PCR–RFLP based genotyping of Indian isolates of *Toxoplasma gondii*

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Abstract *Toxoplasma gondii* is an apicomplexan parasite capable of infecting a wide variety of warm-blooded animals, including birds and humans and is zoonotically important too. Felidae serve its definitive hosts and most infections are inoccuous while in various intermediate hosts (e.g. sheep), it is responsible for abortion, still births. Humans which are immune compromised are also susceptible to toxoplasmosis. Most of the epidemiological studies have revealed it to be belonging to three clonal types with exceptions in South Africa having atypical isolates. Current genotyping was carried out at 11 genetic loci (SAG1, 5'-SAG2, 3'-SAG2, alt. SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358 and PK1) using multiplex-nested polymerase chain reaction restriction fragment length polymorphism (Mn-PCR–RFLP). SAG1, alt SAG2, SAG3, BTUB, GRA6, C22-8, C29-2, L358 and PK1 could differentiate our strain/isolates as type I (*T. gondii* RH) and type III (*T. gondii* isolates from Chennai and Izatnagar). 5'SAG2 and 3'SAG2 in combination confirmed these as above mentioned genotypes. Further, the *T. gondii* RH was assigned Toxo DB#10 and local isolates of *T. gondii* were assigned Toxo DB#2. The present study is the first report on existence of Type III *T. gondii* lineage from animal population of Indian subcontinent based on PCR–RFLP.

Keywords *Toxoplasma gondii* · Genotype · Indian isolate · PCR–RFLP

Introduction

Toxoplasma gondii is an obligate intracellular zoonotic apicomplexan parasite capable of infecting all warm-blooded vertebrates including birds and humans. The parasite causes huge economic losses to the livestock industry in the form of abortions and still births besides being a potential threat to immunocompromised patients (Spycher et al. 2011). *Toxoplasma gondii* isolates were first considered a single species without geographical boundaries and with little genetic diversity (Dubey and Beattie 1988; Ajzenberg et al. 2002). With the establishment of the fact that most animal isolates are from human infective lineage and responsible for huge economic loss to livestock industry through abortion (Dubey 2010; Al-Kappany et al. 2010), the need for sensitive detection and typing of animal isolates of *T. gondii* through molecular techniques was emphasized. The diagnosis of *T. gondii* infection is based on specific detection of the parasite using standard conventional techniques as well as through real time PCR, while molecular characterization of the parasite lineages is possible by multilocus PCR–RFLP, microsatellite, and multilocus sequence typing (MLST). Genetic analysis based on PCR–RFLP using six independent markers classified *T. gondii* isolates into three predominant clonal lineages (types I–III) which occur in both animals and humans (Howe and Sibley 1995). The data thus generated through PCR RFLP clearly demarcate the isolates of *T. gondii* in two groups, viz. the distinctly virulent *T. gondii* strains forming a group, while the field isolates cluster into other avirulent group (Sibley and Boothroyd 1992).

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Subsequently, the virulent lineage strain assigned to clonal lineage Type I and the avirulent isolates to clonal lineages of Type II and III (Howe and Sibley 1995).

In India, studies till date have been focused using single genetic marker. Currently, limited information is available regarding the genotypes of *T. gondii* in India. Hence, the present study was undertaken to know the Clonal lineages of *T. gondii* present for a better insight to epidemiology of this important disease of man and animals.

Materials and methods

Laboratory animals and parasite

The Swiss albino mice were maintained inside PVC cages under standard conditions and were provided pellet feed and enough of water. Cryostocks of *T. gondii* RH strain maintained in Protozoology laboratory of IVRI in liquid Nitrogen were used for the study. Further, two strains were obtained from domestic chicken of Chennai (Tamil Nadu) and Izatnagar (U.P), after their hearts and brain were fed to cats and the oocysts obtained from the feces of cat upon purification were feed to mice and subsequently from mice, tachyzoites were isolated using the standard protocol. Approval for this study was granted by the Institute Animal Ethics Committee (IAEC) of Indian Council of Agriculture Research (ICAR)-Indian Veterinary Research Institute (IVRI), Izatnagar.

Maintenance of the parasite in laboratory

Three groups of four Swiss albino mice each, one infected intra peritoneally by 1×10^2 tachyzoites of *T. gondii* RH strain and the other two with local isolates obtained from Chennai and Izatnagar, were observed daily for any sign of peritonitis. The peritoneal lavage was harvested from the mice under euthanasia and it was mixed with equal quantity of phosphate buffer saline (PBS), pH 7.2. The procedure was repeated till a clear fluid was obtained. The peritoneal fluid lavage was subjected to mechanical disruption upon passage through a needle (18 gauge) under pressure and the procedure was repeated many times to release the tachyzoites from macrophages. The peritoneal lavage was centrifuged at 5000 rpm for 10–15 min and pellet was suspended in 5 ml of PBS and washing was repeated three times. Final resuspension was made in 2 ml for enumeration of tachyzoites.

Extraction of genomic DNA from the parasite

Extraction of genomic DNA from the tachyzoites was carried out using Genomic DNA Mini kit (Geneaid, India)

following manufacturer's protocol. The genomic DNA concentration was determined and a working concentration of 10 µg/µl was prepared.

Primers

External and internal primers for SAG1, 5'-SAG2, 3'-SAG2, alt. SAG2, SAG3, BTUB, GRA 6, c22-8, c29-2, L358 and PK1 were custom synthesized (Su et al. 2010).

Genotyping of *T. gondii* isolates

The multiplex PCR reaction for SAG1, 5'-SAG2, 3'-SAG2, alt. SAG2, SAG3, BTUB, GRA 6, C22-8, C29-2, L358 and PK1 markers was carried out in a thermo cycler (Biotron, India) in a volume of 50 µl containing 1X PCR buffer, 2 mM MgCl₂, 200 mM each of the dNTPs, 10 pico mole of each of the external forward and reverse primers of all these markers, 1 unit of Taq DNA polymerase (Fermentas, USA) and 20 ng of DNA samples of *T. gondii* RH strain and two local isolates. The reaction mixture was treated at 95 °C for 4 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 1 min and 72 °C for 1.5 min followed by final extension at 72 °C for 10 min. Multiplex PCR amplified products were diluted (1:1) by adding 50 µl of nuclease-free water. The nested PCR reaction was carried out individually for each marker viz SAG1, 5'-SAG2, 3'-SAG2, alt. SAG2, SAG3, BTUB, GRA 6, C22-8, C29-2, L358 and PK1, in a volume of 25 µl containing 1X PCR buffer, 2 mM MgCl₂, 200 mM each of the dNTPs, 10 pico mole each of internal forward and reverse primers of a particular individual marker, 1 unit Taq DNA polymerase (Fermentas, USA) and 1 µl of diluted multiplex PCR product. The reaction mixture of all markers was treated at 95 °C for 4 min, followed by 35 cycles of 94 °C for 30 s, 60 °C for 1 min and 72 °C for 2 min followed by final extension at 72 °C for 7 min.

PCR–RFLP analysis of SAG1, 5'-SAG2, 3'-SAG2, alt. SAG2, SAG3, BTUB, GRA 6, c22-8, c29-2, L358 and PK1 markers of *T. gondii* RH strain and two local isolates

Nested PCR amplified products were subjected to RFLP analysis using a set of restriction enzymes as given in Table 1. Restriction enzyme digestion reaction mixture consisted of 10 µl of nested PCR, 2 µl of 10X Cut Smart buffer, 1 µl (10U) each of restriction enzyme(s) (New England Biolabs, UK) and NFW was added to make 20 µl volume. The digestion mixture was incubated at 37 °C. The digested PCR products were analyzed by electrophoresis along with low molecular weight Ladder (Fermentas, USA) using 2.5 % super fine resolution

Table 1 Restriction enzyme digestion carried out for the markers SAG 1, 5' SAG 2, 3' SAG 2, alt SAG 2, SAG 3, BTUB, GRA 6, c 22-8, c 29-2, L358 and PK1

Marker	n PCR (bp)	Restriction enzymes, incubation temperature and time	References
SAG 1	390	Sau96I + HaeII (double digest). Cut smart buffer, 37 °C 1 h	Grigg and Boothroyd (2001)
5'SAG 2	242	MboI, Cut smart buffer, 37 °C 1 h	Howe et al. (1997), Su et al. (2006)
3'SAG 2	222	HhaI, Cut smart buffer, 37°C1 h	Howe et al. (1997)
alt SAG 2	546	HinfI + TaqI, Cut smart buffer, 37 °C 30 min, 65 °C 30 min	Khan et al. (2005), Su et al. (2006)
SAG 3	225	NciI, Cut smart buffer, 37°C1 h	Grigg and Boothroyd (2001)
BTUB	441	BsiEI + TaqI (double digest), Cut smart buffer, 60 °C 1 h	Khan et al. (2005), Su et al. (2006)
GRA 6	344	MseI, Cut smart buffer, 37°C1 h	Khan et al. (2005), Su et al. (2006)
c 22-8	521	BsmAI + MboII (double digest), Cut smart buffer, 37 °C 30 min, 55 °C 30 min	Khan et al. (2005), Su et al. (2006)
c 29-2	446	HpyCH4IV + RsaI (double digest), Cut smart buffer, 37 °C 1 h	Khan et al. (2005), Su et al. (2006)
L358	418	HaeIII + NlaIII (double digest), Cut smart buffer, 37 °C 1 h	Khan et al. (2005), Su et al. (2006)
PK1	906	AvaI + RsaI (double digest), Cut smart buffer, 37 °C 1 h	Khan et al. (2005), Su et al. (2006)

agarose gel (Fermentas, USA) and data was subsequently documented by Alpha Image Viewer documentation system.

The standard digestion patterns generated for each marker of *T. gondii* local isolates were obtained and compared with eight standard positive control reference strains of *T. gondii*, viz. PGT, CTG, Cr1, Ca1, GT, MAS, Br64, and Br5 (Su et al. 2010).

Results

Nested PCR for all the markers was successfully carried out (including 5'SAG2 for which no separate marker was used in multiplex PCR but was covered by the external primer of alt. SAG2) at 60 °C annealing temperature following 1:1 diluted Multiplex PCR product which contained primers for all the markers except 5'SAG2. Individual amplification of each marker followed by restriction enzyme digestion using above mentioned restriction enzyme panel revealed fragmentation pattern (Fig. 1).

The SAG1 amplicon on digestion using *Sau96I* and *HaeII* produced a polymorphic digestion pattern that discriminated virulent RH strain of *T. gondii* as Type I from moderately virulent local isolates as Type III. Following digestion of PCR products with *MboI* restriction enzyme, the 5'-SAG 2 marker amplified from *T. gondii* RH strain and local isolates (Izatnagar and Chennai) produced a polymorphic pattern that was indicative of the *T. gondii* RH strain as Type I and the local isolates viz. Izatnagar and Chennai isolates as Type II or III. 3'-SAG 2 amplified product of *T. gondii* RH strain and local isolates (Izatnagar and Chennai) remained undigested up on treatment with *HhaI*. Absence of the restriction site for *HhaI* suggests for either Type I or Type III genotype of the strain/isolate.

With two markers put together (viz. 5'-SAG 2 and 3'-SAG 2), *T. gondii* RH strain was assigned Type I and two local isolates of *T. gondii* (Izatnagar and Chennai isolate) as Type III. The PCR products up on digestion by *HinfI* and *TaqI* produced a polymorphic digestion pattern that discriminated highly virulent RH strain of *T. gondii* as Type I from local isolates (Type III). Similarly restriction enzyme digestion of PCR products free of any non-specific amplification were subjected to restriction enzyme digestion with *NciI* and the fragments up on resolution in 2.5 % agarose gel revealed a Type I pattern for *T. gondii* RH, while local isolates of *T. gondii* Izatnagar and Chennai were characterized as Type III. Similarly, digestion of BTUB, GRA6, C22-8, C29-2, L358 and PK1 was performed with the enzymes mentioned in Table 1. The restriction fragment pattern revealed a type I pattern for *T. gondii* RH strain while local isolates of Chennai and Izatnagar revealed type III pattern at all the loci.

The RFLP pattern was compared with 8 positive control reference strains of *T. gondii*, viz. PGT, CTG, Cr1, Ca1, GT, MAS, Br64, and Br5 (Su et al. 2010) and the genotype was determined. It was revealed that *T. gondii* RH strain has genotype I with ToxoDB #10 as data base while the local isolates (from Chennai and Izatnagar) obtained from chicken had genotype III belonging to ToxoDB #3. The summary of the genotyping for each of the individual marker is given in Table 2.

Discussion

Although the genus *Toxoplasma* consists of one species, i.e. *T. gondii*, molecular biology tools have provided the evidence of distinct clonal lineages of the organism. There are three distinct clonal lineages of *T. gondii* (Su et al.

Fig. 1 Multiplex nested PCR–RFLP analysis of *Toxoplasma gondii* with 11 different genetic markers. Here *M* represents low molecular weight genetic marker (Fermentas, USA), *1* represents *T. gondii* RH strain, *2* and *3* represent Izatnagar and Chennai isolates of *T. gondii*,

respectively. All the nested PCR products were digested with the restriction enzymes as given in Table 1 and the digested products were separated in 2.5 % agarose gel

Table 2 Summary of genotyping results for local isolates from chicken obtained using PCR–RFLP

Sample	SAG1	5' SAG 2	3'SAG2	alt SAG 2	SAG 3	BTUB	GRA 6	c 22-8	c 29-2	L358	PK1	Toxo DB PCR–RFLP genotype
<i>T. gondii</i> (RH strain)	I	I	I	I	I	I	I	I	I	I	I	#10, Type I
<i>T. gondii</i> (Izatnagar isolate)	II/III	III	III	III	III	III	III	III	III	III	III	#2, Type III
<i>T. gondii</i> (Chennai isolate)	II/III	III	III	III	III	III	III	III	III	III	III	#2, Type III

2003), though atypical cases have also been reported (Dubey et al. 2011; Khan et al. 2011). The isolates of *T. gondii* not only differ in genotype, which shows less than 1 % differences, but substantially in virulence. Earlier, genotyping studies were usually focused on single locus, however, in order to determine clear lineage of parasite prevalent at a place, an increased need to include more markers was felt. Currently world over, 10 standard markers are used and new ones are continuously being introduced. However, SAG1, 3'-SAG2, alt. SAG2, SAG3, BTUB, GRA6, C22-8, C29-2, L358 and PK1 are frequently being used for genotyping of the isolates worldwide. The markers individually or in combination with other markers

can differentiate all the three clonal lineages mostly present, besides some of them are able to differentiate unique genotype as well (Su et al. 2010; Ferreira et al. 2011). Such group of markers has a great potential in rapid discrimination of field isolates as virulent or otherwise, without resorting to time consuming pathogenicity trials.

Numerous studies have shown that most isolates obtained from animals belong to Type II and III (Howe and Sibley 1995; Mondragon et al. 1998; Owen and Trees 1999; Jungersen et al. 2002; Burrells et al. 2013). The types I and II variant strains circulate in wild birds in China (Huang et al. 2012) and type III and II in Iranian birds (Khademvatan et al. 2013). Other studies have shown that

genotype ToxoDB PCR–RFLP #9 is widespread and infects pigs, cats, and human patients in China and is likely the major *T. gondii* lineage in China (Chen et al. 2011). Toxoplasmosis has worldwide prevalence and a few reports on prevalence of the infection from animals and birds exist from India. A prevalence rate of 17.9 % in chickens was recorded using Modified Agglutination Test (MAT) in Chennai (Sreekumar et al. 2003). However, little is known of the biological and molecular characters of isolates of *T. gondii* from animals or humans from India.

In the present study, local isolates of *T. gondii* were genotyped along with *T. gondii* RH strain. *T. gondii* isolates from chicken obtained from Chennai and Izatnagar were studied using the standard markers and restriction fragment length polymorphism study was carried out using specific restriction enzymes. Specific amplicons of 390 bp for SAG1, 242 bp for 5'-SAG2, 222 bp for 3'-SAG2, 546 bp for alt. SAG2, 225 bp for SAG3, 411 bp for BTUB, 344 bp for GRA 6, 521 bp for C22-8, 446 bp for C29-2, 418 bp for L358 and 906 bp for PK-1 were obtained. The RFLP pattern was generated using the panel of restriction enzymes.

In the present study, the target sequence of SAG1 marker was amplified from multiplex PCR product of *T. gondii* RH strain and local isolates (from Izatnagar and Chennai) using the SAG1 marker specific primers. The *Sau96I* and *HaeII* produced a polymorphic digestion pattern from the PCR product that discriminated virulent RH strain of *T. gondii* as Type I from moderately virulent local isolates as Type III. The findings clearly show that SAG1 locus can be used to discriminate between Type II and III genotypes of *T. gondii* (Su et al. 2010).

At 5'-SAG2 locus, *T. gondii* RH had a pattern matching to both Type I and Type II, whereas the Izatnagar and Chennai isolates of *T. gondii* showed RFLP pattern matching to Type III. At 3'-SAG2 locus, *T. gondii* Izatnagar and Chennai isolates absence of restriction site for *HhaI* was suggestive of their genotype I or III. When the data of the 5'SAG2 and 3'SAG2 locus were combined, it revealed Type I genotype of *T. gondii* RH strain, whereas the *T. gondii* Izatnagar and Chennai isolates were typed as genotype III. The 5'-SAG2 and 3'-SAG2 markers though individually could not differentiate the three genotypes, (5'-SAG2 could not differentiate Type I and Type II and 3'-SAG2 could not differentiate Type I and Type III), their combination allowed typing of all three lineages based on a unique combination of allele (Khan et al. 2005).

The alt. SAG2 marker revealed *T. gondii* RH as Type I, while Izatnagar, Chennai isolates of *T. gondii* revealed Type III pattern. The PCR products up on digestion by *HinfI* and *TaqI* produced a polymorphic digestion pattern that discriminated highly virulent RH strain of *T. gondii* as Type I from local isolates (Type III). Similarly, SAG 3,

BTUB, GRA 6, C22-8, C29-2, L358, PK-1 markers could discriminate the three isolates i.e., *T. gondii* RH as Type I and *T. gondii* Izatnagar, Chennai isolates as Type III. The finding is in agreement with the earlier reports where three way markers (Su et al. 2006) were used to differentiate the three genotypes commonly found in most parts of world (Howe and Sibley 1995; Ajzenberg et al. 2002; Dubey et al. 2007; Jiang et al. 2014).

The RFLP pattern of *T. gondii* Izatnagar and Chennai when compared with that of other published reference strains (Su et al. 2010) matched to Type III (ToxoDB #2), while *T. gondii* RH strain matched to Type I (ToxoDB #10).

Earlier, Sreekumar et al. (2003) reported the *T. gondii* isolates from chicken in India were of Type II and III clonal lineages. The lineage composition of *T. gondii* (based on SAG2) from the Indian chicken were shown to be more similar to that of Egypt and the USA than that of Brazil (Sreekumar et al. 2003). However, a single locus based typing runs the risk of being erroneous and may misclassify mixed strains or exotic genotypes as simple genotype (Khan et al. 2005). In order to overcome the risk, a multiplex nested PCR was laboratory standardized to genotype the local isolates of *T. gondii* correctly (Su et al. 2010).

Diagnostic sensitivity of a detection assay plays an important role. Most of the highly repetitive markers that provide sensitive detection (viz., B1, rRNA genes) are not useful for genotyping the parasite due to a relative absence of polymorphisms (Khan et al. 2005). The present study has generated data on the clonal lineage of local isolates of *T. gondii* prevalent in India using three way nested PCR markers either individually or in combination. The method adopted is simple, easy to perform and ready to be adopted for genotyping study of *T. gondii* from unknown samples where a dual purpose of diagnosis and genotyping will be solved (Su et al. 2010).

In summary, molecular genotyping in the present study has established the local isolates, viz. Izatnagar and Chennai of *T. gondii* are of Type III clonal lineage, while RH strain of *T. gondii* is of Type I lineage and assigned the local isolates to ToxoDB #2 while ToxoDB #10 for *T. gondii* RH strain. The present investigation is the first report on existence of Type III *T. gondii* lineage from animal population of Indian subcontinent based on PCR–RFLP study employing a set of 11 markers viz. SAG 1, 5'SAG 2, 3'SAG 2, alt. SAG 2, SAG 3, BTUB, GRA 6, C22-8, C29-2, L358 and PK1.

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Compliance with ethical standards

Conflict of interest The authors have no conflict of interest to declare.

References

- Ajzenberg D, Banuls AL, Tibayrenc M, Darde ML (2002) Microsatellite analysis of *Toxoplasma gondii* shows considerable polymorphism structured into two main clonal groups. *Int J Parasitol* 32:27–38
- Al-Kappany YM, Rajendran C, Abu-Elwafa SA, Hilali M, Su C, Dubey JP (2010) Genetic diversity of *Toxoplasma gondii* isolates in Egyptian feral cats reveals new genotypes. *J Parasitol* 96:1112–1114
- Burrells A, Bartley PM, Zimmer IA, Roy S, Kitchener AC, Meredith A (2013) Evidence of the three main clonal *Toxoplasma gondii* lineages from wild mammalian carnivores in the UK. *Parasitology* 140:1768–1776
- Chen ZW, Gaob JM, Huoa XX, Wanga L, Yu L, Halm-Laia F, Xu YH, Song WJ, Hide G, Shen JL, Lun ZR (2011) Genotyping of *Toxoplasma gondii* isolates from cats in different geographic regions of China. *Vet Parasitol* 183:166–170
- Dubey JP (2010) *Toxoplasmosis of animals and humans*, 2nd edn. CRC Press, Boca Raton
- Dubey JP, Beattie CP (1988) *Toxoplasmosis of animals and man*. CRC Press, Boca Raton
- Dubey JP, Cortes-Vecino JA, Vargas-Duarte JJ, Sundar N, Velmurugan GV, Bandini LM, Polo LJ, Zambrano L, Mora LE, Kwok OCH, Smith T, Su C (2007) Prevalence of *Toxoplasma gondii* in dogs from Colombia, South America and genetic characterization of *T. gondii* isolates. *Vet Parasitol* 145:45–50
- Dubey JP, Velmurugan GV, Rajendran C, Yabsley MJ, Thomas NJ, Beckmen KB, Sinnott D, Ruid D, Hart J, Fair PA, McFee WE, Shearn-Bochsler V, Kwok OC, Ferreira LR, Choudhary S, Faria EB, Zhou H, Felix TA, Su C (2011) Genetic characterization of *Toxoplasma gondii* in wildlife from North America revealed widespread and high prevalence of the fourth clonal type. *Int J Parasitol* 41:1139–1147
- Ferreira IM, Vidal JE, de Mattos Cde C, de Mattos LC, Qu D, Su C (2011) *Toxoplasma gondii* isolates: multilocus RFLP-PCR genotyping from human patients in Sao Paulo State, Brazil identified distinct genotypes. *Exp Parasitol* 129:190–195
- Grigg ME, Boothroyd JC (2001) Rapid identification of virulent type I strains of the protozoan pathogen *Toxoplasma gondii* by PCR restriction fragment length polymorphism analysis at the B1 gene. *J Clin Microbiol* 39:398–400
- Howe DK, Sibley LD (1995) *Toxoplasma gondii* comprises of three clonal lineages: correlation of parasite genotype with human disease. *J Infect Dis* 172:5561–5566
- Howe DK, Honore S, Derouin F, Sibley LD (1997) Determination of genotypes of *Toxoplasma gondii* strains isolated from patients with toxoplasmosis. *J Clin Microbiol* 35:1411–1414
- Huang SY, Cong W, Zhou P, Zhou DH, Wu SM, Xu MJ, Zou FC, Song HQ, Zhu XQ (2012) First report of genotyping of *Toxoplasma gondii* isolates from wild birds in China. *J Parasitol* 98:681–682
- Jiang HH, Zhang WB, Zhao L, Zhou DH, Song HQ, Xu CM, Deng SZ, Zhu XQ (2014) Seroprevalence of *Toxoplasma gondii* infection in pigs in Jiangxi province. *Foodborne Pathog Dis*, Southeastern China. doi:10.1089/fpd.2013.1686
- Jungersen G, Jensen L, Rask MR, Lind P (2002) Non-lethal infection parameters in mice separate sheep Type II *Toxoplasma gondii* isolates by virulence. *Comp Immunol Microbiol Infect Dis* 25:187–195
- Khademvatan S, Saki J, Yousefi E, Abdizadeh R (2013) Detection and genotyping of *Toxoplasma gondii* strains isolated from birds in the southwest of Iran. *Br Poultry Sci* 54:76–80
- Khan A, Su C, German M, Storch GA, Clifford DB, Sibley LD (2005) Genotyping of *Toxoplasma gondii* strains from immunocompromised patients reveals high prevalence of type I strains. *J Clin Microbiol* 43:5881–5887
- Khan A, Dubey JP, Su C, Ajioka JW, Rosenthal B, Sibley LD (2011) Genetic analyses of atypical *Toxoplasma gondii* strains reveals a fourth clonal lineage in North America. *Int J Parasitol* 41:645–655
- Mondragon R, Howe DK, Dubey JP, Sibley LD (1998) Genotypic analysis of *Toxoplasma gondii* isolates from pigs. *J Parasitol* 84:639–641
- Owen MR, Trees AJ (1999) Genotyping of *Toxoplasma gondii* associated with abortion in sheep. *J Parasitol* 85:382–384
- Sibley LD, Boothroyd JC (1992) Virulent strains of *Toxoplasma gondii* comprise a single clonal lineage. *Nature* 39:82–85
- Spycher A, Geigy C, Howard J, Posthaus H, Gendron K, Gottstein B (2011) Isolation and genotyping of *Toxoplasma gondii* causing fatal systemic toxoplasmosis in an immunocompetent 10-year-old cat. *J Vet Diag Invest* 23:104–108
- Sreekumar C, Grahamb DH, Dahl E, Lehmann T, Raman M, Bhalerao DP, Vianna MCB, Dubey JP (2003) Genotyping of *Toxoplasma gondii* isolates from chickens from India. *Vet Parasitol* 118:187–194
- Su C, Evans D, Cole RH, Kissinger JC, Ajioka JW, Sibley LD (2003) Recent expansion of *Toxoplasma* through enhanced oral transmission. *Science* 299:414–416
- Su C, Zhang X, Dubey JP (2006) Genotyping of *Toxoplasma gondii* by multilocus PCR-RFLP markers: a high resolution and simple method for identification of parasites. *Int J Parasitol* 36:841–848
- Su C, Shwab EK, Zhou P, Zhu XQ, Dubey JP (2010) Moving towards an integrated approach to molecular detection and identification of *Toxoplasma gondii*. *Parasitol* 137:1–11