

Original Research Paper

Development of Improved Preventive and Treatment Strategies for Controlling of Toxoplasmosis in Saudi Arabia

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Abstract: Toxoplasmosis causes severe public health problems and has great veterinary and economic importance worldwide. The present study aims mainly to develop an improved preventive and treatment program for controlling toxoplasmosis in sheep and goats. This study was performed on sheep and goats farm containing 357 animals (300 sheep and 57 goats). This farm had a large numbers of barren ewes, history of abortions (below 4 months), stillbirths, malformations and neonatal losses. Blood samples were collected to determine humeral antibody responses and prevalence of toxoplasmosis. Sections of normal and abnormal tissues were fixed using 10% buffered formalin for histopathological examination. Different and new drug regimens and strategies were used for controlling toxoplasmosis. Sixty-four out of 357 sheep and goats were clinically affected with 17.9% overall morbidity rate. These animals showed certain clinical symptoms of toxoplasmosis, such as abortions and stillbirth. The seroprevalence rate was 15.4%. The titers of ELISA were high before treatment in positive cases while after treatment the titers were decreased for certain level that continued to the end of study. The field treatment revealed that the affected sheep and goats treated with camel lactoferrin (cLf) and chemotherapy combination regimen significantly reduced the overall severity of toxoplasmosis with reduction in IgG titers better than those treated with chemotherapy alone (Pyrimethamine and sulfamethazine) or cLf alone. In conclusion, cLf and chemotherapy combination regimen was success in control of toxoplasmosis in small ruminants.

Keywords: Toxoplasmosis, Diagnosis, Sheep and Goats, Camel Lactoferrin, Treatment, Control

Introduction

Toxoplasmosis is a global zoonotic disease caused by the apicomplexan coccidian *Toxoplasma gondii*, an obligatory intracellular protozoan that can infect humans and all warm-blooded animals. This wide-ranging host makes it one of the most successful protozoan parasites (Radostits *et al.*, 2007). In human, it is a dangerous disease in immunocompromised and immunosuppressed patients and congenitally infected children (Tenter *et al.*, 2000; Montoya and Liesenfeld, 2004). Primary infection

in pregnant seronegative women can cause neonatal death, abortion and severe congenital abnormalities, such as hydrocephalus, blindness, chorioretinitis and mental retardation (Petersen *et al.*, 2001). On the other side, toxoplasmosis in animals has great economic importance worldwide because it can cause stillbirth, abortions and neonatal loss especially in sheep and goats (Buxton, 1998; Buxton *et al.*, 2007). Moreover, viable *T. gondii* tissue cysts inside raw or undercooked meat of sheep and goats may be important sources of infection to humans and carnivores (Dubey, 2009).

Although several studies of human toxoplasmosis were carried out in Kingdom of Saudi Arabia (Al-Harathi *et al.*, 2006; Tonkal, 2008; Al-Mohammad *et al.*, 2010; Bin Dajem and Almushait, 2012), a little data is available about the *T. gondii* prevalence in small ruminants under field condition in that country (Sanad and Al-Ghabban, 2007; Al-Mufarrej *et al.*, 2011). Serological diagnosis considers the most commonly approach used to define the stage of infection, whether current, recent, or past (Nagaty *et al.*, 2009).

Despite the seriousness of this disease, there are no effective commercial vaccines to combat it today. Indeed, a live attenuated *Toxoplasma tachyzoites* S48 vaccine is previously available and it can partially (60%) protect pregnant sheep against toxoplasma infection (Buxton *et al.*, 1991), but now it is difficult to manufacture because it has a short shelf life. Interestingly, we succeeded in development of a potent, successful and effective vaccine against toxoplasmosis that provides highly significant protection against congenital and acquired *T. gondii* infection in female OF1 mice (more than 96% protection) (Ismael *et al.*, 2006) and in sheep (90% protection) (Mévélec *et al.*, 2010). This vaccine is currently in the approval period and this work was realized in France. The absence of effective commercial vaccine invites the need to develop an improved preventative and treatment strategies for controlling toxoplasmosis.

Chemotherapy as antibiotics and/or sulphonamides are commonly used for treatment of toxoplasmosis in farm animals and human. In addition to antibiotics residues, the antibiotic therapy has numerous complications as direct toxicity, hypersensitivity, super-infections and antibiotic-induced immunosuppression (Adesiyun *et al.*, 2007). This is highlighting the need for a new strategy for non-antibiotic therapy using novel immunomodulators. We succeed in purification and characterization of camel lactoferrin (cLf) from camel milk whey (Ismael *et al.*, 2013). We proved that cLf is a potent natural antimicrobial and novel immunomodulator agent (Ismael *et al.*, 2013). The present study aimed at, (i) determination of the occurrence of toxoplasmosis in small ruminants in studied farm (ii) make confirmatory diagnosis of toxoplasmosis in abortive small ruminants and (iii) comparison of different and new drug regimens and strategies for controlling toxoplasmosis.

Material and Methods

History of Animals and Location

This study was performed on sheep and goats farm containing 357 animals (300 sheep and 57 goats) and located in Riyadh region, KSA. This farm had a large

numbers of barren ewes, history of abortions (below 4 months), stillbirths, malformations and neonatal losses. The animals had been reared under similar management, feeding systems and environmental conditions.

Clinical Examination

Animals were subjected to general clinical examination including pulse, temperature, respiration, mucous membrane color and others. All managements of the animals and the sample collections were in agreement with the rules for the animal's welfare and those of the concerned ethical authorities.

Samples

Blood Samples

The selected animals were subjected to blood collection via Jugular vein into plain vacutainer tubes. The blood samples were centrifuged at 3000 rpm/15 min for separation the serum that stored at -20°C into 1.5 mL Eppendorf tubes until assay.

Tissue Samples

Sections of normal and abnormal placenta, uterus, liver, fetus, lung and trachea tissues were collected just after abortion or death and fixed in 10% formalin buffer for histopathological examination.

Diagnosis of Toxoplasmosis

Ultrasonography

The animals were subjected to full ultrasonography reproductive examination using multi-frequency linear trans-rectal probe (Prosound 2, Model UST-660-7.5, Aloka, Japan).

Measurement of Humeral Antibody Responses to Natural Infection by Enzyme-Linked Immunosorbent Assay (ELISA)

The antigen-specific IgG antibodies levels in serum samples were identified pre and post-treatment as previously described (Ismael *et al.*, 2003). The TAg of *T. gondii* at 10 µg mL⁻¹ was used to coat micro titer plates. The antigen-specific antibody titer is given as the reciprocal of the highest dilution producing an absorbance (OD) that was 2.5-fold greater than that of control serum at the same dilution. Results were expressed as the means of titers ± Standard Deviations (SD).

Histopathological Examinations

A gross necropsy was performed on the placenta and aborted fetus. The fixed tissues were embedded in paraffin then sectioned by microtome. The resulted sections were stained with Hematoxylin-Eosin (HE) stain.

Field Treatment and Synergy Trials

The affected sheep and goats were divided into 7 groups based on the clinical manifestations, 4 groups of 10 sheep and 3 groups of 8 goats (Table 3 and 4). Group I received no treatment and used as a control group. Group II was intramuscularly injected with Pyrimethamine (100 mg/kg/3 days, then 50 mg) + sulfamethazine or sulfadiazine (73 mg/kg for 3 days then 100 mg) (Radostits *et al.*, 2007). Group III was treated with cLf (7%, 10 mL/50 kg), per Os, for 3-5 days of three periods with an interval of 5 days. Group IV received Pyrimethamine, sulfadiazine and cLf combinations regimen. The infected goats received the same therapeutic regimen of groups I, III and IV in sheep. All treated sheep and goats were examined daily for monitoring any clinical improvement. Sera samples were collected several times pre-, during and post-treatment for serological examination. The total treatment period was 25 days. The predisposing factors were monitored and controlled.

Statistical Analysis

The Statistical Products and Service Solutions program was used for all analysis. Comparisons among groups were tested using the χ^2 (Chi-square) test. A difference was considered to be a statistically significant at $p < 0.05$.

Results

Clinical Findings and Ultrasonography

Clinical summary of the animals before and after treatment was illustrated in Table 1. Clinical reproductive examination of barren ewes revealed normal genital tract and no any abnormalities or pathology in reproductive tract (Fig. 1). Additionally, 15% of animals diagnosed as pregnant at day 23 post insemination (Fig. 2) was diagnosed non-pregnant (aborted) at day 60 post insemination. Sixty-four out of 357 sheep and goats were clinically affected (Table 1) with 17.9% overall morbidity rate. These animals showed certain clinical symptoms of toxoplasmosis, such as abortions in different stages especially in mid-gestation ($n = 35$) (Fig. 3), stillbirth ($n = 9$) (Fig. 4), malformation ($n = 2$) (Fig. 5) and neonatal loss. In addition, fever and other systemic signs were observed on 7 out of 64 clinically affected animals (10.9%). Two cases (goats) out of 64 were died with 3.13% case fatality rate. The post-mortem findings in dead animals revealed septicemia (Fig. 6). A gross necropsy was performed on the aborted fetus and placenta. The abdominal cavity was usually filled with fluid tinged with blood. The placenta was purple-gray in color and the cotyledons were pale tan and friable, with a gritty texture as though mineralized.

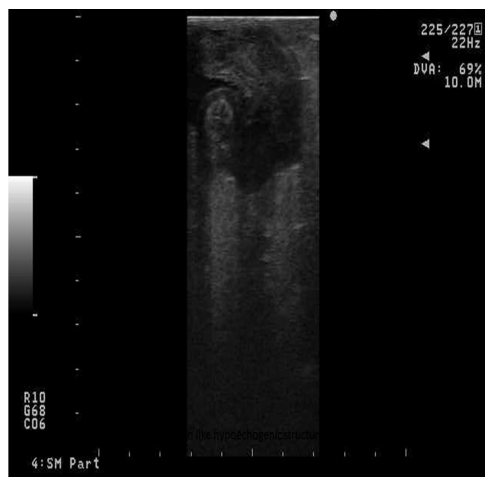


Fig. 1. Non pregnant uterus appeared horn like hypoechoic structure using transrectal probe



Fig. 2. Pregnant uterus had fetus which appeared as as hyperechogenic structure surrounded by fetal membrane and fetal fluid that appeared as anechoic structure using transrectal probe in pregnant goat at 23 day



Fig. 3. Abortion due to toxoplasmosis in sheep

Results of ELISA in Naturally Infected Animals by Toxoplasmosis Pre- and Post-Treatment

Analysis of serum antibody responses in naturally infected sheep and goats elicits serum IgG responses (Table 2). The IgG responses varied from strong positives, weak positive, suspected cases to negatives cases (Table 2). The seroprevalence rate was 15.4%. The titers of ELISA were high before treatment in positive cases while after treatment the titers were decreased for certain level (weak) that continued to the end of study (Table 3 and 4).

Histopathological Results

Large numbers of small basophilic to amphophilic bradyzoites were detected in tissue cyst in the heart without prominent inflammatory reaction (Fig. 7 and 8). Microscopic examination of aborted fetus tissues and placenta indicated sign of necrotic placentitis. Diffuse edema with a population of mononuclear and segmented leukocytes was detected throughout the amnion of the placenta. Necrosis along the chorion and basophilic granular debris, supposed to be mineralization. Histopathological examination of the cotyledon revealed multifocal, mineralized and necrotic lesions.



Fig. 4. Stillbirth due to toxoplasmosis in sheep

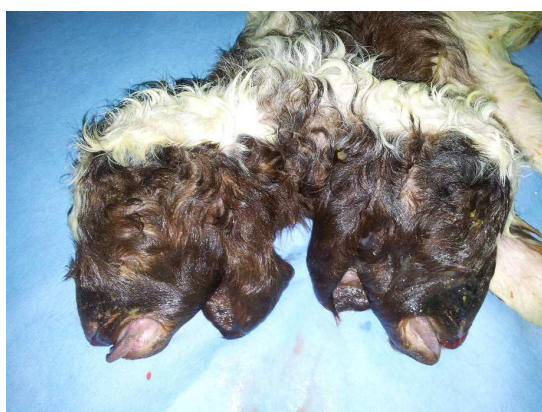


Fig. 5. Malformation due to toxoplasmosis in sheep

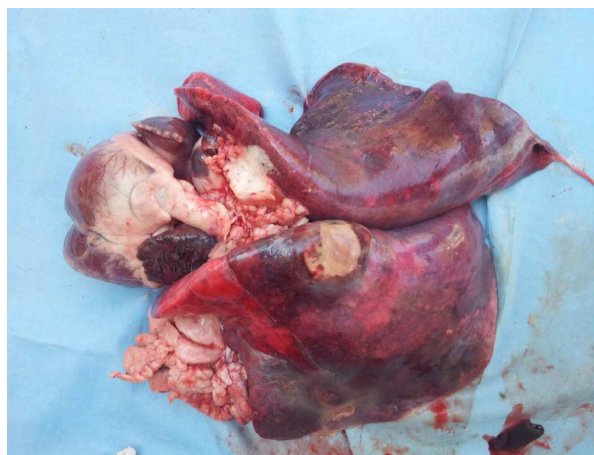


Fig. 6. Septicemia in post-mortum findings in sheep infected with *Toxoplasma gondii*

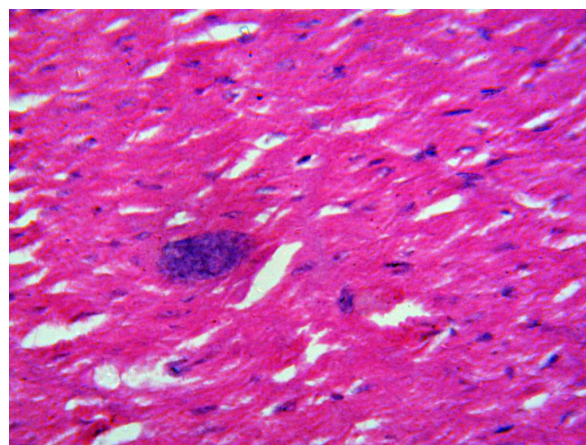


Fig. 7. *Toxoplasma gondii* cyst among cardiac myocytes with no significant inflammation associated with the infected cells. HE X300

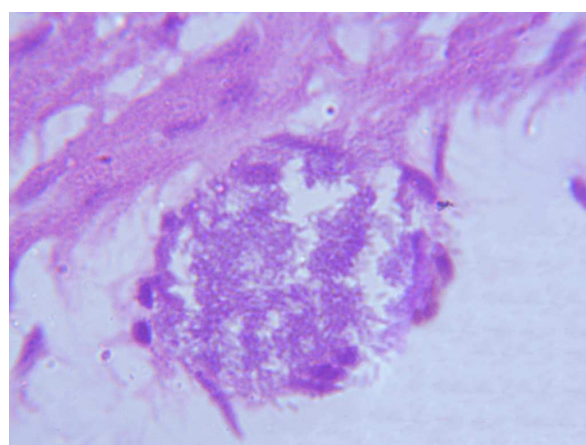


Fig. 8. *Toxoplasma gondii* cyst with greater detail of the small basophilic to amphophilic bradyzoites present in the cyst among myocytes. HE X3000

Table 1. Clinical summary of the infected animals pre- and post treatment

Parameter	Pre-treatment		Post-treatment	
	Sheep (n = 40)	Goats (n = 24)	Sheep (n = 40)	Goats (n = 24)
Temperatures ^a	40±1	41±1	39±1	40±1
Ruminations ^b	3±0	3±1	2±0	2±1
Pulse ^c	98±3	105±5	76±5	88±5
Respiration ^d	26±3	39±4	16±3	27±5
Mucus membrane	Pale	Icterus	Normal	Normal

^a: Rectal temperatures in °C; ^b: Ruminations per minute; ^c: Pulse in beats per minute; ^d: Respiration in breaths per minute; Results are expressed in mean ± SD (Standard deviations).

Table 2. Results of ELISA in naturally infected sheep and goats by toxoplasmosis before treatment

Item	Total cases	Strong positive	Weak positive	Suspect cases	Negative cases
Number	357 ^a	46 ^b	6 ^c	3 ^d	302
%	100	12.88	1.68	0.84	84.6
Means± SD	-	200±23	80±7	20±2	10±3

^a: 300 (84%) sheep and 57 (16%) goats; ^b: 31 (67.4%) sheep and 15 (32.6%) goats; ^c: 5 (83.3%) sheep and 1 (16.7%) goat; ^d: 3 (100%) sheep; Results are expressed as the means of titers ± standard deviations (SD).

Table 3. Results of field treatment of toxoplasmosis in sheep

Group	No.	Therapeutic regimen	Clinical improvement	ELISA results	
				Pre ^c	Post ^c
Group I	10	No treatment	0/10 (0%) ^a	St + ve	St + ve
Group II	10	Pyrimethamine (100 mg/kg/3 days, then 50 mg) + sulfamethazine or sulfadiazine (73 mg/kg for 3 days then 100 mg)	8/10 (80%) ^b	St + ve	W + ve
Group III	10	cLf alone (7%, 10 ml/50 kg)	7/10 (70%) ^b	St + ve	W + ve
Group IV	10	cLf + Pyrimethamine and sulfadiazine	10/10 (100%) ^b	St + ve	W + ve

^a: Significant difference with ^b (p<0.001) in the same column and non significant to that marked with ^a. cLf: Camel lactoferrin. ^c: Pre and post- treatment. St + ve: Strong positive, W + ve: Weak positive.

Table 4. Results of field treatment of toxoplasmosis in goats

Group	No.	Therapeutic regimen	Clinical improvement	ELISA results	
				Pre ^c	Post ^c
Group I	8	No treatment	0/8 (0%) ^{a*}	St + ve	St + ve
Group II	8	cLf alone (7%, 10 ml/50 kg)	7/8 (87.5%) ^b	St + ve	W + ve
Group III	8	cLf + Pyrimethamine and sulfadiazine	8/8 (100%) ^b	St + ve	W + ve

^a: Significant difference with ^b (p<0.001) in the same column and non significant to that marked with ^a. cLf: Camel lactoferrin. ^c: Pre and post- treatment. St +ve: Strong positive, W +ve: Weak positive. * Two cases were died.

Field Treatment

It revealed that the affected sheep and goats treated with cLf and chemotherapy combination regimen significantly reduced the overall severity of toxoplasmosis with reduction in IgG titers better than those treated with chemotherapy alone (Pyrimethamine and sulfamethazine) or cLf alone (Table 3 and 4). The cLf alone was more effective in goats than in sheep (Table 3 and 4).

Discussion

This is the first study reporting the role of camel lactoferrin (cLf) in treatment of ovine and caprine toxoplasmosis in veterinary field. In the present study, the clinical picture of toxoplasmosis was appeared in 64 animals with 17.9% overall morbidity rate and 3.13% case

fatality. Approximately similar results were described by Radostits *et al.* (2007). Abortions, stillbirth, malformation, neonatal loss and fever were observed in infected animals that indicated the appearance of acute phase of infection. Moreover, the systemic disease, with a slightly high case fatality rate occur in young goats (Radostits *et al.*, 2007). Severity of infection is associated with the stage of gestation at which the animals become infected, the earlier in gestation, the more severity of infection (Dubey, 2009). Fetal death and resorption can be resulted from early infection while infection in the latter part of gestation may have no clinical effect with the lambs being born normal but infected and immune (Radostits *et al.*, 2007).

The toxoplasmic infection was confirmed by macroscopic and microscopic pathological lesions. Although DNA detection and serology are useful tools, histopathology is important to establish a cause-effect

association, because *T. gondii* can be passively transmitted transplacentally and fetuses can die of other causes (Dubey, 2009).

The seroprevalence rate was low (15.4%) in our study, as detected by ELISA, that might be attributed to the presence of toxoplasmosis in this region from long time. The furthestmost seroprevalence in sheep were found in Brazil (29.41%) (Clementino *et al.*, 2007) and southern Italy (49.9%) (Vesco *et al.*, 2007). The higher seroprevalence in southern Italy might be attributed to farm size and the presence of cats on the farm (Vesco *et al.*, 2007). Various ELISA methods using recombinant, fractionated or crude antigens have been used to detect *T. gondii* antibodies in ovine sera that gave higher specificity in comparison with other serological tests (Dubey, 2009).

The field treatment revealed that the affected sheep and goats treated with cLf and chemotherapy combination regimen significantly reduced the overall severity of toxoplasmosis better than those treated with chemotherapy alone (Pyrimethamine and sulfamethazine or cLf alone. Moreover, the cLf alone may offer a good alternative treatment. The selection of treated animals was based on clinical findings because the animals in early acute infection showed clinical signs without appearance of antibody titers in serum. We succeed in purification and characterization of camel lactoferrin (cLf) from camel milk whey (Ismael *et al.* 2013). We proved that cLf is a novel immunomodulator and potent natural antimicrobial agent (Ismael *et al.* 2013). The role of Lf in sequestering iron from bacterial pathogens is one of the first antimicrobial properties discovered for Lf as in case of *S. aureus* which is known to be resistant to antimicrobials (Bhimani *et al.*, 1999). This was believed to be the important antimicrobial action of Lf because apo-lactoferrin possessed antibacterial activity (Yamauchi *et al.*, 1993). Spatial orientation and structural characteristics of the molecule are life-threatening factors in the functionality of an antimicrobial compound. Occurrence in several conditions strongly highlights the significance of the structure-function relationship in the multifunctionality of the Lf (Farnaud and Evans, 2005). It was later established that Lf's antimicrobial function has been attributed to an iron-independent mechanism and direct interaction with microbial surfaces (Farnaud and Evans, 2005; Valenti and Antonini, 2005). The cLf increased lymphocyte transformations and capacity to stimulate the proliferation of allogeneic lymphocyte. Moreover, it increased the production of proinflammatory cytokines (Zimecki *et al.*, 2001; Ismael *et al.*, 2013).

Conclusion

The results of current study record that cLf has a potent natural anti-*T. gondii* effect. This is the first study reporting the role cLf in treatment of ovine and caprine toxoplasmosis in veterinary field. This study

recommended using the cLf in the treatment of various infectious diseases in humans and animals. Nevertheless, a lot of effort is required to obtain a better understanding of cLf activities.

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Author's Contributions

Alaa B. Ismael: Contributed to conception and design, clinical diagnosis of infected animals, carrying out the field treatment, draft and critically revised manuscript with its submission.

Ayman A. Swelum: Contributed to acquisition, carrying out ultrasonography and ELISA, interpretation, data analysis and manuscript revision.

Mohammed A. Nassan: Performed the experiments of histopathological examinations and manuscript revision.

Conflicts of Interest

The authors declared no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

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