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Full Length Research Paper

SAG2 locus genotyping of *Toxoplasma gondii* in meat products of East Azerbaijan Province, North West of Iran During 2010-2011

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Toxoplasmosis is an infection caused by *Toxoplasma gondii*, an intracellular obligate parasite. Its transmission is usually attributed to ingestion of undercooked or raw meat. The aim of this study was the detection and genotyping of *T. gondii* in meat products using the molecular method in East Azerbaijan. DNA was extracted from 164 meat products sample obtained from 15 commercial establishments. Nested polymerase chain reaction with specific primers for *Toxoplasma gondii* SAG2 locus was used for detection of the parasite in samples. Genotyping was carried out by digestion of PCR products with restriction enzymes Cfo1 and Sau3AI. *T. gondii* DNA was detected in 16.6, 19.1, 15 and 56.6%, in salami, sausages, and hamburger and kebab samples, respectively. Genotyping by restriction fragment length polymorphism (RFLP) analysis of SAG2 locus revealed that all of the samples belonged to genotype I. The detection of the parasite in uncooked meat and commercial meat products, and the high ratio of seropositive slaughtered animals, emphasis that the risk still exists for food -born toxoplasmosis.

Key words: *Toxoplasma gondii*, SAG2, genotyping, meat products.

INTRODUCTION

Toxoplasma gondii infections are widely prevalent in human beings and animals with a world-wide distribution (Dubey et al., 1988). Infection with the parasite occurs via ingesting tissue cysts from undercooked meat, consuming food contaminated with Oocysts, or by accidentally ingesting Oocysts from the environment (Dubey et al., 2004). However, only a small percentage of exposed adult humans develop clinical signs. The severity of *T. gondii* infection in humans depends on different factors (Sibley et al.,1996), including host factors (immunity and genetic background) and parasite factors (strain, inoculums size, and parasite life-cycle stage). This parasite has been classified into three genetic types (I–III) based on restriction fragment length polymorphism (RFLP) (Howe and Sibley, 1995 and Howe at al., 1997). Epidemiological screening is important to detect *T. gondii* strains from various sources of infection, in order to evaluate possible relations between type of parasite and severity of the disease in humans and animals (Da silva et al., 2005). Meat producing animals can be infected with *T. gondii*, and considered as the most important source of *T. gondii* for humans, (Tenter et al., 2000). Older animals with high prevalence of this organism are used for production of sausages, salami and cured meats (Dubey et al., 2000). Furthermore, such products often contain meat from multiple animals in a single serving. Together, these factors result in a higher potential risk of infection after consumption unless these foodstuffs are very well cooked. *T. gondii* prevalence in Iran is high, (Assmar et al., 1997; Ghorbani and Samii, 1973; Hashemi, 1996; Keshavarz and Ebrahimi, 1994; Sedaghat et al., 1978). However, there is few report of genetic characterization of Toxoplasma isolates from this

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Table1. Names and sequences of the polymerase chain reaction primer pairs used.

Primer name	Sequence
SAG2 -F1	CTCGAACAGGAACACAAAGG
SAG2-R1	AGGGTGCCTCCAACAGTCTTC
SAG2-F2	GAAATGTTTCAGGTTGCTG
SAG2-R2	AAGAGCGAACTTGAACACAAC
SAG2-F3	TCTGTTCTCCGAAGTGACTCC
SAG2-R3	GGTATTCAAAGCGTGCATTATC
SAG2-F4	ATTCTCATGCCTCCGCTTC
SAG2-R4	AACGTTTCACGAAGGCACAC

country. In this study, isolation and genetic analysis of *T. gondii* obtained from meat products in Iran was done.

MATERIALS AND METHODS

Sample collection and DNA extraction from tissue samples

A total of 164 samples including 48 salami, 46 sausage, 40 hamburger and 30 kebab samples were collected from different factories from East Azerbaijan provinces. Samples were kept in the freezer at -12°C before been used.DNA was extracted from tissue sample using Accu Prep® Genomic DNA Extraction Kit (Bio Neer) according to the manufacturer's instruction. All DNA extracts were stored at -20°C until use.

Detection of *T. gondii* infection by nested-PCR

Specific primers were obtained from MWG (Germany) and PCR amplification of SAG2 gene was performed as described by Dudy et al. (1989). Briefly, the extracted DNA was used as template for the first PCR reaction to amplify the region corresponding to 5' and 3′ flanking sequences of *T. gondii* surface antigen P22. In the second set of reactions, the product of the first reaction was employed as template to amplify a fragment corresponding to bases 1-19 and 202-221 in 5′ and 3′ flanking region of antigen P22 antigen, respectively (khodaai et al., 1988; Burg et al., 1989).

Amplification of SAG2 locus as a target for genotyping

SAG2 locus has two polymorphic sites at 3´ and 5´ ends for type II and type III (Howe et al., 1997). So, amplification of this locus was performed separately. The 5´end of *SAG2* locus was amplified using standard PCR with outer primers. 1 µl of the first PCR amplicons was subsequently used as a template for nested-PCR with specific internal primers. The protocol for temperature cycling included 30 cycles that consisted of 4 min of denaturation at 94°C, 1 min at the annealing temperature of 58°C, and 30 s of extension at 72°C. The final extension step continued for an additional 5 min. The 3´ end of *SAG2* locus was similarly amplified by standard PCR with outer primers. 1 µl of this PCR product was directly used for nested-PCR with inner primers (Table 1).The protocol for nested-PCR condition was as described for the 5´ end of *SAG2* locus. The reaction mixture (25 µl) contained sterile distilled H_2O, dNTPs, 10x PCR buffer, $MgCl_2$, primers (forward and reverse), *Taq* DNA polymerase and template DNA. The reaction mixture was covered with 100 µl of mineral oil. Amplifications were performed in a thermal cycler (Master cycler Gradient Eppendorf Germany). *T. gondii* positive control (genomic DNA from strain RH) was included in all PCR experiments to ensure that the PCR was functioning correctly, and a further negative control (water template) was included to ensure that contamination of the PCR components had not occurred. Products were electrophoresed on 1.2% agarose gel, stained with 0.5 µl of ethidium bromide and then visualized under UV illuminator.

RFLP genotyping

10 µl of the nested-PCR products of the 3´and 5´ end regions were digested using 5 U of each *cfoI* and *Sau3AI* restriction enzymes (Fermentas) respectively in separate reactions in a total volume of 20 µl at 37°C. All digestions were performed according to the manufacturer's instructions.

RESULTS

Identification of *T. gondii* positive meat-products samples

A total of 164 samples including 48 salami, 46 sausage, 40 hamburger and 30 kebab samples were collected from a variety of commercial sources, and DNA was extracted from each. Howe et al. (1997) previously described a method for the typing of *T. gondii* by restriction analysis of two PCR fragments derived from 5′ and 3′ regions of the SAG2 gene with the enzymes Sau3A and CfoI, respectively. In this way, *T. gondii* can be classified as type I (not cut by either Sau3A or CfoI), type II (cut by CfoI but not by Sau3A) or type III (cut by Sau3A but not by CfoI). PCR amplification of the SAG2 5′ and 3′ fragments thus allowed us to both identify which meat samples were *T. gondii* positive and also to type them (Howe et al. 1997). In this study, amplification of 5′flanking region was successful in 52 (32%) of the 164 samples whereas 3′ flanking amplification was positive in 65 (40%) of the 164 cases. As shown in Figure 1, samples that were found to be positive for either 5′ or 3′ SAG2 PCR were classified as positive for *T. gondii*

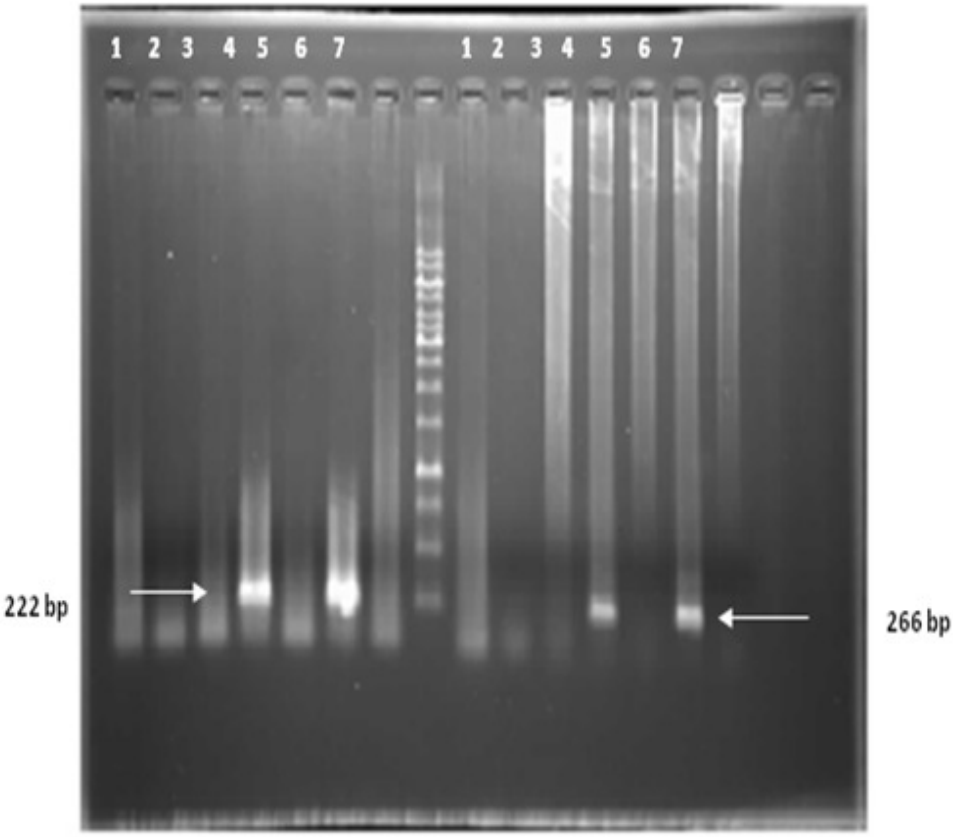

Figure 1. Nested-PCR amplification of *SAG2* locus. A and B, represent the 5´ and 3´ ends respectively that were amplified by nested-PCR. Lane 6 corresponds to the RH strain as a positive control, lane 7 corresponds to negative controls and lane 4 is the positive sample.

contamination. The PCR products amplified from positive samples were purified and digested with restriction enzymes in order to determine the SAG2 type of the contaminating parasite. Any sample found to be positive for only one fragment was re-amplified in order to confirm status, and only samples found positive for both fragments were pursued further for genotyping. In this way, 56 of the 164 meat samples (34%) were found to be contaminated with *T. gondii*, including 15 of the salami samples, 13 of the sausage sample; 14 of the hamburger and 30 of the kebab samples (Table 2).

SAG2 typing of *T. gondii* contaminated meat products samples

In order to determine the SAG2 type of contaminating parasite, the SAG2 PCR products were restricted and their type determined according to the restriction pattern observed after separation by agarose gel electrophoresis. This RFLP analysis revealed that none of the samples were cut with specific enzymes therefore they were classified as genotype I (Figure 2).

DISCUSSION

One-third of the human world population is infected with the protozoan parasite *T. gondii*, (kijlstra et al., 2008). High prevalence of toxoplasmosis has been shown among animals which are raised for meat production; therefore undercooked meats are an important risk factor for transmission of toxoplasmosis (Ergin et al., 2009). This is the first report of the detection of *T. gondii* by the PCR technique in meat and meat products in Iran. Since this molecular method detects DNA of parasite in samples and unable to distinguish viable from non-viable parasites (Holliman et al., 1994), the results obtained do not indicate the presence of a living *T. gondii* in samples. However, it is well known that cyst forms of the parasite may survive several weeks between 1 and 4°C and can be inactivated only in the processes over 67°C or below -12°C (Aspinall et al., 2002). In our study, 40 meat-products samples (24.3%) including 8 salami, 9 sausage, 6 hamburger and 17 kebab samples were contaminated with *T. gondii*. Our results are consisted with several previous reports. A recent study in Turkey (Ergin et al., 2009) confirmed the presence of *T. gondii* in slaughtered

Table 2. Genotypes of *T. gondii* samples detected in meat products from North West of Iran.

Meat product	Number of sample studied	Number of PCR positive sample	Type of SAG2	Number of SAG2 type II and III samples
Salami	48	8	I	0
Sausage	46	9	I	0
Hamburger	40	6	I	0
Kebab	30	17	I	0

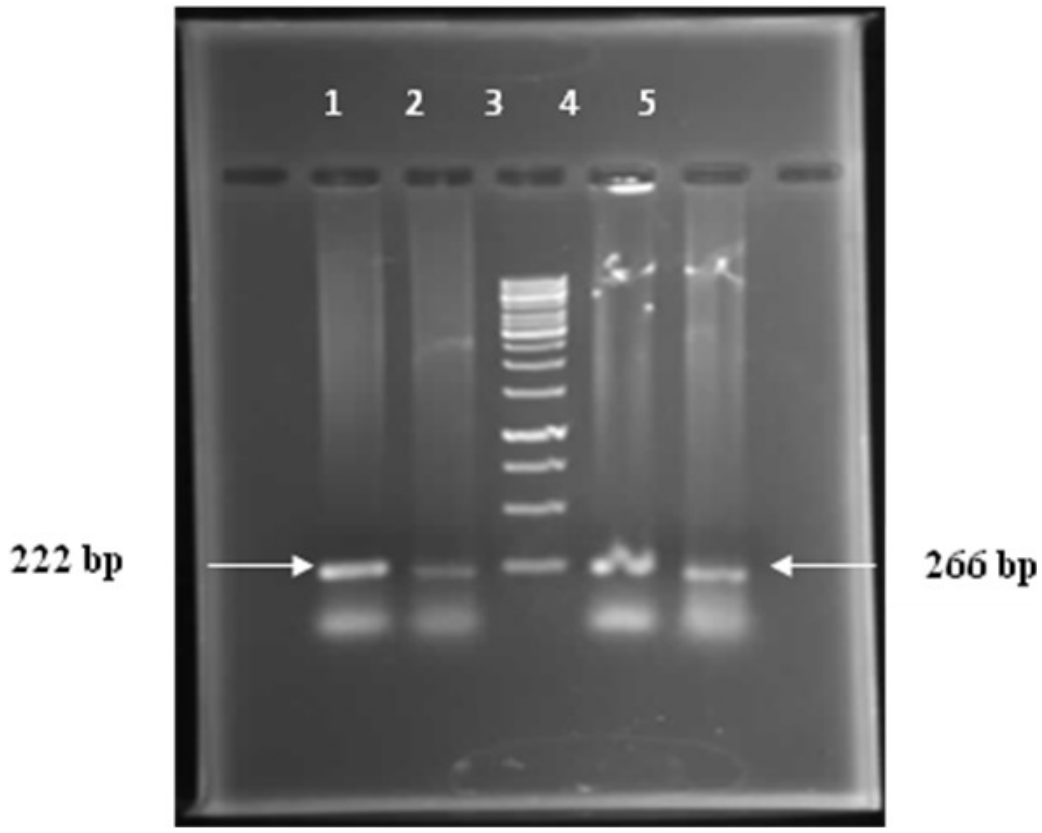

Figure 2. PCR-RFLP analysis of the 3´and 5´end flanking region of *SAG2* locus. The 3´ end of *SAG2* locus was digested with *cfo1* (lane 1). The 5´ end of *SAG2* locus was digested with *Sau3AI* (lane 4); lanes 2 and 5, undigested sample. No digestion occurred in the 5´ and 3´ end, which indicates the presence of a type I genotype. In this type,,3´ and 5´ ends of *SAG2* locus resisted cleavage of *cfo1* and *Sau3AI,* respectively. Lane 3 corresponds to the DNA molecular weight marker (100 bp DNA ladder).

animals and commercially-available uncooked meat products. Warnekulasuriya et al. (1998) detected one viable *T. gondii* in 67 cured meat samples, including dried and semi-dried sausages and hams in UK. Another similar study by Aspinall et al. (2002) showed that 27 meats sample were *T. gondii* positive by PCR detection and determined a much higher level of contamination (38%). Da silva et al. (2005) also reported that *T. gondii* DNA was found in 19 (27.14%) of 70 sausage samples examined. These findings may be related to various prevalence of infection in meat-producing animals or different eating habits. Only few studies reported genotypes of *T. gondii* from meat and meat products. Aspinall et al. (2002) reported that 34% samples were positive for *T. gondii* DNA among meat products with 85% SAG2 type I samples and 15% mixed SAG2 type I + II samples. In a study that was conducted by Dubey et al. (2002), the presence of type I and type III *T. gondii* in brain and heart samples of poultry from Brazil was reported .The difference in these results is due to genetic distribution of *T. gondii* in different geographic regions. In this work, no type II and III strains were found, and type I

was the most prevalent. This indicates that all of our infected samples were carrying SAG2 type I parasites. This finding is different from previously published reports in which majority of the animals were found to be carrying SAG2 type II and III parasites (Zia et al., 2007). This controversial result is probably due to various genotypes of Toxoplasma in different geographic regions. In the other hand, in our study, amplification of 5′ and 3′ flanking region was successful respectively in 31.7 and 40% of the samples that may cause a bias in the results of genotyping. Similar findings were observed in some other studies. In the study of Fuentes et al. (2001) in Spain no amplification of 5′ and 3′ regions was observed in 6 and 18%, whereas in a study carried out by Gallego et al. (2006) in Colombia, amplification failed in 9 and 57% for 5′ and 3′ flanking regions . It is thought that polymorphism in SAG2 flanking regions might be the cause for this phenomena. Hence, it suggested further investigation for the confirmation of genotyping results. In summary, our results confirm the presence of *T. gondii* in meat products. Therefore, the potential risk of the transmission of the disease through *T. gondii* containing meat should still be considered a public health threat. In view of recent developments in the epidemiology of the disease, it is suggested that not only pregnant women should be addressed but the whole population should be informed on how to prevent infection.

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