *T. evansi* (Table 1). Upon comparing the genetic distance between reference database and study sequences, it was observed that six species showed 83%-98% similarity between the region of 18S rDNA (Table 2). Based on current study findings, *T. brucei* and *T. evansi* are more closes than the rest of the species as they showed a genetic identity of 98%.

Table I: 18S rDNA sequences based identification of Trypanosoma species haplotypes in the reference database and those identified in this study

Species	Public database		This study	
	18S rDNA sequences	Haplotypes	18S rDNA sequences	haplotypes
T. vivax	13	3	-	-
T. theileri	35	11	8	5
T. simiae	3	3	-	-
T. evansi	26	6	22	4
T. congolence	13	10	-	-
T. brucei	36	15	-	-
Total	126	48	30	9

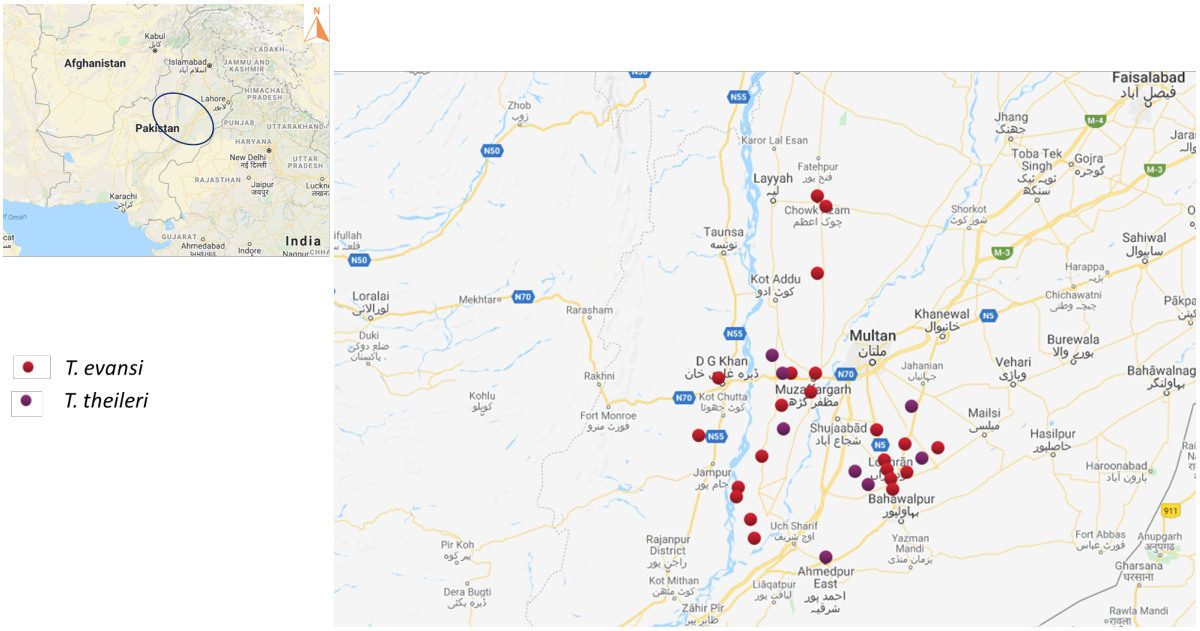

Fig. 1: Geographical distribution of sampling sites and places where each of the studied sample was found positive corresponding to *T. theileri* and *T. evansi.*

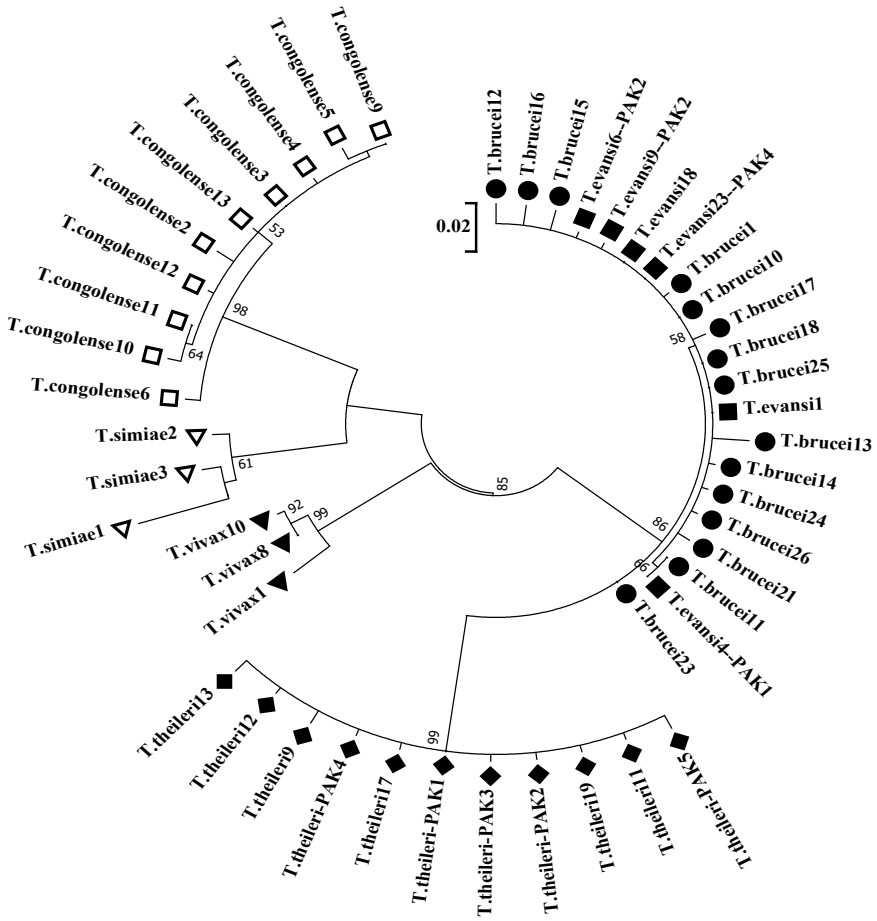

Fig. 2: A Phylogenetic analysis of study haplotypes with reference haplotypes reported worldwide. The study-identified haplotypes [(*T. theileri*, n = 5) (*T. evansi*, n = 4)] are marked "PAK" in the analysis for their unique identification. The tree was made using the gamma-distributed Kimura 2 parameter (K2+G) model. The maximum likelihood statistical tool with a bootstrap value of 1000 was used. Different shading was used to represent each species. Bootstrap values >70 was considered significant.

T. theileri and *T. evansi* were also studied for inter-specific and intra-specific species variation. The SNPs analysis revealed significant inter-species variation among the *T. theileri* and *T. evansi* haplotypes but minimal intra-species variation within the *T. theileri* and *T. evansi* haplotypes. (Table 3). The slight genetic variation seen in *T. theileri* sequences shows that the isolates from Pakistan are highly similar to *T. theileri* isolates found in the NCBI database. Furthermore, the genetic differences between *T. evansi* and *T. theileri* haplotypes imply two different species.

Table 2: The 18S rDNA-derived relative genetic divergence among haplotypes of Trypanosoma species in the reference database and those identified in this study

	T. brucei	*T. congolence*	*T. evansi*	*T. simiae*	*T. theileri*
T. vivax	85	84	86	86	86
T. theileri	88	83	89	84	
T. simiae	84	88	86		
T. evansi	98	87			
T. Congolence	86				

Table 3: A comparative analysis of intra-species specific variation (SNPs) within the *T. theileri* and *T. evansi* haplotypes identified in Pakistan and those published in the NCBI database

Nucleotide Position	*T. theileri*	*T. evansi*	Nucleotide position	*T. theileri*	*T. evansi*
16	A/C	A	285	-	T
37	A	A/G	292	-	C
47	G/A	A	301	-	C
53	T/C	T	304	A	-
60	T	T/A	311	-	T
94	C	C/-	312	-	C
95	-	C	315	A	-
101	C	C/-	317	-	C
103	-	C	318	-	C
152	C/A	A	322	G	T
156	T/C	T	326	-	T
193	C/T	T	327	-	A
197	A/T	T	328	-	C
199	A/G	G	329	-	A
223	G/A	G	333	T	G
248	-	T	334	A	C
249	-	C	335	T	C
251	C	A	339	T/G	A/T
254	T	C	343	T	-
259	T	C	344	-	T
264	A	G	358	-	C
269	C	-	363	-	C
275	-	G	374	G/A	A
280	-	G	377	G/T	T
284	-	A			

Phylogenetic analysis of the Trypanosoma 18S rDNA haplotypes: The presence of *T. theileri* in southern Punjab was confirmed by phylogenetic analysis because it is assumed that only *T. evansi* species infect livestock in this area. About 48 haplotypes were used to construct a phylogenetic tree that shows distinct species-specific clustering of each species. *T. theileri* and *T. evansi* isolates obtained from Pakistan made a cluster and showed similarity with published sequences in the NCBI database (Fig. 2). A bootstrap confidence interval of 82 supports the divergence of the *T. theileri* clade from the *T. brucei* and *T. evansi* clades. Additionally, phylogenetic analysis of the haplotypes showed distinct species clustering in all species, except for *T. evansi* and *T. brucei* (Fig. 2). A bootstrap value with a high confidence interval for clustering is ranging from 53-99 because a node with a bootstrap value greater than 50 is considered distinct and well supported for each species.

DISCUSSION

In Pakistan, animal trypanosomiasis is considered endemic and has caused sporadic epidemics in various country regions. Several studies revealed *T. evansi* infection in buffalo, camels, equines, sheep, and goats (Sobia *et al.,* 2018; Sabir *et al.,* 2018); however, there has been a lack of information on *T. evansi* and *T. theileri* infection in cattle throughout the country. The present study is the first-ever report about the prevalence and genomic characterization of *T. evansi* and *T. theileri* strains reported in indigenous cattle from Southern Punjab, Pakistan, to the best of the acquaintance. Using the microscopic technique, blood samples were positive for trypanosomes species simply because they were taken from clinically positive animals for use in this study. A marked difference in the occurrence of *T. theileri* is observed in the present study and previous study in proportion to *T. evansi* (Rodrigues *et al*., 2003). Such distinction is attributed to the number of infected cattle being assessed correctly using buffy coat layers in microscopic and microhematocrit methods (Verloo *et al.,* 2000; Rodrigues *et al.,* 2003). The PCR-based assay was used to identify further, and analysis of genomic characteristics because previous studies claimed more sensitivity and specificity of PCR-based techniques than conventional microscopic examination (Desquesnes and Davila, 2002; Rodrigues *et al.,* 2006). In Pakistan, mostly Trypanosomiasis is diagnosed by clinical signs and microscopic examination of a blood smear and *T. theileri* infection usually remains unnoticed or undiagnosed because of a low level of parasitemia, closely-related morphology, and non-specific clinical signs. The infection of *T. theileri* may persist for an extended period without detection in blood smears using microscopic and microhematocrit methods. Therefore, genome-based PCR is crucial to detect specific trypanosome infections in all susceptible hosts (Desquesnes and Davila, 2002). Therefore, only PCR-based findings for *T. theileri* and *T. evansi* were considered in the current study.

Compared to gained outcomes of the current study on the prevalence of *T. theileri* in cattle from Southern Punjab, a comparable prevalence of *T. theileri* was reported in indigenous cattle population in Sri Lanka (7.6%) (Yokoyama *et al.,* 2015) and Western Brazil (8.13%) (Pacheco *et al.,* 2018). *T. theileri* prevalence, on the other hand, was far lower than that reported in cattle populations in Cameroon (17%) (Ngomtcho *et al*., 2017), Thailand (26%) (Garcia *et al*., 2011a), and Colombia (Jaimes Duenez *et al*., 2018). Moreover, the current study's finding on the prevalence of *T. evansi* in cattle agrees with previous evidence of *T. evansi* prevalence (16.9%) in the cattle population in the Southern Punjab districts (Sobia *et al.,* 2018). Herein, the prevalence *T. evansi* in cattle population was higher than that reported in buffalo (7.7%) (Shahzad *et al.,* 2010), and equines (8%) (Sabir *et al.,* 2018) and 14.4% (Tehseen *et al.,* 2017) in Northern Punjab, and camel population in Sindh province (11.25%) (Bhutto *et al.,* 2010) and 13.72% (Shah *et al.,* 2004). The prevalence *T. evansi* in the cattle population (20.1%) was lower than that reported in the camel population in Southern Punjab districts (30.5%-31.9%) (Tehseen *et al.,* 2015). Concisely, the differences in the prevalence of *T. theileri* and *T. evansi* might be attributed to animal species, immunological status, disease-endemic region, geographical distribution, climate influence, landscape variations, diagnostic techniques, farming practices, and measures adopted to control the disease (Majekodunmi *et al.,* 2013; Fikru *et al.,* 2015). The possible reason for the prevalence of Trypanosomiasis in Southern Punjab could be related to production and rearing systems, presence of vectors, particularly hematophagous flies (mainly Tabanids and Stomoxes),

livestock mitigation, lack of veterinary and para-veterinary services for proper treatment, lack of diagnostic services, parasitic load, public unawareness, poor socioeconomic environment, and high agricultural activities in the regions (Ashraf *et al.,* 2013; Aslam *et al.,* 2017). Even though these factors reflect the possible acquisition of Trypanosomiasis, a series of demographic and epidemiological investigations are necessary to prove this hypothesis.

An 18S small subunit rDNA (SSU rDNA)-based phylogenetic analysis was conducted for the taxonomic classification of trypanosomes. Internal transcribed spacer (ITS), small subunit, glycosomal glyceraldehyde 3-phosphate dehydrogenase (GADPH), and cathepsin L-like protein genes have been used as a genetic marker to analyse genetic variations in *T. theileri* strains reported in a wide range of hosts worldwide (Garcia *et al.,* 2011b). The phylogenetic analysis clustered the *T. evansi* strains close together with the strain reported from Pakistan. This is not surprising because several studies have reported *T. evansi* infection in a wide range of animals' species in Pakistan (Shah *et al.,* 2004; Bhutto *et al.,* 2010; Shahzad *et al.,* 2010; Tehseen *et al.,* 2015; Tehseen *et al.,* 2017; Sabir *et al.,* 2018; Sobia *et al.,* 2018). Notably, the current study provides the first-ever genomic information on *T. theileri* strain reported in the indigenous cattle population. Herein, phylogenetic analysis and haplotype distribution clustered *T. theileri* strains together with strains reported in cattle from different parts of the world but away from strains reported in buffalo. Such a pattern of clustering highlighted the host-specific potential of *T. theileri*. Several studies have also claimed the clustering of *T. theileri* strains independently according to the host species (Rodrigues *et al.,* 2006; Garcia *et al.,* 2011b). Previous research proposed that *T. theileri* strains that infect buffaloes and cattle may have genetic differences based on SSU rDNA nucleotide sequence analysis (Rodrigues *et al.,* 2006; Garcia *et al.,* 2011b). Even though the SSU rDNA region is considered a highly conserved and multi-copy locus gene, the evidence of nucleotide polymorphism displaying at SSU rDNA gene likely suggested a host-specific evolutionary potential of *T. theileri* (Garcia *et al.,* 2011b).

Differentiation among different strains of *T. theileri* originating from distinct species has been detected through genetic polymorphism even in the conserved SSU rDNA (Rodrigues *et al*., 2006; Garcia *et al*., 2011b). These findings concluded that *T. theileri* genomic characteristics might be hosts specific. However, genetic potential in *T. theileri* has been studied in a few countries with a limited number of nucleotide sequences. Furthermore, because only partial SSU rDNA gene sequences were used for analysis, thus the findings on *T. theileri*'s host specificity may be inconclusive. Therefore, the current study may not draw any conclusions on *T. theileri*'s host specificity in Pakistan. Further investigations of *T. theileri* prevalent strains, using a more significant number of sequences from diverse host species in endemic regions, are required to elucidate the question of host specificity and get a more comprehensive knowledge of genetic diversity. Conclusively, the current study revealed the prevalence of *T. theileri* in cattle and highlighted the need to incorporate surveillance programs for this parasite better to understand its epidemiology and economic impact on international trades and develop appropriate and long-term control strategies. Moreover, the current study reported the first SSU rDNA gene sequence obtained naturally in cattle in Pakistan and supported the hypothesis that *T. theileri* may be strongly constrained by hosts' diversity.

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Authors contribution: GY, KA, MIR, and MZS imitated the idea of the manuscript. GY and MZS conducted laboratory experiments and analysed data. GY, KA, MIR and MZS edited the manuscript. All authors approved the final manuscript.

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