大韓獸醫學會誌 (2013) 第 53 卷 第 1 號
Korean J Vet Res(2013) 53(1) : 1~5

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Pathological description and immunohistochemical demonstration of ovine abortion associated with *Toxoplasma gondii* in Iran

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(Received: June 23, 2012; Revised: October 7, 2012; Accepted: November 14, 2012)

Abstract : The obligatory intracellular protozoan parasite *Toxoplasma gondii* is a major world wide cause of infectious ovine abortion. In some different diagnostic techniques that are being used to detect this pathogen in ovine fetuses, immunohistochemistry (IHC) is a very sensitive and expensive one. Histopathology is not truly a specific and sensitive test for *Toxoplasma* infection but it can be helpful to choose some suspected tissues for IHC. In this study 9.5% of 200 samples (aborted ovine fetuses internal organs such as brain, liver, heart, lung, kidney, spleen) (4.6~14.4% with 95% CI) were positive in IHC with a very good logical agreement among different diagnostic techniques ($\kappa = 0.73$, 0.8) and with no significant difference among different fetal age groups ($p > 0.05$).

Keywords : histopathology, immunohistochemistry, Iran, ovine abortion, *Toxoplasma gondii*

Introduction

The obligatory intracellular protozoan parasite *Toxoplasma* (*T.*) *gondii* is a major cause of infectious abortion all over the world. *Toxoplasma* was first reported to be an important pathogen in sheep in 1950s [9, 10].

The route of transmission of the parasite to sheep is devided into two routes: ingestion of the infective oocysts shedding by infectedcats and congenital transmission [11]. Recrudescence of internal ovine infection that recently has been considered, can also play an important role in *Toxoplasma* life cycle. Therefore, congenital transmission is found to be more important than what was previously assumed [5, 11, 14, 15].

Most of the times, various diagnostic techniques are used in *T. gondii* infection. In order to investigate ovine toxoplasmosis in the north-east of Iran, we examined 200 aborted fetal brains by PCR and maternal serology by indirect fluorescent antibody test (IFAT) [17], to complete this study, pathological and immunohistochemical (IHC) diagnosis of *T. gondii* was done on different tissues collected from those aborted fetuses. Histopathology of *T. gondii* is not a decisive test for detection of the infection because it is not suitable for severely decomposed fetuses and it is difficult to diagnose the infection when only a few organisms are present [19], so we combined it with easy IHC technique based on fluorescence labeling of antigen/antibody complex as a more sensitive and specific test.

IHC is a combination of histological, immunological and biochemical techniques used for the identification of the specific tissue components by means of specific antigen/antibody reaction tagged with a visible label.

There are different IHC techniques for *T. gondii* detection such as PAP technique [19], fluorescent labeling [7, 12], Vectastain avidin biotin complex method (Vector Laboratories, USA) and DAKO En Vision (DAKO corporation, USA).

The aim of this study is 1) to determine the ovine abortion prevalence by different diagnostic methods in north-east of Iran 2) to set up an easy IHC technique for detection of the infection 3) to compare the data collected by IHC with the most widely used techniques such as PCR and serology in diagnosis of the infection in ovine fetuses.

Materials and Methods

Pathology

This procedure was done by a thorough sampling, fixation, dehydration, paraffin infiltration, blocking, sectioning

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and mounting as described by Soleimani Rad [18]. The prepared tissues then were stained with haematoxylin and eosin method [13] and were viewed under light microscope.

Monoclonal antibody

Anti-P30 antibody (Serotec, UK) and anti sheep IgG conjugated to FITC complex (Serotec) were used to increase specificity and sensitivity of IHC.

IHC

All available fetal tissues of positive samples in PCR and IFAT [17] and fetal brain tissues of PCR and IFAT negative samples were tested by IHC [7]. A five-micrometer section of each tissue was deparaffinized in xylol for 30 min and rehydrated in 100%, 85%, 70%, 50% ethanol solution and distilled water, respectively, each for 2 to 5 min.

No pretreatment was done for antigen retrieval and between each incubation slides were rinsed 3 times in PBS, pH 7.4, each for 5~10 min.

Blocking was carried out by incubation of slides with 50~100 μL of 1.5% bovine serum albumin (BSA) diluted in PBS and 50 mM glycine for 1 h in humid chamber at room temperature.

Antibody1: antibody was added 1 : 1 to 0.15% BSA diluted in PBS, incubated 30 min in humid chamber at room temperature.

Antibody 2: anti-sheep IgG-FITC (Serotec) added 1 : 1 to 0.15% BSA diluted in PBS, incubated 30 min in humid chamber at room temperature.

Counter stain: Evans blue 1% (Biogene, Iran) diluted 1 : 50 with PBS was used for counterstaining. Glycerin buffer was added to slides and viewed under fluorescent microscope. Tachyzoites or tissue cysts were seen with a green fluorescent shining under fluorescent microscope.

One positive and negative control tissue for each set of tests should be included in each staining run. For positive control we use samples of ovine congenital outbreak (Maryam Rassouli, Iran) that *Toxoplasma* cysts were seen in histopathology. For negative control, PBS was added instead of antibody.

Data analysis

The minimum and maximum range of ovine abortion prevalence and occurrence of congenital transmission were calculated with 95% confidence interval (CI) and the level of agreement (kappa statistics, ver. 16; SPSS, USA) was measured to analyze any association between congenital infection among different diagnostic techniques used [6]. The Chi-square test was used, SPSS16, to analyze the association between congenital infection and gestational age.

Results

Age and macroscopic lesions of aborted ovine fetuses were recorded. Macroscopic lesions were seen in 122 of 200 (61%) samples, some of which were not typical for toxoplasmosis. In suspected cases some macroscopic lesions as they were summarized in Table 1 were recorded.

All of the common pathological lesions (necrosis, hyperemia, hemorrhage, edema and inflammatory cells infiltration)

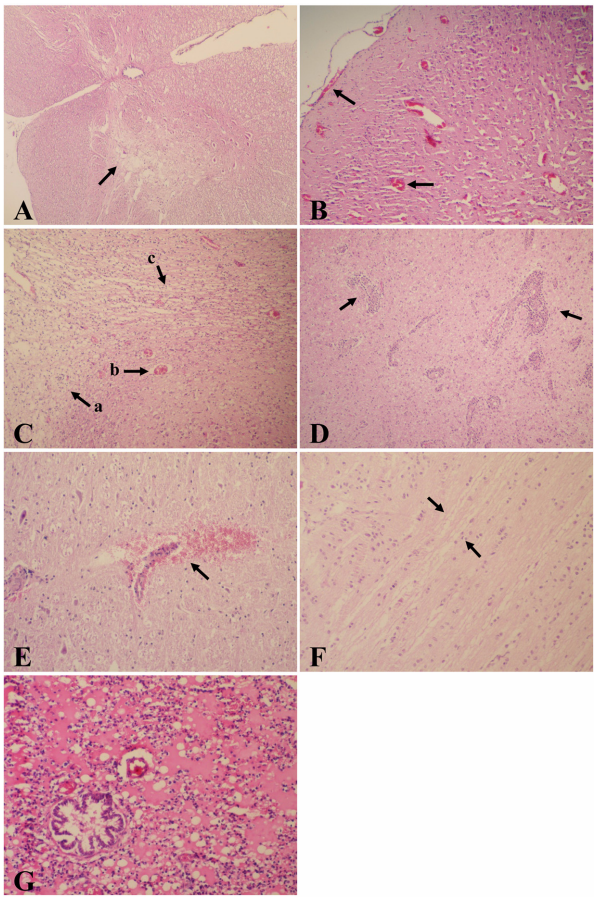

Fig. 1. (A) Spinal cord tissue: Liquefactive necrosis in gray matter. (B) Brain tissue: meninge hyperemia, edema, ischemic cell changes. (C) Brain tissue: a; leukoencephalomalacia, b; severe hyperemia, edema, c; ischemic cell changes, demyelination. (D) Brain tissue: perivascular cuffing of inflammatory cells. (E) Brain tissue: Hemorrhage around vessel. (F) Brain tissue: myelin degeneration in cerebral white matter. (G) Lung tissue: serous pneumonia. H&E stain, A: ×4, B-D: ×10, E and F: ×20.

Table 1. Some of the recorded macroscopic lesions suspected to *Toxoplasma gondii* infection

Tissue	Macroscopic lesions
Liver	Hemorrhage (1 case)
Muscle	Necrosis (1 case)
Heart	Hyperemia, hemorrhage and necrosis (4 cases)
Lung	Hydrothorax, hyperemia and hemorrhage (6 cases)
Kidney	Swelling, necrosis, hypoplasia, hyperemia, necrosis (5 cases)
Brain	Hydrocephalus, encephalomalacia (4 cases)
Placenta	Congestion and swelling (1 case)

Table 2. Common pathological lesions of infectious ovine abortion cases

	Necrosis	Hyperemia	hemorrhage	Inflammatory cells infiltration	Edema
Liver	16	54	10	22	–
Spleen	–	30	4	12	–
Heart	8	42	10	10	–
Lung	–	66	8	–	6
Kidney	20	48	22	–	4
Brain	10	88	14	–	35
Cerebellum	1	7	2	–	3
Fetal membranes	1	–	–	1	–

Table 3. Histopathological lesions in aborted ovine fetuses

Tissue	Lesions	Number
Cerebrum	Meningitis, focal gliosis, ischemic cell change, leukoencephalomalacia and demyelination	23
Spinal cord	Myelin degeneration and liquefactive necrosis in white matter	1
Cerebellum	Ischemic cell changes in Purkinge cell and status spongiosus in white matter	2
Heart	Cell lysis, myocarditis and fibroblast proliferation	2
Liver	Focal necrosis	3
Kidney	Cell swallow, glumerolitis, existence of different casts	24
Spleen	Hemocydrosis	4
Lung	Severe pneumonia, peribronchial cuffing	24

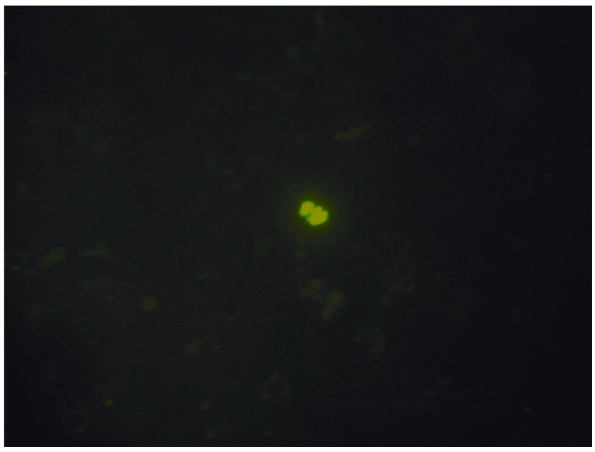

Fig. 2. *Toxoplasma gondii* tissue cyst stained by fluorescent labeling IHC method, ×40.

were summarized in Table 2, but some of the specific lesions were mentioned in details in Table 3, Fig. 1, they were not typical for *Toxoplasma* infection but some of them especially central nervous system (CNS) lesions could be helpful in diagnosis. No *Toxoplasma* cyst was seen in all different tissues of 200 samples.

IHC positivity (Fig. 2) was recorded in 18 brain, 9 kidney, 7 liver, 10 lung and 3 spleen tissues of 19 samples. All of these 19 samples were positive in PCR and maternal serology, so the *Toxoplasma*-induced ovine abortion was estimated 9.5% (4.6~14.4% with 95%CI) but there were no significant difference among various age groups (X^2 value = 1.97, $p > 0.05$, Table 4). There was a very good logical agreement between IHC and serology ($\kappa = 0.73$) and anexcellent agreement between PCR and IHC ($\kappa = 0.8$) [6].

Table 4. Toxoplasmosis in ovine fetuses based on gestation time, diagnosed by IHC technique

Fetal age (days)	Number (n)	IHC positive (%)
< 60	3	0 (0)
60~120	39	3 (10.25)
> 120	81	7 (8.64)
Dead after birth	55	8 (14.5)
Unknown	22	1 (4.54)
Total	200	19 (9.5)

IHC: immunohistochemistry. X^2 value = 1.97, $p > 0.05$.

Discussion

Histopathology could help *T. gondii* diagnosis in tissue sections, especially in placenta and CNS tissues. Some samples of placental cotyledons may show moderate edema of mesenchyme of the fetal villi with a diffuse hyper cellularity due to the presence of large mononuclear cells. Sometimes, a small number of intracellular and extracellular *Toxoplasma*

are visible [16].

Unfortunately, in this study, we did not have access to any placental cotyledons. Brain tissues present primary and secondary lesions in histopathology [1, 2]. In primary lesions, there are inflammatory foci composed of microglia and other mononuclear inflammatory cells and typically with necrotic and sometimes mineralized centers. They represent a fetal immune response following direct damage by local parasite multiplication and are often associated with mild focal lymphoid meningitis. Secondary lesions manifest themselves as focal leukomalacia. They are considered to be due to fetal anoxia in late gestation caused by the progressive multifocal necrosis in the placentome preventing sufficient oxygen transfer from mother to fetus [1].

Leukomalacia most commonly occurs in the cerebral white matter cores, but sometimes it also happens in the cerebellar white matter. When both inflammation and focal leukoencephalomalacia are seen together, diagnosis is reasonably certain, but less commonly it could also be seen in chlamydialabortion [3] or tick-borne fever [4].

Toxoplasma cyst is rarely found, at the periphery of these lesions [16]. It is still not known what determines the possibility of *Toxoplasma* existence in tissue sections.

In this study we couldn't see *Toxoplasma* tissue cyst in any part of thedifferent organs among the 200 samples but it was easily seen in 4 samples submitted as an outbreak (Maryam Rassouli, Iran).

Focal necrosis, as a non specific lesion that can be seen in other organs such as liver, lung and heart. In these suspected tissues *Toxoplasma*-induced lesions should be confirmed by IHC [16].

In this study CNS lesions were more typical than lesions of other organs which could be helpful to the approach used in *Toxoplasma* diagnosis. IHC confirmed *Toxoplasma* infection in 18 brain samples which showed various lesions from mild to severe in histopathology.

IHC is a specific and sensitive method for *T. gondii* detection in tissue sections but the most important disadvantage is that IHC is too expensive and specifically, not economical in lots of samples. Therefore, it seems that there is not a much broad study regarding to this field in the world. Uggla *et al.* [19] could detect *Toxoplasma* cyst or Tachyzoites in heart, lung, muscle, brain, spinal cord, eye, placenta and lymphoid nodes by PAP method.

They could also show that CNS, placenta, lung and heart are the more valuable organs. Furthermore, Gutierrez *et al.* [8] mentioned that lung and placenta were as well as CNS tissues in *Toxoplasma* detection by RT-PCR.

In this study we didn't have any placenta samples but *Toxoplasma* could be detected in 18 brain and 10 lung tissues as the most recorded ones. According to the agreement among different diagnostic techniques, including this study and the previous one [17], the lowest agreement was between IHC and maternal serology and the highest one was between PCR and maternal serology.

Comparison of different diagnostic techniques showed 9.5% were positive in all 3 tests (Nested-PCR and maternal serology [17], and IHC) and 4% were positive in at least one of these tests which confirmed the sensitivity of these tests were different but their specificity were quite similar.

According to the results 4.6~14.4% (95% CI) of these 200 ovine abortion caused by *T. gondii* and by the very good agreement ($\kappa = 0.73$) between IHC and maternal serology we can conclude that congenital toxoplasmosis must be considered more important than what was previously assumed.

As some recent researches in the UK showed that recrudescence of internal infection can also be an important route of transmission in *Toxoplasma* life cycle [5, 11, 14, 15], more researches are needed to clarify these ambiguous aspects of *Toxoplasma* infection.

References

1. **Buxton D, Gilmour JS, Angus KW, Blewett DA, Miller JK.** Perinatal changes in lambs infected with *Toxoplasma gondii*. Res Vet Sci 1982, **32**, 170-176.
2. **Buxton D, Finlayson J.** Experimental infection of pregnant sheep with *Toxoplasma gondii:* pathological and immunological observations on the placenta and foetus. J Comp Pathol 1986, **96**, 319-333.
3. **Buxton D, Anderson IE, Longbottom D, Livingstone M, Wattegedera S, Entrican G.** Ovine Chlamydial abortion: characterization of the inflammatory immune response in placental tissues. J Comp Pathol 2002, **127**, 133-141.
4. **Chianini F, Adams C, Buxton D.** Neuropathological changes in ovine fetuse caused by tickborne fever. Vet Rec 2004, **155**, 805-806.
5. **Duncanson P, Terry RS, Smith JE, Hide G.** High levels of congenital transmission of *Toxoplasma gondii* in a commercial sheep flock. Int J Parasitol 2001, **31**, 1699-1703.
6. **Fleiss JL, Levin B, Paik MC.** Statistical Methods for Rates and Proportions. 3rd ed. pp. 598-626, Wiley, Hoboken, 2003.
7. **Gottstein B, Hentrich B, Wyss R, Thür B, Busato A, Stärk KDC, Müller N.** Molecular and immunodiagnostic investigations on bovine neosporosis in Switzerland. Int J Parasitol 1998, **28**, 679-691.
8. **Gutierrez J, O'Donovan J, Williams E, Proctor A, Brady C, Marques PX, Worrall S, Nally JE, McElroy M, Bassett H, Sammin D, Buxton D, Maley S, Markey BK.** Detection and quantification of *Toxoplasma gondii* in ovine maternal and foetal tissues from experimentally infected pregnant ewes using real-time PCR. Vet Parasitol 2010, **172**, 8-15.
9. **Hartley WJ, Jebson JL, McFarlane D.** New Zealand type II abortion in ewes. Aust Vet J 1954, **30**, 216-218.
10. **Hartley WJ, Marshall SC.** Toxoplasmosis as a cause of ovine perinatal mortality. N Z Vet J 1957, **5**, 119-124.
11. **Innes EA, Bartley PM, Buxton D, Katzer F.** Ovine toxoplasmosis. Parasitology 2009, **136**, 1887-1894.
12. **Kim JH, Kang KI, Kang WC, Sohn HJ, Jean YH, Park BK, Kim Y, Kim DY.** Porcine abortion outbreak associated with *Toxoplasma gondii* in Jeju Island, Korea. J Vet Sci

2009, **10**, 147-151.
13. **Luna LG.** Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology. 3rd ed. pp. 38-39, McGraw-Hill, New York, 1968.
14. **Morley EK, Williams RH, Hughes JM, Terry RS, Duncanson P, Smith JE, Hide G.** Significant familial differences in the frequency of abortion and *Toxoplasma gondii* infection within a flock of Charollais sheep. Parasitology 2005, **131** (Pt 2), 181-185.
15. **Morley EK, Williams RH, Hughes JM, Thomasson D, Terry RS, Duncanson P, Smith JE, Hide G.** Evidence that primary infection of Charollais sheep with *Toxoplasma gondii* may not prevent foetal infection and abortion in subsequent lambings. Parasitology 2008, **135**, 169-173.
16. **Ortega-Mora LM, Gottstein B, Conraths FJ, Buxton D.** Protozoal Abortion in Farm Ruminants: Guidelines for Diagnosis and Control. 1st ed. pp. 124-164, CABI, Wallingford, 2007.
17. **Rassouli M, Razmi GR, Bassami MR, Movassaghi AR, Azizzadeh M.** 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. Parasitology 2011, **138**, 691-697.
18. **Soleimani Rad J**. Histology. 2nd ed. pp. 2-3, Golban Medical Publication, Tehran, 2009. [In Persian]
19. **Uggla A, Sjöland L, Dubey JP.** Immunohistochemical diagnosis of toxoplasmosis in fetuses and fetal membranes of sheep. Am J Vet Res 1987, **48**, 348-351.