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Molecular detection of *Toxoplasma gondii* from aborted fetuses of sheep, goats and cattle in Bangladesh

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ABSTRACT

The study was planned to apply the PCR method for detection of *T. gondii* infection in sheep, goats and cattle aborted fetuses from Mymensingh, Bangladesh. A total of 58 fetal tissue samples (brain, liver, heart, skeletal muscle and placenta) of sheep (5), goats (5) and cattle (2) were selected for study. Aborted fetuses were taken from serologically positive mothers by indirect ELISA. Among them 24 and 34 samples were subjected for PCR assay by using TgB1 and TgTox4 primers respectively. DNA fragments were visualized under UV illumination after gel run. The results demonstrated 15.52% tissue samples from sheep and goat aborted fetuses were positive for *T. gondii* parasite. Among different tissue samples, brain, liver and heart showed presence of *T. gondii* parasite. None of tissue samples showed positive in case of cattle. The results of the PCR exhibited that *T. gondii* infection might be considered as one of the major causative agents for abortion in ewes and does. Further studies are needed to improve our knowledge on different genotypes of *T. gondii* that infect sheep, goat and cattle population in Bangladesh.

1. Introduction

Toxoplasmosis is a parasitic disease caused by the intracellular apicomplexan protozoan *Toxoplasma gondii* with worldwide distribution (Dubey and Beattie, 1988). It is estimated that *T. gondii* infects up to one third of human population in the world (Dubey, 2010) causing congenital disease and abortion both in humans and livestock (Dubey and Beattie, 1988). The infection is major cause of reproductive failure associated with fetal, abortion and prenatal mortality of lambs in pregnant sheep and goats (Buxton, 1990; Buxton and Brebner, 1998; Tenter et al., 2000; Dubey and Schares, 2011). Cattle also become infected, but are considered to be more resistant to *T. gondii*, and the importance of these animals in the epidemiology of toxoplasmosis is still a controversial issue (Dubey, 1986, 2010; Dubey and Jones, 2008). Veterinary Investigation Diagnosis Analysis (VIDA) data from 2014 showed that about 25% of ovine production problems were caused by *T. gondii* (www.gov.uk/government/statistics). Moreover, viable *T. gondii* has been isolated from goat meat, milk and cheese (Dubey et al., 2014a, 2014b). Horizontal transmission occurs frequently after ingestion of cysts in undercooked meat or by consumption of food contaminated with sporulated oocysts (Dubey et al., 2014a). Recently, infection through goat and sheep raw milk has been confirmed (de Santana Rocha et al., 2015; Amairia et al., 2016). The zoonotic role of *T. gondii* has been demonstrated since the consumption of infected raw/undercooked meat or milk causes human infection (Tenter et al., 2000). Infected lamb is considered the main source of toxoplasmosis worldwide (Tenter et al., 2000). Domestic and feral cats are the definitive hosts but other animals including humans can be infected by ingestion of oocysts or tissue cysts. Overwhelming infections, especially in an immunosuppressed or immunologically naïve animal, may be fatal (Jennifer et al., 1995). The diagnosis of *T. gondii* infection is usually based on histopathological examination, serological assay, and isolation of *Toxoplasma* by mouse inoculation (da Silva and Langoni, 2001; Masala et al., 2003; Remington et al., 2004; Garcia et al., 2006). Most of epidemiological studies on *T. gondii* have been done based on extensive serological tests in all over the world including Bangladesh. The seroprevalence of toxoplasmosis was reported ranging from 12 to 27% in cattle, 12–61% in goats and 18–69% in sheep in different parts of Bangladesh (Samad et al., 1993; Shahiduzzaman et al., 2011; Rahman et al., 2014; Sah et al., 2018). However, the classical diagnosis of

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toxoplasmosis based on serological tests is inefficient and inadequate in all the time of patients. Therefore, confirmatory diagnosis is needed and is based on the direct demonstration of the parasite in tissues or biological fluids. This can be achieved by tissue culture or mouse inoculation. However, tissue culture is not very sensitive and inoculation of mice takes more than three weeks to complete (Fircker-Hidalgo et al., 1997). PCR overcomes these shortcomings (Bretagne et al., 1995). PCR is sensitive and the diagnosis can be confirmed within a day. Molecular assays, such as the PCR, make it possible to detect small quantities of target DNA and potentially provide an alternative sensitive diagnostic tool (Muller et al., 1996; Remington et al., 2004). PCR is becoming a favored technique for the detection of *T. gondii* in tissues (Jones et al., 2000) over the conventional mouse bioassay (Villena et al., 2004), as it reduces the detection time from weeks to 1 to 2 days. No studies have reported about isolation of viable *T. gondii* from ruminants in Bangladesh. In this Study, fetal samples were used to assay from sheep, goat and cattle aborted fetuses. *T. gondii* DNA was detected in 54% of brain samples of aborted fetuses in sheep. *T. gondii* infection has been detected in sheep aborted fetuses to range between 13.5% to 61% in different areas of Iran by PCR assays (Satbige et al., 2017; Moazeni Jula et al., 2013). The B1 gene (580 bp) and 529 bp fragment as a target sequence using PCR was applied for detection of the *T. gondii*. The aim of this study was to apply the PCR method in detection of *T. gondii* infection in provided sheep, goat and cattle aborted fetus samples from Mymensingh areas and periphery of Bangladesh.

2. Materials and methods

The study plan was approved by the animal welfare and experimentation ethical committee (AWEEC) of Bangladesh Agricultural University.

2.1. Collection of samples

Tissue samples (brain, liver, heart, skeletal muscle (near thigh and shoulder) and placenta) were collected from each aborted fetuses (sheep fetus-5, goat fetus-5 and cattle fetus-2) aseptically from Kewatkhali, Sheshmore, Chaurkalibari, Digerkanda of Mymensingh, Bangladesh during 2015–2016. The aborted fetuses were collected from farmers of those places after getting information. Blood samples of the mothers were also collected for ELISA. The sera were examined for presence of antibodies against T. gondii by indirect ELISA as described by Gebremedhin et al. (2013). The ELISA was performed using a commercial test kit (ID Screen® Toxoplasmosis Indirect Multi-species, Innovative Diagnostics, France). Mothers of these collected aborted fetuses were found serologically positive by ELISA. A tiny piece of 25 mg of tissue samples of different organs was cut with a sterile knife for DNA extraction and weighed and was kept in eppendorf tube separately. Tissue samples were cut separately one by one and were kept in eppendorf tubes and labeled to avoid contamination of each other. The tissue samples were stored at −20°C for DNA extraction.

2.2. DNA extraction from tissue samples

Eppendorf tubes having tissue samples (each having 25 mg) were taken out from deep freeze and were kept at room temperature for few minutes. The genomic DNA was extracted from tissue samples (brain, liver, heart, muscle and placenta) using PureLink™ Genomic DNA Kit for purification of genomic DNA (PureLink® Genomic DNA Mini Kit, 50 preps, Catalog No. K1820–01, Invitrogen Life technologies) in accordance with the manufacturer's instructions. Briefly, cells were lysed and digested with 20 µl of Proteinase K, RNase A (50 Mm Tris-HCl, Ph 8.0,10 Mm EDTA) and 200 µl of Lysis/Binding Buffer at 55 °C for 10 min. Absolute ethanol (200 µl) was added and the mixture was transferred to the PureLink™ Spin column in a 2 ml collection tube and centrifuged for 1 min. The columns were washed twice, and the DNA was eluted from the columns with 50 µl of elution buffer (10 mM Tris-HCl, pH 9.0, 0.1 mM EDTA).

2.3. Target genes

The multicopy B1 gene (580 bp) and the 529 bp repetitive element were the PCR targets evaluated in this study. The B1 gene consists of 35 copies and it is highly conserved among strains of *T. gondii* (Contini et al., 2002). The 529 bp repetitive element consists of 200 to 300 copies in the genome of *T. gondii* (Homan et al., 2000).

2.4. Polymerase chain reaction (PCR) and gel run

2.4.1. PCR amplification of 529 bp fragment and B1 gene

DNA amplification of *T. gondii* was performed using the method described by Homan et al. (2000); and Reischl et al. (2003). Primers TgTox4F (5-CGCTGCAGGGAGGAAGACGAAAGTTG-3′) and TgTox4R (5'-CGCTGCAGACACAGTGCATCTGGATT-3′) were used, and these flanked a 529 bp fragment of *T. gondii* DNA. PCR reaction was performed in a mixture containing 5 µl of DNA template plus 20 µl (final volume of 25 µl) of mixture [5× PCR My Taq red master mix (100 mM dNTP, 60 mM Tris ± HCl (pH 9.0), 15 mM $(NH_4)_2SO_4$, 2 mM $MgCl_2$), (Bioline), Taq DNA polymerase (Bioline), 25 pmol from each primer (Sigma) and volume completed by nuclease free water]. Each amplification run one negative control (ultra-pure water) and one positive (cDNA). Amplification of DNA from parasites were performed over 35 cycles in Thermocycler (Flex Cycler^2), Biometra GmbH, Germany, using the following cycling conditions: 7 min at 94 °C for denaturation in cycle one, followed by 35 cycles on 60s at 94 °C for denaturation, 60s at 55 °C for annealing and 60s at 72 °C for extension, was followed by a final extension of 10 min at 72 °C. The coding regions of the B1 gene (580 bp) in the positive control strain and also in *T. gondii* cysts, isolated from tissue samples of aborted fetus, were amplified using 25 µl PCR reaction volume by a conventional PCR assay according to Contini et al. (2002). Two oligonucleotides were used from the B1 gene sequences (Bretagne et al., 1993); forward primer, TgB1F; 5'-ACGGGCGAGTAGC ACCTGAGGAGA −3′ and reverse primer, TgB1R; 5'-TGGGTCTACGTC GATGGCATGACAAC -3′. Amplification was performed using Thermo cycler (Flex Cycler^2), Biometra GmbH, Germany. The following PCR components were added in each PCR tube: 5 X PCR My Taq red masters mix (Bioline), DNA polymerase (Bioline), 25 pmol from each primer (Sigma), 5 µl of DNA template and volume completed by nuclease free water. Each amplification run one negative control (ultra-pure water) and one positive (cDNA). PCR amplification consisted of initial denaturation of 95 °C for 5 min; followed by thirty five cycles, each cycle included a denaturation step at 93 °C for 1 min, a primer annealing step at 60 °C for 1 min and an extension step at 72 °C for 3 min. The final elongation step was prolonged for 10 min at 72 °C to ensure a complete extension of amplified DNA. The PCR product of B1 gene in all tissue samples was 580 bp. Aliquots (10 µl) of each PCR products were electrophoresed on 1.5% agarose gel and stained with ethidium bromide (Pereira et al., 1983). DNA fragments were visualized under UV illumination. The images were captured in memory. The size of fragments was based on comparison with a 100-bp ladder.

3. Results

In this study altogether 58 tissue samples from aborted fetuses were selected for isolation of *T. gondii* parasite by conventional PCR. Among them, 24 tissue samples were subjected for PCR by using TgB1 primer and other 34 tissue samples were subjected for PCR by using Tox4 primer. Out of 58, 9 tissue samples were found positive for *T. gondii* parasite (15.52%). Among them, 3 samples showed positive by TgB1 and 6 samples showed positive by TgTox4. Similarly, 6 and 3 tissue samples were *T. gondii* positive in case of sheep and goats respectively. Four fetuses of sheep and three fetuses of goats were found positive for

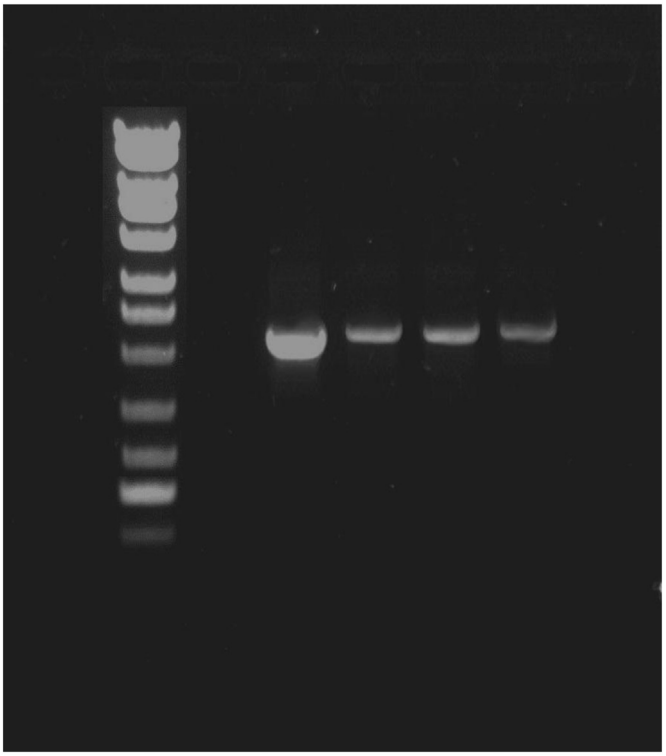

Fig. 1. PCR product (580 bp) from infected sheep fetus with *T. gondii*: Lane M: 100 bp marker, Lane N: negative control, Lane P: positive control, Lane S1-S3: positive samples (brain, liver and heart).

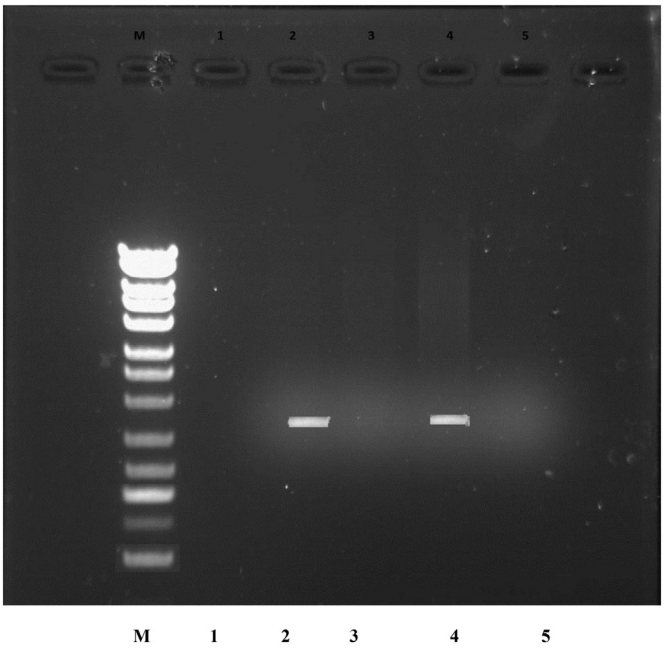

Fig. 2. PCR product (529 bp) from infected sheep fetus with *T. gondii*: Lane M: 100 bp marker, Lane 1: negative control, Lane 2 &4: positive samples (brain, brain), Lane 3–5: negative samples (placenta, skeletal muscle).

T. gondii DNA. Brain, liver and heart of sheep and goat fetuses showed positive for *T. gondii* parasite. A brain and liver samples of aborted fetus was positive for both TgB1 and TgTox4 primers. None of tissue samples showed positive in case of cattle. The sizes of PCR products/bands were given in Figs. 1, 2 and 3. The positive rate was high in sheep and low in goat in this study.

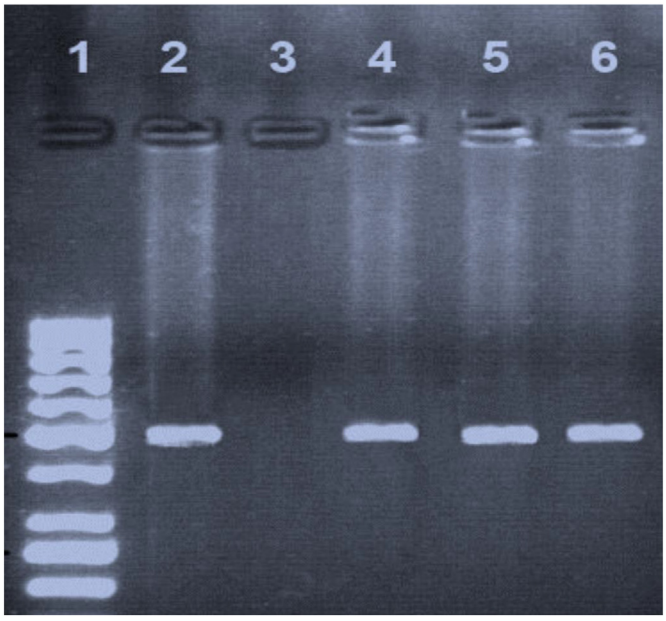

Fig. 3. PCR product (529 bp) from infected goat fetus with *T. gondii*: Lane 1: 100 bp marker, Lane 2: positive control, Lane 3: negative control, Lane 4–6: positive samples (brain, liver and heart).

4. Discussion

In the current study, *T. gondii* DNA was detected in 9 out of 58 fetal tissue samples (15.52%) by conventional PCR. To our knowledge, this study was the first report of molecular detection of *T. gondii* in sheep, goat and cattle in Bangladesh. This finding is high from a report molecularly (PCR) confirming *T. gondii* in 1.69% and 1.35% of the slaughter sheep and goat, respectively from North India (Kalambhe et al., 2017). Similary almost similar finding reported by Satbige et al. (2017) that four samples (heart/brain) consisting of 3 numbers of sheep and 1 number of goat tissues out of 14 samples detected by B1 PCR in south India. Oral ingestion of *T. gondii* oocysts is the main source of infection for sheep and goats, and poses a risk for exogenous transplacental transmission in pregnant animals (Innes et al., 2009). Moreover, a previous study confirmed that reactivation of *T. gondii* cysts in chronically infected sheep and goats serves as another important risk for endogenous transplacental transmission in sheep and goats during pregnancy (Williams et al., 2005; Hide, 2016). In this study, conventional PCR assays based on the B1 gene and 529-bp repetitive element were tested for their sensitivity in detecting *T. gondii* parasite in tissue samples. These genes were selected because of their frequent use for the detection of *T. gondii* in clinical specimens and the high copy number of the targets (Calderaro et al., 2006; Edvinsson et al., 2006; Kompalic-Cristo et al., 2007). Results of the present study confirm the higher sensitivity of PCR assays based on the 529-bp repetitive element compared to those based on the B1 gene (Reischl et al., 2003; Calderaro et al., 2006; Cassaing et al., 2006; Edvinsson et al., 2006). This likely was due to the difference in copy numbers of the two targets, as reflected by the results of analyses (Table 1). This difference was not fully explained by the difference in copy numbers of the targets (35 versus 200 to 300 copies). On the other hand, many studies showed higher or lower prevalence values of the infection. For example, in Ireland, the presence of *T. gondii* DNA was detected by PCR in diaphragm samples from 3.6% (3/83) sheep (Halova et al., 2013), and higher prevalence values (31%, 34.2%, 38%) in sheep were found in Tunisia (Rouatbi et al., 2017), in Iran (Armand et al., 2016) and in Morocco (Azizi et al., 2014) respectively. In the present study, only 25 mg tissue samples

Table 1
Presence of *T. gondii* parasite in different tissue samples based on PCR assay (TgB1 and TgTox4 primers).

Species and no of fetus	Name of tissue sample	Number of tissue	TgB1		TgTox4	
			Tested no.	Positive no.	Tested no.	Positive no.
Sheep - 5	Placenta	5	2	–	3	–
	Brain	5	2	1	3	2
	Liver	5	2	1	3	1
	Heart	5	2	1	3	–
	Skeletal muscle	5	2	–	3	–
Goat - 5	Placenta	5	2	–	3	–
	Brain	5	2	–	3	1
	Liver	5	2	–	3	1
	Heart	5	2	–	3	1
	Skeletal muscle	5	2	–	3	–
Cattle - 2	Placenta	2	1	–	1	–
	Brain	2	1	–	1	–
	Liver	2	1	–	1	–
	Heart	2	1	–	1	–
Total		58	24	3	34	6

were analysed which could induce an under-estimation of *T. gondii* infection prevalence, the ability of gene amplification by PCR allowed detection of small amounts of DNA. This suggests that PCR is a sensitive method for the diagnosis of toxoplasmosis (Wastling et al., 1993). Our result showed negative for *T. gondii* parasite in all fetal samples of cattle. This finding resembles with opinion given in Turkey (Ozkaraca et al., 2017) that none of the aborted bovine fetuses were shown to have *T. gondii* by PCR.

5. Conclusions

PCR is the important diagnostic tool for the detection of *T. gondii* parasite in aborted fetuses of sheep, goats and cattle as revelaed from this study like previous studies. As this organism remains present in the tissues of sheep, goats and therefore people may get infection via consumption of undercooked meat. Sheep and goats pose a risk of *T. gondii* infection and have the potential to impact public health. Further studies are needed to improve our knowledge on different genotypes of *T. gondii* that infect sheep, goat and cattle population in Bangladesh.

Declaration of Competing Interest

We declare no conflicts of interest.

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