Study on ovine abortion associated with *Toxoplasma gondii* in affected herds of Khorasan Razavi Province, Iran based on PCR detection of fetal brains and maternal serology

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SUMMARY

Toxoplasma gondii, an obligatory intracellular protozoan parasite, is one of the causative agents of ovine abortion, as reported in many countries. Different techniques are being used to detect this pathogen in infected ovine fetuses. One of the most sensitive and specific diagnostic techniques is Nested-PCR amplification of the B1 target gene of the organism. In total, 200 brain samples of aborted ovine fetuses and maternal sera submitted from different parts of Khorasan Razavi province, Iran were investigated to track the role of *Toxoplasma gondii* in ovine abortion by a slightly modified Nested-PCR and IFAT assays, respectively. Among all samples, 27 (13·5%) were PCR-positive and 31 (15·5%) were IFAT-positive and the *Toxoplasma*-induced abortion prevalence calculated was 8·8% to18·2% with 95% confidence interval. Results show that high levels of congenital transmission may occur in 27/31(87%) of pregnancies with an excellent logical agreement ($\kappa = 0{\cdot}9$) between 2 different tests. According to the results of this study, the Nested-PCR employed in this investigation could be recommended as an applied routine test for the routine examination and confirmation of *Toxoplasma gondii*-induced ovine abortion.

Key words: *Toxoplasma gondii*, abortion, sheep, Nested-PCR, Iran.

INTRODUCTION

Ovine toxoplasmosis, caused by the obligatory intracellular protozoan parasite *Toxoplasma gondii*, was first described in New Zealand by Hartley *et al*. (1954) as a significant cause of abortion in sheep, and thereafter was recognized in many other countries (Buxton *et al*. 2007). While *T. gondii* does not appear to cause any significant clinical disease in sheep, congenital infection can cause reproduction disorders such as early embryonic death, fetal resorption, mummification, abortion, stillbirth, neonatal and fetal death in sheep (Blewett and Watson 1983; Johnston,1988; Buxton, 1991; Givens and Marley, 2008) and evidence suggests that this route of transmission may be much more significant than previously assumed (Duncanson *et al*. 2001; Williams *et al*. 2005; Hide *et al*. 2009). Further, more recent molecular studies in England showed that repeated ovine transmission of *T. gondii* may be more common than previously believed (Morley *et al*. 2005, 2007; Hide *et al*. 2009; Innes *et al*. 2009; Dubey, 2009). Consumption of infected meat from sheep is considered as a source of *T. gondii* infection in humans and carnivores (Dubey, 2009).

Different techniques have been used to detect the infection in aborted fetuses. One of the most widely used diagnostic tests is the PCR assay on fetal brains of infected animals (Ortega-Morta *et al*. 2007; Dubey, 2009). Comparison of different conventional PCR methods has shown that Nested-PCR amplification of the B1 gene of the organism is one of the most sensitive and specific (Burg *et al*. 1989; Jones *et al*. 2000; Mason *et al*. 2010). Detection of anti-*Toxoplasma gondii* antibodies in ewe's serum is also a very useful method for comparison of the data achieved for fetuses (Hurtado *et al*. 2001). However, detection of anti-*Toxoplasma gondii* antibodies in maternal serum may not be interpreted as an indicator of cause and effect phenomenon for the abortion associated with this pathogen. In contrast, identification of the genome of the organism in the brain, achieved by molecular approaches, definitely confirms an active infection process.

The aims of this study were 3-fold. (1) To set up an easy, rapid and economical conventional molecular technique for the detection of *Toxoplasma*-induced

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Fig. 1. Map of the Khorasan Rasavi Province, in north-east Iran.

abortion in laboratories around the world, more specifically in developing countries which can not afford to use more modern molecular assays, such as Real-time PCR. (2) To track the involvement of *T. gondii* in ovine abortion in Khorasan Razavi Province, Iran. (3) To assess the correlation of data achieved by molecular methods with the serological response of infected ewes.

MATERIALS AND METHODS

Samples and tissue collection

This investigation was conducted on samples collected from 200 aborted ovine fetuses all across the Khorasan Razavi Province, north-east of Iran (Fig. 1). These samples were submitted to the Center of Excellence in Ruminant Abortion and Neonatal Mortality, School of Veterinary Medicine, Ferdowsi University of Mashhad, Iran, from 2006 to 2010. The general status of each fetus such as freshness, autolysis, mummification and presence of macroscopic lesions in the fetus and fetal membranes was examined and recorded. To estimate the conception age of the fetuses, crown-rump length was measured (Evans and Sack, 1973). One-half of fetuses' brains were collected in such a way that no cross-contamination occurred, which was followed by storage at −20 °C for PCR analysis. Furthermore, maternal serum samples of these 200 aborted fetuses were collected and stored at −20 °C to be assayed by the indirect fluorescent antibody test (IFAT).

IFAT on maternal sera

The presence of *Toxoplasma* antibodies in maternal serum was assessed by IFAT, as previously described by Razmi and Rahbari (2001). The IFAT was run on 10-well glass slides, with each well covered with *in vitro*-produced tachyzoites of the RH strain of *T. gondii* (Bio gene®, Iran). Sera were diluted 1:20 in PBS, then 5 μl of a positive-control, 5 μl of a negative-control and 5 μl of diluted test samples were added to the wells. The slides were then incubated for 30 min in a humid chamber at room temperature. After washing the slides 3 times for 10 min in PBS, 5 μl of rabbit-anti-sheep IgG conjugated to FITC (Sigma®, St Louis, Missouri) were added (conjugate dilution was 1:500 in 0·2% filtered Evan's blue dye in PBS). Incubation with the secondary antibody was carried out for 30 min at room temperature in a humid chamber. The slides were subsequently washed again 3 times in PBS for 10 min. For fluorescence microscopy, buffered glycerol was added to the slides and they were then covered by cover-slips.

DNA extraction and nested-PCR assay

Frozen fetal brain tissues were homogenized by mortar and pestle in Tris-HCl (pH 8·0) followed by treatment with proteinase K (Fermentas®, Lithuania) at a final concentration of 200 μg/ml. DNA was extracted by use of the phenol-chloroform and ethanol precipitation method. The purified DNA samples were resuspended in 20 ml of TE (10 mM Tris and 1 mM EDTA, pH 8·0) and stored at −20 °C. DNA extracted from 10^6 particles of the RH strain of *T. gondii* (kindly provided by The School of Public Health and Institute of Public Health Research, Tehran University of Medical Sciences, Tehran, Iran) were used as a source of positive control sample. DNA samples were tested by Nested-PCR based on the amplification of high copy number B1 target gene (35 copies per parasite) using 2 sets of oligonucleotide primers as described by Burg *et al.* (1989), as presented in Table 1. Amplification was conducted in 20 μl reaction volumes (Accupower PCR premix kit, Bioneer®, South Korea) with a final concentration of each dNTP of 250 μM in 10 mM Tris-HCl pH 9·0, 30 mM KCl and 1·5 mM $MgCl_2$, 1 U *Taq* DNA polymerase and 10 pmol of each PCR primer (Takapouzist Co. Iran). Then 1 μl of DNA template (250–500 ng) was added to each reaction and the remaining 20 μl reaction volume was filled with sterile distilled water. Slight modifications including the PCR conditions and elimination of the gelatin were employed. The reactions were subjected to the following cycling conditions using a Bio-Rad thermocycler: 94 °C for 3 min, 40 cycles of 94 °C for 1 min, 50 °C for 1 min and 72 °C for 1 min, followed by a final extension at 72 °C for 7 min. The PCR products were electrophoresed through a 2% agarose gel to assess the presence of a 193 bp band (if any) in the first round. All PCR products, regardless of the presence or absence of a visible band were subjected to a second round of PCR. The Nested-PCR reaction condition was the same as the first round, except

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Table 1. Oligo-nucleotide primers specific for B1 gene used in the experiments

(The primer sets of F1 & R1 and F2 & R2 were used for the first round and nested-PCR round, respectively.)

Oligonucleotide primer	Sequence	Sequence position
Forward primer 1	5′-GGAACTGCATCCGTTCATGAG-3′	694–714
Reverse primer 1	5′-TCTTTAAAGCGTTCGTGGTC-3′	887–868
Forward primer 2	5′-TGCATAGGTTGCAGTCACTG-3′	757–776
Reverse primer 2	5′-GGCGACCAATCTGCGAATACACC-3′	853–831

Table 2. Infection by *Toxoplasma gondii* in ovine fetuses aborted at different times of gestation by different diagnostic techniques

Positives (%)					
PCR (Total)	PCR (first band invisible)	PCR (first band visible)	Maternal serology (IFAT)	Number (*n*)	Fetal age (days)
0 (0)	0 (0)	0 (0)	0 (0)	3	<60
5 (12·8)	0 (0)	5 (12·8)	7 (17·9)	39	60–120
10 (12·4)	2 (2·5)	8 (9·9)	11 (13·6)	81	>120
9 (16·3)	1 (1·8)	8 (14·5)	9 (16·4)	55	Dead after birth
3 (13·6)	0 (0)	3 (13·6)	4 (18·2)	22	Unknown
27 (13·5)	3 (1·5)	24 (12)	31 (15·5)	200	Total

for the annealing temperature which was 52 °C, the number of cycles was 30. The template for the nested PCR was a 1:10 dilution of first PCR products. The Nested-PCR products were also electrophoresed through a 2% agarose gel to assess the presence of a 96 bp band. A 100 bp molecular marker was used for size evaluation (Fermentas®, Lithuania).

Analysis of data

Serological samples that showed fluorescence in at least 50% of coated RH tachyzoites were considered positive, and those that showed less than 50% fluorescence in coated RH tachyzoites were considered negative. In PCR, the positivity and negativity was based on detection of special visible bands (193 bp for the first run and 96 bp for the second run) on 2% agarose gel. Both samples– with the193 bp visible band and without– were tested for the second run of PCR to detect the 96 bp band. Both samples, either with 2 bands visible and or just one 96 bp band visible, were recorded as positive. Negative samples displayed neither of the 2 special bands.

The minimum and maximum range of ovine abortion prevalence and occurrence of congenital transmission were calculated with 95% confidence interval (+/−) and the level of agreement (kappa statistics, SPSS 16) was measured to analyse any association between congenital infection by *T. gondii* and between the 2 different diagnostic techniques used (Fleiss *et al*. 2003). The Chi-square test was used (SPSS 16) to analyse the association between congenital infection and gestational age.

RESULTS

Age and macroscopic lesions

The age of aborted fetuses had been recorded in all cases except 4 (Tables 2 and 3). Macroscopic lesions were recorded in 122 out of 200 cases (61%). In 18 out of 31 positive cases, a swollen and congested placenta was recorded in 1 sample, central nervous system (CNS) lesions were recorded as hydrocephalus, encephalomalachia and small cerebelli (4 cases). Cardiac lesions included congestion, haemorrhage and necrosis (4 cases). Thoracic lesions consisted of hydrothorax, pulmonary congestion and haemorrhage, (6 cases). There was necrosis and haemorrhage in the liver, (1 case). Kidneys showed swelling, necrosis, hypoplasia, congestion and haemorrhage (5 cases). Skeletal muscle necrosis was recorded in 1 case (Tables 2 and 3). Not one sample was typical for toxoplasmosis (i.e. necrotic placenta).

Serology

Indirect fluorescent antibody test (IFAT) showed positive titres ⩾1:20 in 31 (15·5%) out of 200 examined maternal sera. Titration was 1:20 (16 samples), 1:80 (3 samples), 1:160 (4 samples), 1:320 (3 samples), 1:640 (1 sample), 1:1280 (3 samples), 1:2560 (1 sample).

PCR

Toxoplasma infection was diagnosed in 27 out of 200 (13·5%) ovine fetuses' brain samples based on PCR.

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Table 3. Distribution of different IFAT titres, macroscopic lesions and PCR results in positive samples

PCR (First band invisible but second one visible)	PCR (First and second bands visible)	Maternal serology titre (IFAT)	Macroscopic lesions	Positive fetuses
	+	1:20	C, K**	1
	+	1:20	C	2
	+	1:20	Lu**	3
	–	1:20	Autolysed	4
	+	1:2560	Lu	5
	+	1:160	–	6
	–	1:1280	–	7
	–	1:20	S**	8
+	+	1:20	H**	9
	+	1:20	C	10
	+	1:20	K**	11
	+	1:20	Lu	12
	–	1:20	Lu, K	13
	+	1:80	K	14
	+	1:20	–	15
	–	1:80	–	16
+	+	1:640	–	17
	+	1:1280	–	18
	+	1:20		19
	+	1:20	H, K, M**	20
	–	1:80	P**	21
+	+	1:160	K, L	22
	+	1:320	H, Lu, C	23
	+	1:1280	H, Lu	24
	+	1:320	–	25
	+	1:20	–	26
	+	1:20	C	27
	–	1:20	–	28
	+	1:160	–	29
	+	1:160	–	30
	+	1:320	–	31

* DB, Dead after birth; UKn, Unknown; **C, CNS; H, Heart; L, Liver; K, Kidney; Lu, Lung; P, Placenta; S, Small-sized fetus; M, Muscles.

In 24 of the positive samples both the 193 bp and the 96 bp bands were detectable (Fig. 2), but in 3 positive specimens, just the 96 bp band was detected in Nested-PCR (Fig. 2). Four maternal sera were positive in IFAT but not in the PCR attempted on the reported fetal brains. (Tables 2 and 3).

Correlation between age and infection

No significant association was found between the infection and 3 different fetal age categories (60–120, >120 and DB) (χ^2 value = 0·439 for the serological test and χ^2 value = 0·481 for PCR test; $P > 0{\cdot}05$, Table 2).

Prevalence and agreement

Maternal seroprevalence was 10·6% to 20·4% (95% confidence interval) and the estimated ovine abortion prevalence associated with *T. gondii,* based on PCR, was calculated as being from 8·8% to 18·2% with 95% confidence interval. These results show that the congenital levels of transmission constitute a high percentage of lambing, 27/31(87%) with an excellent logical agreement between the 2 different tests ($\kappa = 0{\cdot}9$) (Fleiss *et al.* 2003).

DISCUSSION

In the course of this study a slightly modified Nested-PCR assay based on previously described primers was set up and employed for molecular detection of *Toxoplama gondii* in brain tissues of ovine aborted fetuses. Brain, skeletal muscle, cardiac muscle, liver, spleen, lung and placenta are valuable tissues for *T. gondii* detection but the brain is a preferred tissue for diagnosis of *Toxoplasma*-induced ovine abortion because it is the most frequently infected tissue compared with the others (Esteban-Redondo and Innes, 1998; Hurtado *et al.* 2001; Pereira-Bueno, 2004; Dubey, 2009). On the other hand, Gutierrez *et al.* (2010) discussed the fact that placenta and fetal lung could also be good tissues and they detected the infection from these organs approximately as well as from the brain. However, Duncanson *et al.* (2001) and Sreekumar *et al.* (2004) failed to detect the parasite in lung.

Furthermore, the maternal serology of reported cases was tested by IFAT to assess the agreement between the maternal serological response and detection of the parasite genome in the reported fetal brain tissue. Overall, in this survey a reasonable

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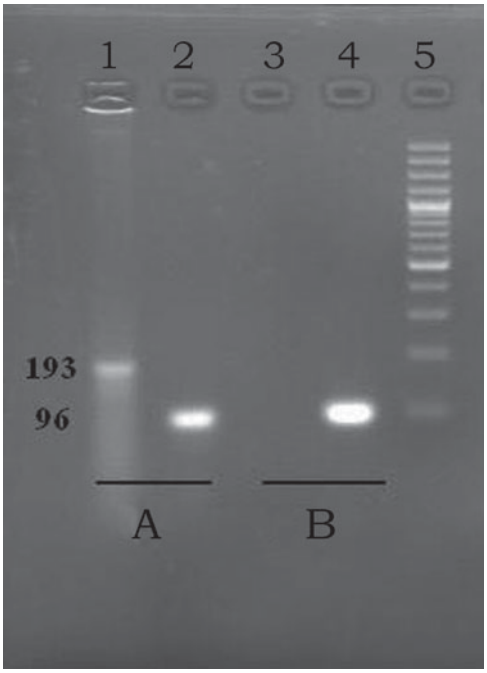

Fig. 2. PCR products electrophoresed on a 2% agarose gel.

number of ovine abortions were examined to assess the role of *T. gondii* as the causative agent in economical losses and potential public health threats in sheep herds in Khorasan Razavi Province, Iran. According to the result of this study, about 13·5% (8·8%–18·2% with a 95% confidence interval) of ovine abortions and fetal losses were associated with this organism, although the involvement of other pathogens could not be ruled-out. According to our findings the rate of ovine *Toxoplasma*-induced abortion in the province was similar to rates found previously using various techniques, including surveys based on serology in different countries such as Australia, 10% (Plant *et al*. 1972), UK, 14% (Linklater, 1979), USA, 15·4%–16·7% (Sheryl, 1989), Oregon, USA, 14–18%, (Dubey *et al*. 1990; Dubey and Kirkbride, 1990), South Dakota, USA 10·7% (Clyde and Kirkbride, 1993), New Zealand, 14–32% (Charleston, 1994), Italy, 28·4% by serology and 11·1% by Nested-PCR on ITS1 gene amplification (Masala *et al*. 2003) and in Spain, 15% in a pathological study (Oporto *et al*. 2006). In evaluation by different diagnostic techniques such as histopathology, serology and Nested-PCR on B1 gene amplification, a rate of 23·1% was obtained by at least 1 of the diagnostic techniques used (Pereira-Bueno *et al*. 2004). Prevalence in a pathological study in Hungary was 5·2% (Szeredi *et al*. 2006) and in Egypt was, 4 (50%) of 8, as meausured by Nested-PCR on B1 gene amplification and histopathology (Ahmed *et al*. 2008).

In Iran, little is known about the relative prevalence of *Toxoplasma*-induced abortion in sheep and most of the data are based on serology, including a report describing a rate of 72·6% seropositivity by ELISA and MAT (modified agglutination test) in a single sheep herd with a history of severe abortion (Hamidinejat *et al*. 2008) and 5·2% seropositivity by IFAT in 325 aborted ovine fetuses in the Mashhad area (Razmi *et al*. 2010).

In this present study, macroscopic lesions were seen in 18 of 31 maternal IFAT-positive cases and none of them was typical for toxoplasmosis (i.e. necrotic placental lesions were not observed in any of the placenta) but it was interesting that most of the lesions were observed in those organs that were most frequently used for detection of the parasite such as CNS, lungs, liver, kidneys (Table 3).

There were 7, 11 and 9 cases in the different age groups (60–120, >120 days and the ones that died shortly after birth), respectively that showed no significant difference amongst them ($P>0·05$). No case was recorded in the age group <60 days after gestation because fetal resorption was the most frequent event at this time (Buxton, 1991; Johnston, 1988; Blewett and Watson, 1983; Givens and Marley, 2008). A second reason might be that if fewer cases of aborted fetus and placenta are observed by the owners, then fewer cases will be referred to the Center of Excellence in Ruminant Abortion and Neonatal Mortality.

In *Toxoplasma*-induced abortion, a combination of diagnostic techniques is necessary for accurate diagnosis. Among them is the Nested-PCR assay, based on the B1 target gene having 35 copies per parasite, which is one of the most sensitive and specific tests (Jones *et al*. 2000; Mason *et al*. 2010). Although there are some new quantitative tests for *Toxoplasma* detection (Jauregui *et al*. 2001; Wu *et al*. 2009), the one recently used by Gutierrez *et al*. (2010) as a specific and sensitive test for detection of the infection in ovine maternal and fetal tissues was unfortunately not cost-effective for our study and we conclude that it is better to consider all advantages and disadvantages of a test before making a decision.

To prevent cross-contamination during sample collection and processing, efforts were made to prevent carry-over contamination during amplification steps in both rounds of PCR. The use of pre-prepared reagents, such as Accupower PCR premix kit (Bioneer®, South Korea) reduced the extent of manipulation and the time needed for the assays. Negative controls, included in each PCR experiment ensured us that no contamination had occurred during the experiment. Increasing annealing temperature and decreasing the number of cycles in the second PCR gave a sharp and intense band. As previously described, there were 3 positive specimens that yielded only the 96 bp band in Nested-PCR. Consequently, we conclude that because there may be a very low copy number in these samples, the importance of the Nested-PCR is greatly increased as a more specific test compared with single PCR.

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As previously discussed, the prevalence of congenital toxoplasmosis is somewhat higher than previously supposed, i.e. 61% (Duncanson *et al*. 2001), 69% (Williams *et al*. 2005) and 66% (Hide *et al*. 2009) and in our study it was estimated at 87% (82% to 91% with 95% confidence interval) with an excellent agreement between the 2 different tests ($\kappa = 0{\cdot}9$) (Fleiss *et al*. 2003) These findings increase the necessity for further work to clarify the precise mechanisms of this route of transmission and its occurrence from generation to generation following a primary infection as described by Morley *et al*. (2005, 2007), Hide *et al*. (2009) and Innes *et al*. (2009). These latter studies also suggest the need for further investigation of the processes that provide lifelong immunity to ewe and offspring after an initial infection.

According to the results of this study, it is concluded that *T. gondii* can be considered a common cause of abortion in Khorasan Razavi Province, north-east of Iran and that the level of congenital transmission is sufficiently high that it necessitates further research. It can also be concluded that the slightly modified Nested-PCR assay used in this study might be considered as a rapid, sensitive, specific and highly affordable conventional assay for molecular diagnosis of *T. gondii* worldwide.

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