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Sequence Analysis of SAG2 of Feline *Toxoplasma gondii* Oocysts in Pakistan

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Authors' Contribution
IR conceived and designed the study. HuN, SI and ARB acquired, analyzed and interpreted the data. IR wrote the article. All others helped in preparation of manuscript.

Key words
***Toxoplasma gondii*, SAG2, Cat, PCR, Pakistan.**

ABSTRACT

Toxoplasmosis is caused by coccidian parasite, *Toxoplasma gondii*. One third of human population of the world is believed to be infected with *T. gondii*. Cats serve as final host of *Toxoplasma gondii* and are the main source of contamination of soil and water. Fecal samples from cats at Pet center of UVAS (Lahore, Pakistan) were screened for coccidian parasites through microscopy examination. DNA was extracted from positive fecal samples for coccidian parasites and a *T. gondii* PCR was performed. Five sets of primers were designed using PrimerSelect tool for PCR to amplify SAG2 gene 5' and 3' regions. Sequences of 5 fragments of SAG2 were annotated and analyzed using DNASTAR Lasergene. After phylogenetic analysis with 3 clonal types and atypical strains, and on the basis of restriction map of HhaI and Sau3AI, our 3 isolates of *T. gondii* were found more closely linked to a typical strain. This is the first genetic analysis of *T. gondii* in Pakistan. In order to develop our knowledge about the toxoplasmosis epidemiology, further genotype analyses of *T. gondii* from animals and man need to be performed in Pakistan.

INTRODUCTION

Toxoplasmosis is caused by an intracellular protozoan parasite, *Toxoplasma gondii* that may result in life-long colonization in animals and humans (Ali *et al*., 2017; Nicolle and Manceaux; 1909). This protozoa has cosmopolitan distribution and it is the most common human zoonotic infection in many geographic regions of the world (Scott *et al*., 2007). The infection can be acquired by three primary routes: ingestion of tissue cysts in undercooked infected meat; ingestion of food or water contaminated with sporulated oocysts shed in the feces of a cat; and congenitally, across the placenta from the mother to the fetus when she is infected through one of the previous two routes during pregnancy (Remington *et al*., 1985; Dubey, 1994). Please mention Remington before Dubey People may acquire infection by accidental intake of oocysts present in environment (soil, water, vegetables and fruits) contaminated with feces defecated by infected felines (definite host) (Benenson *et al*., 1982; Coutinho *et al*., 1982; Dubey et al., 2007; Baldursson and Karanis, 2011; Karanis *et al*., 2013). The role of cats in the contamination of environment with their fecal oocysts has been emphasized by health professional. Feral and owned cats contribute to spread *T. gondii* to humans and animals, as well as maintaining wildlife reservoirs (Frenkel *et al*., 1995; Weigel *et al*., 1995; Lehmann *et al*., 2003). A wide variability of fecal oocysts varying from 3 to 810 million, are shed by cats during 3 to 5 days after initial infection with *T. gondii*, and the shedding period lasts for a median of 8 days, although it may be as long as 3 weeks (Dubey, 1976, 2001, 2002, 2005). Oocysts may survive for months in soil and water, thereby enhancing the probability of transmission to intermediate hosts such as birds, rodents and humans (Yilmaz and Hopkins, 1972; Frenkel *et al*., 1975).

The genotyping studies on *T. gondii*'s led to the description of a clonal population structure with three main lineages, designated as type I, II and III, related to mouse-virulence (Darde *et al*., 1988, 1992; Howe and Sibley, 1995; Sibley and Boothroyd, 1992). Please rank the references Genotypes not belonging to the three main lineages were found predominant in Brazil where the population structure of *Toxoplasma* was more complex, with a higher genetic diversity than initially described.

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These "new" genotypes, or more exactly newly discovered genotypes, were designated, depending on the authors, as atypical, exotic, recombinant, or non-archetypal genotypes (Darde, 2008).

Several markers like SAG2, SAG3, PK1, BTUB, APICO, GRA6, c22-8, c29-2 and L358 have been used for *T. gondii* genotyping (Darde, 2004; Boughattas *et al*., 2014). However, the Surface Antigen 2 (SAG2) marker has been extensively used for strain identification (Sibley and Boothroyd, 1992; Parmley *et al*., 1994; Howe and Sibley, 1995; Howe *et al*., 1997; Mondragon *et al*., 1998; Owen and Trees, 1999; da Silva *et al*., 2005; Dubey *et al*., 2005a, b, 2006) into three clonal lineages and atypical strains (Behzadi *et al*., 2003; Wang *et al*., 2013). A preliminary study of the SAG2 gene, using restriction fragment length polymorphism (RFLP) method with HhaI restriction enzyme (Sibley and Boothroyd, 1992), found two alleles at this locus, including virulent specific and avirulent specific. Later, researchers developed the SAG2-RFLP marker using two enzymes, HhaI and Sau3AI, in PCR-RFLP that could distinguish the three major genotypes I, II and III (Howe *et al*., 1997).

We have collected fecal samples from cats and identified *T. gondii* using non-invasive method (Dabritz *et al*., 2007; Wendte *et al*., 2011). To the best of our knowledge, sequence analysis of SAG2 of *T. gondii* fecal oocyst was the pioneer study in Pakistan.

MATERIALS AND METHODS

Fecal samples screening

Fecal samples from diarrheal cats were collected at Pet center, University of Veterinary and Animal Sciences (UVAS), Lahore, Punjab, Pakistan. The samples were screened microscopically for *T. gondii* on the same day and preserved in 70% ethanol at -20°C for further analysis.

Oocysts were used for DNA extraction using AxyPrep™ Multisource Genomic DNA mini-Prep kit following manufacturer instructions. Reference DNAs (RH) of *Toxoplasma* were used as positive samples. Five sets of primers were designed by PrimerSelect program (Wan and Fang, 2003) of DNASTAR Lasergene (Ahern, 1993; Burland, 1999) (DNAstar, Madison, WI) and used to amplify 5' and 3' regions of SAG2 gene (Table I). SAG2 gene was amplified from 5' and 3' end by performing polymerase chain reaction (PCR). Amplification was performed in thermo-cycler in a final volume of 20 µl of reaction mixture consisting of 2 µl of each primer (1 pM each), template (100 ng/µl), 10x PCR buffer with $MgCl_2$ (25 mM), dNTPs (2 mM each) and 1 µl of Taq (5 U/µl) polymerase. The protocol for temperature cycling included 5 min at 94°C for initial denaturation, 35 cycles (45 sec of denaturation at 94°C, 1 min at annealing temperature of 64.6°C and 1 min of extension at 72°C). The final extension continued for an additional 10 min. The 3' locus of SAG2 gene was similarly amplified by this standard PCR with specific primers. Products were electrophoresed in 1.2% agarose gel.

Table I.- Five sets of primer pairs for amplification of 5' and 3' regions of *Toxoplasma gondii* SAG2 gene.

Locus	Forward (F) and reverse (R) primers used
5' Locus_171	F: 5'-AGTGACCCATCTGCGAAGAA-3' R: 5'-TTCTCAAAGACCACGAGCCT-3'
5' Locus_242	F: 5'-TTCTCAAAGACCACGAGCCT-3' R: 5'-TGCACAGACTCGAGGAAGTT-3'
5' Locus_211	F: 5'-CAGTGGCGAAGGTGATGTCT-3' R: 5'-CTCTCACGGGCAAGGTTCTT-3'
3' Locus_288	F: 5'-CTCTCACGGGCAAGGTTCTT-3' R: 5'-CGAAGTTGGTGGTAACGGGA-3'
3' Locus_230	F: 5'-CGCAGTTCTGTTCTCCGAAG-3' R: 5'-AGGAACTTGTTTGCCGACAC-3'

Genetic analysis

Before PCR products sequencing, amplified DNA were purified using ethanol precipitation. Briefly, 80% ethanol was added to PCR products and kept in dark for 30 min, followed by centrifugation at 12,000 rpm for 15 min. Pelleted material was air-dried overnight and diluted in distilled water.

The sequencing results were subjected to blast using Basic Local Alignment Search Tool (BLAST) provided by NCBI (McGinnis and Madden, 2004). Amplified DNA samples were sequenced by Sanger sequencing method at 1st BASE DNA Sequencing Services (Singapore) (Xie *et al*., 2004).

Sequenced fragments of SAG2 were annotated. Restriction enzyme analysis for HhaI and Sau3AI was carried out by using Sequence Builder program (Morales *et al*., 2012) of DNASTAR Lasergene. Alignment of sequences were performed using CLUSTAL W (Thompson *et al*., 1994) to quantify genetic distances among isolates of Type I, II, III and atypical ones found in Genbank as shown in Table II. We used MegAlign program (Fukushima *et al*., 2002) of DNASTAR Lasergene for phylogenetic tree construction and Bootstrapping analysis. The P (uncorrected distance often referred as p-distances or dissimilarity distance) and Jukes–Cantor distances were used to construct a neighbor-joining tree. We applied parsimony method with bootstrapping (1000 replicates) with MegaAlign program. SAG2 gene sequence of *Neospora caninum* was used as out-group (Ajzenberg *et al*., 2004).

Table II.- List of published SAG2 sequences along with their accession numbers.

Genotype	Accession number	Reference	Strain name
Type I	AK317818.1	(Wakaguri *et al*., 2008)	RH
	M33572.1	(Prince *et al*., 1990)	RH
	JX045478	(Khan *et al*., 2009)	RH
	EU053942.1	(Ferreira *et al*., 2008)	RH
	EU258520	(Dubey *et al*., 2008b)	RH
Type II	AF249697	(Lehmann *et al*., 2000)	Beverley
	AF357578	(Fazaeli and Ebrahimzadeh, 2007)	LGE96-1
	JX045473	(Khan *et al*., 2009)	DEG
	JX045474	(Khan *et al*., 2009)	ME49
	AB667974	(Tavalla *et al*., 2013)	Tehran
	EF585695	(Lindstrom *et al*., 2008)	TgUgCh2
	EF585696	(Lindstrom *et al*., 2008)	TgUgCh52
	EU053943.1	(Ferreira *et al*., 2008)	ME49
	EU258521	(Dubey *et al*., 2008b)	PTG
	EU258523	(Dubey *et al*., 2008b)	TgWtdUs4
	KC928258	(Burrells *et al*., 2013)	Pc10
	KM246841	(Donahoe *et al*., 2014)	NZfs8825
	KM246837	(Donahoe *et al*., 2014)	NZfs8825
	KJ754425.1	(Vilares *et al*., 2014)	G2992316
	KJ754389.1	(Vilares *et al*., 2014)	P1509306
	KJ754409.1	(Vilares *et al*., 2014)	P454205
Type III	AF249698	(Lehmann *et al*., 2000)	C56
	AF357577	(Fazaeli and Ebrahimzadeh, 2007)	S48
	AF357579	(Fazaeli and Ebrahimzadeh, 2007)	NED
	AB667973	(Tavalla *et al*., 2013)	S4
	AB667972.1	(Tavalla *et al*., 2013)	S7
	AB667975	(Tavalla *et al*., 2013)	S5
	DQ000461	(Sreekumar *et al*., 2005)	Skunk SAG2
	EF585703	(Lindstrom *et al*., 2008)	TgUgCh52
	EU053944.1	(Ferreira *et al*., 2008)	VEG
	EU258522	(Dubey *et al*., 2008b)	CTG
	EU258528	(Dubey *et al*., 2008b)	TgWtdUs13
	KJ754400.1	(Vilares *et al*., 2014)	P3884739
	KJ754396.1	(Vilares *et al*., 2014)	P3216020
Atypical	AF249696	(Lehmann *et al*., 2000)	COUGAR TC751G34
	AF357582	(Fazaeli and Ebrahimzadeh, 2007)	CASTELLS
	AF357580	(Fazaeli and Ebrahimzadeh, 2007)	MAS
	AF357581	(Fazaeli and Ebrahimzadeh, 2007)	RUB
	JX045494	(Khan *et al*., 2009)	COUG
	JX045491	(Khan *et al*., 2009)	GUYKOE
	JX045492	(Khan *et al*., 2009)	RUB
	JX045493	(Khan *et al*., 2009)	GUYDOS
	JX045489	(Khan *et al*., 2009)	GUYMAT
	JX045472	(Khan *et al*., 2009)	CASTELLS
	JX045470	(Khan *et al*., 2009)	TgCatBr1
	JX045471	(Khan *et al*., 2009)	MAS
	EU258519	(Dubey *et al*., 2008b)	TgCgCa1
	EU258531	(Dubey *et al*., 2008b)	MAS
	EU258533	(Dubey *et al*., 2008b)	TgDgCo11
	EU650329	(Velmurugan *et al*., 2008)	TgCkGh1
	EU650330	(Velmurugan *et al*., 2008)	TgCkNg1
	JX045490	(Khan *et al*., 2009)	VAND

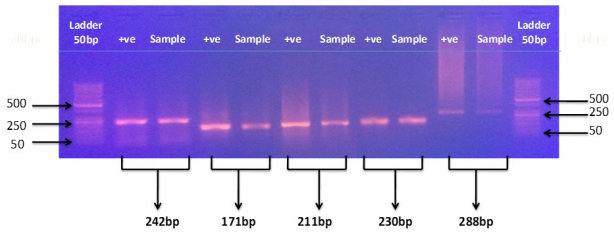

Fig. 1. Agarose gel eletrophoresis of SAG2 gene products (242, 171, 211, 230 and 288bp).

RESULTS

Sequence analysis of T. gondii

Five regions were amplified at 5' and 3' loci of SAG2 gene as shown in Figure 1. Five fragments of SAG2 were purified and got sequenced and were annotated. Clustal W analysis and then bootstraping were performed to find genotype comparison of our 3 laboratory isolates from cat fecal samples with published strains of *Toxoplasma* in GenBank. The 3 isolates of *Toxoplasma* were found to be more close to atypical strain AF357581 (Fig. 2). Three representative strains of each genotype from type I, II or III was taken after Clustal W analysis. The alignment of our 3 isolates was done with SAG2 sequences from GenBank of SAG2 *T. gondii* Type I, II and III strains at 5'. Dissimilarities among sequences after comparison at some points were observed (Fig. 3). Thus, it was found that our 3 isolates were near to atypical. Restriction enzyme analyses of Hha1 and Sau3AI were done by using Sequence Builder tool of DNASTAR Lasergene at 5' and 3' loci of SAG2 of our 3 isolates with representative isolates of Type I, II and III strains of *T. gondii* (Table III). Dissimilar pattern of restriction enzymes; Hha1 and Sau3A1 sites were observed in our isolates at 5' and 3' loci of SAG2 gene with the representative *T. gondii* strains of Type I, II and III.

DISCUSSION

This study reveals new information on *T. gondii* genotype shedding from cat feces in the metropolitan city of Lahore, Pakistan. We confirmed the presence of *T. gondii* oocysts in cats' feces through PCR, genetically characterized them at SAG2 locus and found diversity for atypical strain.

SAG2 polymorphic gene has been extensively used solely to characterize *Toxoplasma* genotype into 3 archetypal types (I, II and III) (Owen and Trees, 1999; Honore *et al*., 2000; Gallego *et al*., 2006; Abdel-Hameed and Hassanein, 2008; Asgari *et al*., 2013; Tavalla *et al*., 2013; Elamin, 2014) and atypical or recombinant types (Gallego *et al*., 2006; Lindstrom *et al*., 2006; Elamin, 2014).

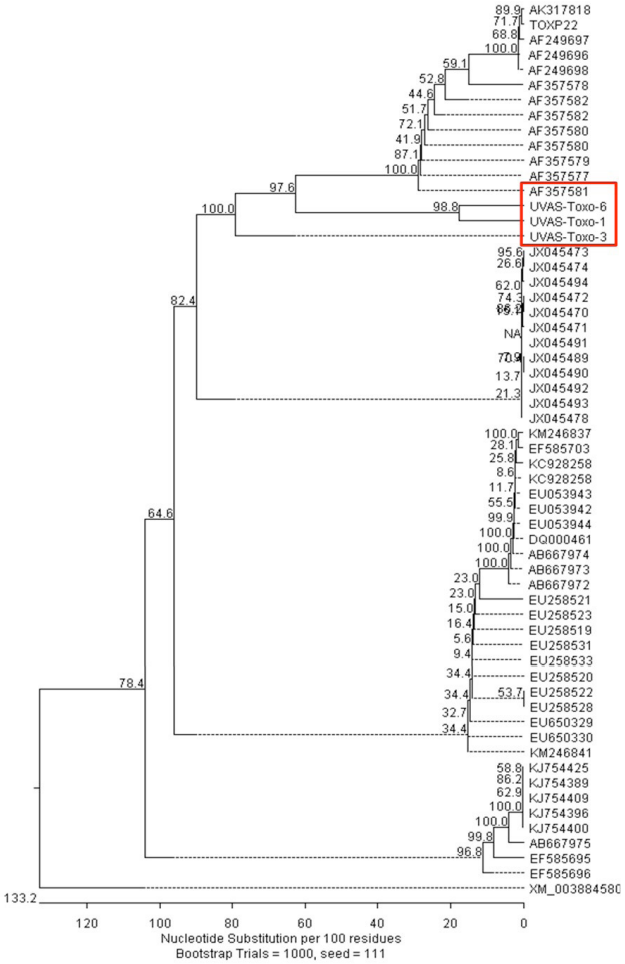

Fig. 2. Phylogenetic and bootstrapping analysis of 3 isolates with published archetypal lineages and atypical strains. UVAS-Toxo 1, 3 and 6 are our isolates from cat feces. XM_00384580 is the SAG2 sequence of *Neospora caninum* served as out-group. AF357581 is the SAG2 sequence of atypical *T. gondii* strain.

We designed 5 primer sets to amplify 5' and 3' loci of SAG2 to see the polymorphic changes in the loci. Similarly, the researchers adopted this strategy to amplify separately SAG25' and 3' loci for genotyping analysis of *Toxoplasma* from clinical samples of patients and tissue samples of mice in Iran (Fuentes *et al*., 2001; Behzadi *et al*., 2003; Fallah *et al*., 2013).

Previously, most of the studies for genotyping analysis were done through ingestion of oocysts by rodents or from tissue cysts in mammals (Araujo *et al*., 2010; Cabral *et al*., 2013; Yan *et al*., 2014). All the tested fecal samples gave correct genotypes at least once for each locus when referenced against blood-derived genotypes (Lathuilliere *et al*., 2001).

Table III.- Restriction enzyme analysis of Hha1 and SauA3I. Analysis of restriction enzymes at 5' SAG2 locus (A) and 3' SAG2 locus (B).

A.- At 5'SAG2 locus.

Toxoplasma strains	HhaI position					
	11	12	61	83	167	168
EU053942 (RH)	I	-	-	-	-	I
EU053943 (Me49)	-	II	-	-	-	II
EU053944 (VEG)	-	-	-	-	III	-
UVAS-Toxo-3	-	-	-	-	-	-
UVAS-Toxo-1	-	-	UVAS-Toxo-1	UVAS-Toxo-1	-	-
UVAS-Toxo-6	-	-	UVAS-Toxo-6	UVAS-Toxo-6	-	-

B.- At 3'SAG2 locus.

Toxoplasma strains	3 locus													
	Sau3A1													Hhal
	29	58	63	73	107	152	164	165	173	186	198	199	207	120
AY895019 (RH)	-	I	-	I	I	-	-	-	-	-	-	-	-	-
EF585695 (TgUgCh2)	II	-	II	-	-	-	-	-	-	-	-	-	-	II
AB667975 (S5)	-	-	-	-	-	III	-	-	-	III	-	-	-	-
UVAS-Toxo-3	-	-	-	-	-	-	-	UVAS-Toxo-3	-	-	-	UVAS-Toxo-3	-	-
UVAS-Toxo-1	-	-	-	-	-	-	-	-	UVAS-Toxo-1	-	-	-	UVAS-Toxo-1	-
UVAS-Toxo-6	-	-	-	-	-	-	UVAS-Toxo-6	-	-	-	UVAS-Toxo-6	-	-	-

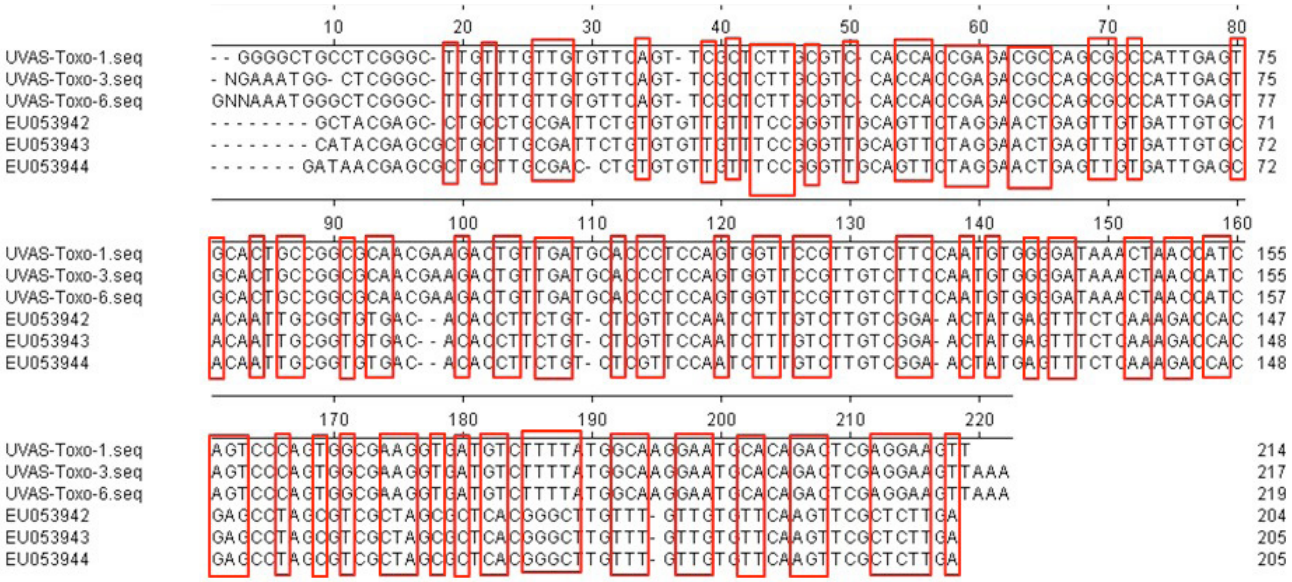

Fig. 3. Clustal W alignment of 3 isolates of this study with the representative 3 clonal strains of Type I (EU053942), Type II (EU053943) and Type III (EU053944). Red rectangular marked sequences show dissimilarity of our isolates at 5' locus of SAG2 with Type I, II and III strains.

No study in Pakistan has been carried out to identify the genotype of *Toxoplasma* strain. The attempt to amplify SAG2 gene was unsuccessful by using the primers described by Howe and Sibley (1995) to amplify 1196 bp product for genotyping analysis. It might be due to the large size of fragment to be amplified. Experiencing the difficulties to optimize PCR on the published primers, we designed 5 sets of primers (Table I) to amplify 5' and 3' loci separately and contiguous sequence was constructed for alignment analysis with SAG2 published sequences of archetypal clonal and non-archetypal lineages. The 3 isolates from cat feces in Lahore metropolitan city were atypical. Vaudaux *et al*. (2010) showed that atypical strains possessed dissimilar sequences due to polymorphism in GRA6 and GRA7 genes that encoded different epitopes identified in Brazilian *Toxoplasma* isolates in chickens and cats from Santa Isabel (Vaudaux *et al*., 2010).

Different studies showed that the *T. gondii* population structure consists of three major clonal lineages designated types I, II, and III which are predominant in Europe and North America (Darde *et al*., 1992; Howe and Sibley, 1995), whereas atypical genotype is dominant in South America and Asia (Lehmann *et al*., 2006; Dubey *et al*., 2007, 2008a), some of which were also shown to be highly virulent in mice (Pena *et al*., 2008). *T. gondii* atypical genotypes were found to be associated with a number of severe cases of toxoplasmosis in immunocompetent individuals (Carme *et al*., 2009). Experimental studies showed that such atypical genotypes can develop when a cat ingests prey infected with *T. gondii* of more than one clonal Type, followed by sexual recombination in the gut of the cat which can result in progeny representing a mixture of the two parental genotypes (Su *et al*., 2002; Saeij *et al*., 2006). It is most likely that these atypical genotypes are the result of sexual recombination in cats (Herrmann *et al*., 2010). It is obviously similar to the prevalence of atypical strains in different ecological and geographical regions like South America.

Hha1 and Sau3AI were used for PCR-RFLP analyses at SAG2 loci to interpret genotyping studies of *Toxoplasma* (Howe *et al*., 1997; Fuentes *et al*., 2001; Grigg *et al*., 2001; Behzadi *et al*., 2003; Pena *et al*., 2006; Sabaj *et al*., 2010; Lass *et al*., 2012). We used Sequence Builder and MegAlign tools of DNASTAR Lasergene to characterize the restriction sites of Hha1 and Sau3AI of our isolates comparing with the representative *T. gondii* strains of 3 archetypical clonal lineages. Our isolates were atypical strains since dissimilar distribution pattern of restriction enzyme sites of Hha1 and Sau3AI of our isolates (Table III). These results are in comparison with Pena *et al*. (2006), who found 2.1% of mixed or recombinant *T. gondii* strains from tissue homogenates of 47 cats. They found dissimilar pattern of restriction digestion of Hha1 and Sau3AI of SAG2 locus as compared to Type I, II and III (Pena *et al*., 2006). Further studies are need to assess the genotype studies of *T. gondii* in intermediate hosts in humans and animals in Pakistan.

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Statement of conflict of interest

Authors have declared no conflict of interest.

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