

SEROLOGICAL PREVALENCE OF TOXOPLASMA GONDII AND ITS ASSOCIATION WITH ABORTION IN SHEEP IN SAUDI ARABIA

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The purpose of this study was to investigate serological prevalence and titers of anti-Toxoplasma gondii antibodies in ewes following waves of abortion and stillbirths in a commercial flock in Riyadh, Saudi Arabia. Serum samples from 168 aborted ewes and 52 breeding rams, were tested for toxoplasmosis using an indirect enzyme-linked-immunosorbent assay (ELISA) and indirect haemagglutination test (IHA). 71 randomly sampled sheep from an abortion-free flock (60 ewes and 11 rams) were also tested, which served as controls. 149 (88.7%) ewes and 42 (80.8%) breeding rams from the flock where abortions and stillbirths occurred were positive for anti-T. gondii antibodies by ELISA. 155 ewes (92.3%) and 44 rams (84.6%), including all of the ELISA positive cases, were also positive by indirect haemagglutination test (IHA). More than 80% of the ELISA-positive ewes had O.D. exceeding 100% and nearly 25% of them had O.D. of $\geq 150\%$. The IHA results, on the other hand, indicated that more than 75% of the seropositive ewes had antibody titers $\geq 1:1024$, including 58 (37.4%) ewes with IHA titer ranging between 1:4096-1:8192. Pyrexia, depression and vaginal discharge were recorded in some ewes shortly prior to abortion. Post-mortem examination of 5 aborted fetuses revealed blood-stained fluid in the abdominal and thoracic cavities and small inflammatory and necrotic foci in the brain, liver and lungs while the placenta was reddish and friable, and its cotyledons were speckled with small whitish foci of necrosis and mineralization. T. gondii tachyzoites were demonstrated in placental sections of two ewes. By contrast, only 7 (9.9%) out of 71 randomly sampled sheep from an abortion-free flock (60 ewes and 11 rams), were positive for anti-T. gondii antibodies by ELISA and 6 (8.5%) by indirect haemagglutination (IHA) test and most of these had significantly lower titers compared to the flock where abortions and stillbirths were recorded. These results constitute the first detailed serological study of ovine toxoplasmosis in Saudi Arabia and strongly implicate toxoplasmosis as the cause of the abortions and stillbirths in these sheep.

Key words: abortion, ELISA, IHA, Saudi Arabia, sheep, Toxoplasma gondii

INTRODUCTION

Toxoplasmosis is a major zoonosis caused by *Toxoplasma gondii*, a ubiquitous protozoan parasite that uses felids as definitive hosts and numerous species of vertebrate animals as intermediate hosts. Despite its vast range of intermediate hosts, however, *T. gondii* is clinically and economically most important in two host species: human beings and sheep. Human infections usually occur following consumption of undercooked or raw meat infected with tissue cysts, or food and water contaminated with oocysts from infected cats' feces. The infection may also be transmitted congenitally and rarely through blood transfusion and organ transplantation. About 60% of all human infections are asymptomatic. However, serious clinical disease occurs in congenitally infected babies and in post-natally infected immunocompromised individuals (Dubey, 2009a; Becker *et al.*, 2010). In sheep, on the other hand, *T. gondii* continues to be a major cause of economic loss, and is considered the second most important cause of ovine reproductive failure worldwide (Hamidinejat *et al.*, 2008; Dubey, 2009b). In addition to abortion, stillbirth and perinatal mortality, ovine toxoplasmosis may also cause other manifestations including hepatitis and myocarditis (OIE, 2008). Human toxoplasmosis, with or without associated clinical manifestations occurs widely throughout the Kingdom of Saudi Arabia. By contrast, very sparse information is available regarding toxoplasmosis in animals in that country, and that information is based almost exclusively on serological testing for anti-*T. gondii* antibodies in asymptomatic animals of unknown origin and clinical history slaughtered in municipal abattoirs (Sanad and Al-Ghabban, 2007).

Mutton is the preferred meat in Saudi Arabia, and sheep are the most numerous meat producing animals in the country, totaling more than 7.7 million heads. These animals comprise several indigenous breeds of which the most prevalent and most favorite is the fat-tailed *Najdi* sheep. However, the local sheep population provides only part of the annual demand for mutton and lamb, and to fill that gap, more than 5 million sheep are imported annually, especially during pilgrimage to Makkah and other religious occasions. Although most of the indigenous Saudi sheep are still reared under traditional nomadic conditions, a number of modern sheep fattening enterprises have been established around major Saudi cities to provide additional sources of mutton and lamb. In addition to their inherent low prolificacy (Abuheif *et al.*, 2011), one of the most important challenges facing these enterprises is reproductive failure, especially high incidence of abortions and stillbirths. Despite the role of *T. gondii* as a major cause of ovine abortion throughout the world, no attempt has been made to investigate the prevalence of this parasite in sheep under field conditions in Saudi Arabia nor to determine its relevance during ovine abortion outbreaks, most of which are presumed to be caused by brucellosis. In the recent years, there have been several storms of late abortions and stillbirths in commercial farms in Riyadh area in the Central Region of Saudi Arabia in flocks in which serological results, clinical history and placental and fetal changes were consistent with toxoplasmosis, while tests for other possible causes of ovine infectious abortion were negative apart

from a small proportion (<5%) of ewes exhibiting low titers of anti-chlamydial antibodies.

The following study constitutes the first report on serological prevalence, antibody titers and pathology in a flock of commercial sheep in Saudi Arabia during outbreaks of abortion and stillbirths, in which *T. gondii* is suspected to be the primary underlying cause.

MATERIALS AND METHODS

Animals

The sheep in which abortions and stillbirths occurred (Farm A) belonged to the indigenous *Najdi* breed. They were kept under intensive management in units assigned according to their status of production. Feeding consisted of Rhodes grass, alfalfa and concentrate cubes (13% crude protein) offered according to the animal's weight and production needs. Another group of *Najdi* sheep from King Saud University farm (Farm B) with no history of abortion or other reproductive disorders served as controls. These animals were also housed in pens and fed on a diet similar to that described above. All sheep were vaccinated against foot and mouth disease, sheep pox, Pest des petits ruminant, pasteurellosis, enterotoxaemia and brucellosis. They were also given anthelmintic medication and coccidiostats as necessary.

Sampling

Serum samples were collected from a total of 162 aborted ewes and 52 breeding rams in Farm A. The sampled ewes constituted about one third of all the ewes in which abortion or stillbirth has recently occurred. Samples were also obtained from 71 control sheep, comprising 60 ewes and 11 rams (Farm B). For serum separation, 7 mL blood samples were collected by jugular venipuncture from each animal into plain vacutainer tubes (Becton, Dickinson and Co., Franklin Lakes, N.J., USA). The blood was allowed to clot at room temperature for 3 hours and the sera were separated by centrifugation (1,500 g for 15 min) and stored at -20°C until analysis. Five of the aborted fetuses were necropsied on the farm for gross lesions while pieces of their placentae, brains, livers and lungs were fixed in 10% formalin, embedded in paraffin, sectioned and stained with hematoxylin and eosin. Fetal serum was not available for examination.

Serological tests

Tests for *T. gondii* antibodies were performed using an indirect ELISA immunoassay designed to screen ovine toxoplasmosis (Chekit-Toxotest, IDEXX laboratories, Bommeli Diagnostics, AG, Bern, Switzerland). A horseradish peroxidase-labeled monoclonal IgG conjugate was used, and the tests were performed according to manufacturer's procedure. Known positive and negative sheep sera were included in each test plate. Briefly, test and control sera were diluted 1:400 and dispensed in 100 µL quantities into the wells of microtiter plates pre-coated with inactivated *T. gondii* antigen. The plates were gently mixed then incubated for 1 hr at 37°C. 100 µL of the peroxidase-labeled IgG conjugate was

then added into each well and the plates incubated for another hour at 37°C. 100 µL of substrate solution was then added and incubated for 15 min followed by addition of 100 µL of the stopping solution. The tests were performed in duplicates and the optical density was determined at 450 nm using a microtiter plate reader. The percent optical density (%O.D.) was expressed according to the following equation:

$$\%O.D. \text{ of the test sample} = 100 (S - N)/(P - N)$$

where S, N and P are the O.D. values of the test, negative and positive sera, respectively. Samples with O.D. <30% were considered negative. Samples giving O.D. ≥30% were considered positive. The test was validated based on the positive and negative control O.D. values.

The same samples that were tested by ELISA were also tested by indirect hemagglutination (IHA) test (Wampole Laboratories, LLC, Princeton, NJ, USA), using stabilized sheep red blood cells (SRBCs), sensitized with *T. gondii* antigen. The tests were performed in microtiter plates following manufacturer's instructions. Sera containing anti-*T. gondii* antibodies agglutinate SRBCs forming a mat at the bottom of the microtiter well. For detection of non-specific agglutinins, the samples were first tested with unsensitized RBCs and if non-specific agglutinins were detected, they were removed with an absorbent provided with the test kit. Two-fold serial dilutions of the test sera were used for quantitative analysis. Initially, each serum sample was diluted from 1:16 to 1:64. Samples giving titers of 1:64 were considered positive (Nieto and Melendez, 1998) and further diluted through 1:8912. Negative, positive and diluent (buffered saline) controls provided by the manufacturer were included in each test. Sera from the control sheep were similarly tested for toxoplasmosis, using ELISA and IHA tests.

Statistical analysis

Statistical analysis of the results was carried out using SAS 8.1 program for Windows. Chi square test of independence was used to analyze the relation between serological prevalence and the farm and sex of the animals. The relation between ELISA and IHA results was investigated using Spearman's correlation test (Sprinthall, 2006).

RESULTS

Clinical history

Between January and April 2010, ewes in Farm A witnessed successive waves of abortions and stillbirths. Out of 1093 ewes confirmed pregnant by ultrasonography (Ovi-Scan 6; BCF Ultrasound Australasia Pty Ltd, Melbourne, Australia), 336 abortions and 135 stillbirths were recorded. This high incidence (~43%) indicated an infectious cause. Most abortions occurred during late gestation (>4 month old fetuses) in 1-1½ yr old ewes and some of the aborted ewes showed pyrexia, depression and vaginal discharge shortly prior to abortion. Serological tests revealed a high prevalence of antibodies against toxoplasmosis while no other infectious cause of ovine abortion was confirmed by serological

and bacteriological examinations, other than a small proportion (<5%) of ewes in which only low titers of anti-chlamydial antibodies were detected serologically.

Serological results

Of 168 aborted ewes and 52 breeding rams tested for toxoplasmosis in the abortion group (Farm A), 149 (88.7%) ewes and 42 (80.8%) rams were found to be positive for anti-*T. gondii* antibodies by the indirect ELISA test (Table 1). When the same animals were tested with IHA, 155 (92.3%) ewes and 44 (84.6%) rams, including all ELISA-positive animals, were also found to be positive for anti-*T. gondii* antibodies (Table 2). More than 80% of the ELISA-positive ewes had ELISA O.D. exceeding 100% and nearly 25% of those had ELISA O.D. of $\geq 150\%$, including 5 ewes with O.D. exceeding 200%. The IHA results, on the other hand, indicated that more than 75% of the seropositive ewes had antibody titers $\geq 1:1024$, including 48 (30.9%) ewes with IHA titer of 1:4096 and 10 (6.5%) with IHA titer of 1:8192, the highest dilution tested. Of the ELISA positive rams, on the other hand, the highest O.D. was 170%, which was recorded in 1 animal only, while 7 rams (16.7%) had O.D. ranging between 100-150%. All other rams, totaling 34 (81.0%), had ELISA O.D. <100%. Also the IHA antibody titers of more than 80% of the breeding rams ranged between 1:64-1:512, while only 3 rams had titers of 1:4096 and 1 had a titer of 1:8192.

By contrast, only 2 ewes (5%) and 5 rams (36.4%) of the control sheep (Farm B) were ELISA-positive for anti-*T. gondii* antibodies, giving an overall prevalence of less than 10%. The same animals, except 1 ram, were also IHA-positive, with titers of 1:512 and 1:1024 in the ewes and 1:64-1:1024 in the rams.

Statistical analysis showed a highly significant difference ($p < 0.0001$) in serological prevalence in the abortion versus the control group. There was also a significant agreement between ELISA and IHA results ($r = 0.83$; $p \leq 0.005$). On the other hand, no significant difference in prevalence was recorded between female versus male sheep in the abortion group in Farm A, while control males (Farm B) had a significantly higher prevalence than females.

Pathology and histopathology

Some of the aborted fetuses showed blood-stained fluid in their abdominal and thoracic cavities while the liver was congested and the placenta was swollen, reddish and friable and its cotyledons were speckled with whitish foci of necrosis and mineralization. Histopathological examination revealed small areas of necrosis and focal mononuclear cellular infiltration in the liver and lungs, while the placenta showed multiple foci of coagulative necrosis and calcification. Intra-cellular *T. gondii* tachyzoites were demonstrated in placental sections of two animals. Examination of fetal brains revealed no gross pathological lesions; however, histopathological examination showed focal areas of necrosis, perivascular lymphocytic infiltration and focal gliosis.

Table 1. Results of ELISA test for toxoplasmosis in sheep in Riyadh (Saudi Arabia)

Animals	Total tested	No.		No. positive	Positive (O.D.%)					
		negative	positive		30-69	70-100%	101-150%	151-200	<200	
Abortion group (Farm A)										
Ewes	168	19 (11.3)	149 (88.7%)	4 (2.4%)	24 (14.3%)	94 (56.0%)	22 (13.1%)	5 (3.1%)		
Rams	52	10 (19.2%)	42 (88.8%)	13 (25.0%)	21 (40.4%)	7 (13.5%)	1 (1.9%)	-	-	
Total	220	29 (13.2%)	191 (86.8%)	17 (7.7%)	45 (20.4%)	101 (45.9%)	23 (10.5%)	5 (2.3%)		
Control group (Farm B)										
Ewes	60	58 (96.7%)	2 (3.3%)	-	-	-	-	2 (3.4%)		
Rams	11	6 (54.4%)	5 (45.4%)	-	1 (9.1%)	1 (9.1%)	2 (18.2%)	1 (9.1%)		
Total	71	64 (90.1%)	7 (9.9%)	-	1 (1.4%)	1 (1.4%)	2 (2.8%)	3 (4.2%)		

Table 2. Results of indirect Haemagglutination test for toxoplasmosis in sheep in Riyadh (Saudi Arabia)

Animals	Total tested	No. negative	No. positive	Antibody titers							
				1:64	1:128	1:256	1:512	1:1024	1:2048	1:4096	1:8192
Abortion group (Farm A)											
Ewes	168	13 (7.7%)	155 (92.3%)	5 (3.0%)	3 (1.8%)	9 (5.4%)	20 (11.9%)	22 (13.1%)	38 (22.6%)	48 (28.6%)	10 (6.0%)
Rams	52	8 (15.4%)	44 (84.6%)	4 (7.7%)	6 (11.5%)	7 (13.5%)	19 (36.5%)	2 (3.8%)	3 (5.8%)	3 (5.8%)	-
Total	220	21 (9.5%)	199 (90.5%)	9 (4.1%)	9 (4.1%)	16 (7.3%)	39 (17.7%)	24 (10.9%)	41 (18.6%)	51 (23.2%)	10 (4.5%)
Control group (Farm B)											
Ewes	60	58 (96.7%)	2 (3.3%)	-	-	-	1 (1.7%)	1 (1.7%)	-	-	-
Rams	11	7 (63.6%)	4 (36.4%)	1 (9.1%)	-	-	2 (18.2%)	1 (9.1%)	-	-	-
Total	71	65 (91.5%)	6 (8.5%)	1 (1.4%)	-	-	3 (4.2)	2 (2.8%)	-	-	-

DISCUSSION

Little is known about ovine toxoplasmosis in Saudi Arabia. So far only four publications have been made on the subject during the past 25 years. All of these reports comprised preliminary records of serological prevalence in slaughtered sheep of unknown origin and history, and none of them provided evidence of reproductive impairment in the animals. This study is the first report of *T. gondii*-associated abortions in sheep in Saudi Arabia. The diagnosis was based on detecting exceptionally high prevalence (around 85%) and much higher titers of anti-*T. gondii* antibodies in aborted ewes and breeding rams in comparison to controls, in addition to clinical history of the affected flock and the occurrence of typical pathological lesions in the placenta and other fetal tissues. The rate of ELISA seropositivity in these animals was similar to that recorded during outbreaks of *T. gondii* abortions in sheep elsewhere (Hamidinejat *et al.*, 2008). It was also noted that the prevalence of toxoplasmosis by ELISA was similar in ewes and rams in the abortion group. However, the percentage of ewes with 100% O.D. was more than four times higher than that of the breeding rams. Furthermore, much higher prevalence and antibody titers were recorded in ewes than in rams using the IHA test. In control sheep, on the other hand, the overall prevalence of *T. gondii* antibodies was less than 12% with ELISA and 8.5% with IHA tests, and there was a significantly higher prevalence in rams than in ewes. It should be noted, however, that the number of control rams was much smaller than that of control ewes (11 and 60, respectively). Other investigators reported that serological prevalence was similar in both sexes during surveys of sheep toxoplasmosis (Oncel and Vural, 2006) while others found a higher prevalence in ewes as compared to rams (van der Puije *et al.*, 2000).

According to reproduction records in the affected sheep (Farm A), nearly one fifth of the ewes that originally entered mating were found to be empty by repeated ultrasonographic examination. The cause of their failure to conceive was unknown but the possibility that some might have experienced early embryonic deaths and fetal resorption cannot be ruled out. According to Dubey (2009b), *T. gondii* might cause early abortion in some ewes. It was also noted that most of the aborted ewes in the abortion group subsequently conceived and lambed normally, thus confirming the view that ewes usually develop immunity against the parasite which protects them from disease during subsequent pregnancies (Dubey, 2009b).

The present study showed close agreement between ELISA and IHA results. Both tests are easy to perform and relatively inexpensive, and could therefore be used for the screening of *T. gondii* abortion in sheep. However, it is important to support serological diagnosis with other diagnostic means such as histopathology of fetal and placental tissues or isolation of the parasite.

The present results are important not only to sheep producers in Saudi Arabia, but also to feed manufacturers, since feeds (and water) may be contaminated with *T. gondii* oocysts which are shed in a huge number by infected cats. Schares *et al.* (2008) reported that one gram of infected cat's feces may contain up to 13 million oocysts, which is sufficient to infect hundreds of

thousands of animals, causing abortion or stillbirth in most of them. The situation is further complicated by the fact that oocysts can survive in feeds, water and moist soil for extended periods. It should also be borne in mind that while eliminating stray cats and rodents from a farm is an essential part of toxoplasmosis control, it does not completely eliminate the infection since oocysts can be easily introduced by flies, cockroaches and various fomites. Another important consideration is that congenital transmission of toxoplasmosis in sheep is apparently much more prevalent than originally thought. Studies in the U.K. indicated that in some flocks, congenital transmission might occur in more than 60% of all ovine pregnancies, including a significant proportion of lambs which are born live but infected (Duncanson *et al.*, 2001).

In conclusion, *T. gondii* appears to be a major cause of reproductive failure in sheep in Saudi Arabia. In addition to its surveillance in these animals, it is important to investigate its impact on sheep production in that country, and to evaluate the hygienic standards in farms, the factors that increase the risk of transmission and the zoonotic implications of the disease.

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SEROLOŠKA PREVALENCIA *TOXOPLASMA GONDII* I ABORTUSI OVACA U SAUDIJSKOJ ARABIJI

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SADRŽAJ

Svrha ove studije je bila da se ispita serološka prevalenca kao i titar antitela na *Toxoplasma gondii* kod ovaca, praćenjem talasa abortusa i pojave mrtvorodne jagnjadi u jednom komercijalnom stadu u Rijadu, Saudijska Arabija. Indirektnim ELISA testom i indirektnim hemaglutinin testom (IHA) ispitano je 168 uzoraka seruma ovaca sa abortusom i 52 uzoraka seruma rasplodnih ovnova. Metodom slučajnog izbora, iz stada bez pojave abortusa, izdvojeno je 71 grlo (60 ovaca i 11 ovnova) i ova grupa je služila kao kontrolna.

U stadu sa abortusima i pojavom mrtvorodne jagnjadi dokazano je, indirektnim ELISA testom, prisustvo antitela na *T. gondii* kod 149 (88,5%) ovaca i 42 (80,8%) ovna. U istom stadu, 155 ovaca (92,3%) i 44 ovna (84,6%), takođe je ispoljavalo pozitivnu reakciju u indirektnom IHA testu. Više od 80% ELISA-pozitivnih uzoraka imalo je O.D. preko 100%, a skoro 25% je imalo O.D. \geq 150%. Rezultati IHA testa ukazuju da je više od 75% seropozitivnih ovaca imalo titar antitela \geq 1:1024, uključujući 58 (37,4%) ovaca sa IHA titrom u opsegu od 1:4096 i 1:8192.

Kod nekih ovaca, neposredno pre abortusa, registrovane su pireksija, depresija i vaginalne promene. Postmortalnim ispitivanjem 5 abortiranih fetusa otkriven je krvav eksudat u abdominalnoj i torakalnoj šupljini kao i manji inflamatorni i nekrotični fokusi na mozgu, jetri i plućima. Placenta bila crvenkasta i trošna, a njeni kotiledoni su bili tačkasti sa malim beličastim mineralizovanim nekrotičnim fokusima. Tahizoiti *T. gondii* su dokazani u placentama dve ovce.

U stadu bez pojave abortusa samo je 7 (9,9%) jedinki bilo pozitivno na *T. gondii* ELISA testom i 6 (8,5%) indirektnim IHA testom. U većini uzoraka vrednosti titra su bile značajno niže u poređenju sa prvim stadom.