Frequency of *Toxoplasma gondii* infection in sheep from a tropical zone of Mexico and temporal analysis of the humoral response changes

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SUMMARY

An indirect ELISA and an immunoblot were standardized to detect anti-*Toxoplasma gondii* antibodies in sheep, and were compared with a commercial ELISA as reference. Sensitivity was 92·0 and 96·0%, and specificity 88·0 and 75·0%, respectively. Then the serum samples of 103 sheep on a ranch located in the Eastern region of Mexico were analysed. A frequency ranging from 77 to 84% was observed, with a heterogeneous pattern among the animals by immunoblotting. Ten months later 56 sheep were sampled and tested again. Six animals became negative while 1 case was negative the first time and strongly positive 10 months later. Considering this to be a new case we calculated an incidence rate of 2·1% ($CI_{95\%}$ 0·6–4·8%). IgG avidity ELISA was performed on 36 positive samples, 33 being of high-avidity at both times; slight increases in 2 samples and conservation of low-avidity in 1 sheep were also observed. Higher prevalence rates of toxoplasmosis in a moist warm compared to a cold atmosphere was attributed to the long viability of *T. gondii* oocysts. This may explain the high frequency of *T. gondii* in this region, which apparently has favourable climatic conditions for the transmission of this protozoan, besides the presence of both domestic and wild cats.

Key words: frequency, immunoassay, Mexico, sheep, *Toxoplasma gondii*.

INTRODUCTION

Toxoplasma gondii is an obligate intracellular parasite distributed worldwide. It infects many warm-blooded animal species, including sheep, with its definitive host being Felidae family members (Dubey, 2004). Toxoplasmosis transmission occurs by ingestion of sporulated oocysts released with the faeces of infected cats and other felids or of cysts present in the tissues of an infected animal. This infection can cause severe life-threatening disease in immunocompromised patients and in newborns with congenital toxoplasmosis (Montoya and Liesenfeld, 2004). Besides, *T. gondii* infection is important in veterinary medicine due to fetal absortion, abortions and neonatal losses of livestock, especially in sheep; thus, it was recognized as an economic problem a long time ago (Hartley and Marshall, 1957; Dubey and Welcome, 1988; Buxton, 1990). The most likely source of infection for sheep kept on pasture are the oocysts shed by cats in their faeces; thus, these animals can also be sentinels for transmission studies (Blewett and Watson, 1983).

The prevalence of *T. gondii* in sheep varies considerably in different parts of the world, from 15% to 85%, depending on raising conditions and weather (van der Puije *et al*. 2000; Figliuolo *et al*. 2004; Hove *et al*. 2005; Sawadogo *et al*. 2005; Sevgili *et al*. 2005; Dumètre *et al*. 2006; Gaffuri *et al*. 2006; Klun *et al*. 2006; Sharif *et al*. 2007; Clementino *et al*. 2007; Romanelli *et al*. 2007; Vesco *et al*. 2007). In Mexico, two studies performed during the 1990's revealed a prevalence of 20% in Guanajuato, 39% in San Luis Potosí and from 38–55% in Morelos, as determined by the indirect fluorescent antibody test (IFAT) (García Vázquez *et al*. 1990; Cruz-Vazquez *et al*. 1992). These values were geographically related to the frequency of infection in humans around those years (Velasco-Castrejón *et al*. 1992). Besides these reports, nothing is known about the presence of *T. gondii* in other regions of Mexico. Each year, more than 40 000 tons of ovine meat are produced in the States of the central part of Mexico (De Lucas-Tron and Abiza Aguirre, 2005; www.sagarpa.gob.mx); thus, ovine toxoplasmosis is probably causing economical losses of high impact, besides the risk for

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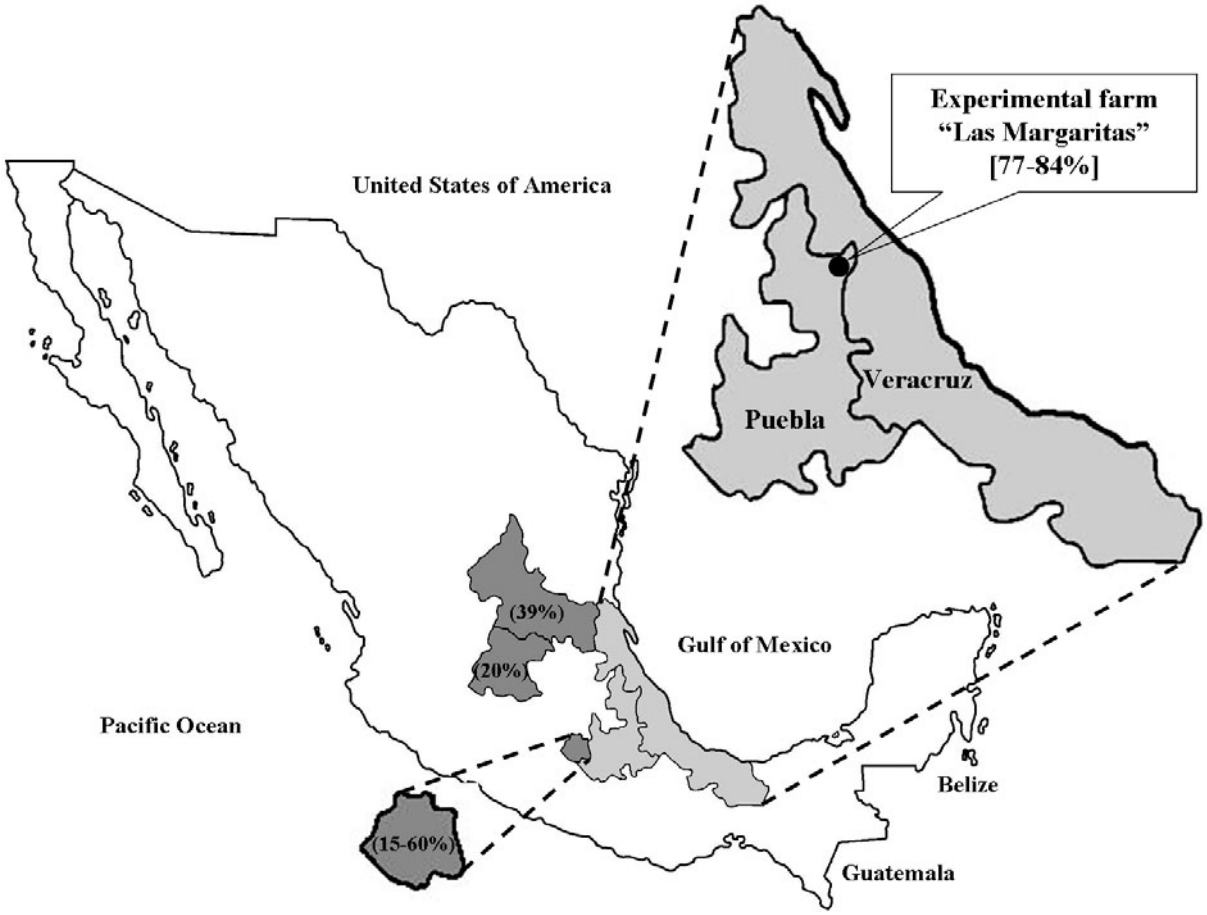

Fig. 1. Map of Mexico showing the areas where *Toxoplasma gondii* antibodies have been searched for in sheep, including the present study. Numbers in brackets and parentheses represent frequency and prevalence values, respectively. Data from other studies were taken from García-Vázquez *et al*. (1990) and Cruz-Vazquez *et al*. (1992).

humans who eat this type of meat. Therefore, the aim of this study was (i) to determine the frequency of anti-*T. gondii* seroantibodies in sheep fed with pasture and living in a high risk zone of Central-Eastern Mexico, and (ii) to analyse changes occurring both in this frequency as well as in the response pattern and avidity after 10 months.

MATERIALS AND METHODS

Sheep

The experimental farm 'Las Margaritas' of the National Institute of Forest, Agriculture and Animal Research (INIFAP) is in the borderline between the States of Puebla and Veracruz (Fig. 1). In total, 103 sheep between 1 and 4 years of age were included in the study, from a total population of 180 present in the farm in November 2005. Fifty-six of these animals, still living in the ranch after 10 months were sampled again (September 2006). Five ml samples of peripheral blood were taken from the jugular vein. After clotting, the serum was separated by centrifugation at 2000 ***g*** for 10 min, aliquoted and stored at −20 °C until use.

Immunoassays

In order to detect anti-*T. gondii* IgG antibodies in sheep, a home-made ELISA (hmELISA) and an immunoblot were developed and evaluated. A commercial ELISA kit (*IP* kit) was used as reference test (*Institut Pourquier*®*, France*, version P00710/03).

hmELISA

A crude extract antigen was prepared with tachyzoites of the RH strain as described by Figueroa-Castillo *et al*. (2006). Polystyrene ELISA plates (Maxisorp, Nunc) were incubated with 2 μg/ml of antigen diluted in 15 mM carbonate buffer, pH 9·6, overnight at 4 °C. Non-specific binding sites were blocked with 200 μl/well of 0·01 M phosphate-buffered 0·15 M NaCl, pH 7·2 (PBS) containing 0·05% Tween 20 (PBS-T) and 1% of bovine serum albumin (Euro-Clone, Italy). Plates were washed thrice with PBS-T, 5 min each time. Immediately, 100 μl/well of the serum samples (diluted 1 : 1000 in PBS-T) were incubated for 2 h at 37 °C. The plates were washed as above, and incubated with 100 μl/well of a donkey anti-sheep IgG peroxidase conjugate

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(Sigma-Aldrich Corp., St Louis, MO, USA, product A3415) diluted 1 : 10 000 in PBS-T overnight at 4 °C. After further washing, the reaction was revealed by addition of 100 µl/well of the substrate/chromogen solution (5 ml of 0·1 M citric acid plus 5 ml of 0·1 M sodium citrate, added with 5 mg *O*-phenylenediamine (Sigma-Aldrich) and 4·5 µl of 30% hydrogen peroxide). After 10–20 min incubation in the dark at room temperature, the reaction was stopped by addition of 50 µl/well of 1 N sulphuric acid. The absorbance values were read at 492 nm on a *Wallac Victor² 1420* micro-ELISA autoreader (Wallac Oy, Turku, Finland). The cut-off was set as the mean plus 3 times the standard deviation of the absorbance obtained with 11 negative samples, which were tested by the 3 methods. The absorbance average of each serum was divided by the cut-off to establish the reactivity index (RI). Serum samples with RI $\geqslant$1 were considered positive, including undetermined samples (RI from 1·0 to 1·1).

Immunoblot

Immunoblot (IB) was adapted for sheep, from a technique previously performed for human serum (Vela-Amieva *et al*. 2005). Briefly, 1×10^7 tachyzoites were electrophoresed in SDS-PAGE under reducing conditions and electroblotted onto nitrocellulose membranes (Hybond-C pure, Amersham Pharmacia Biotech), which were blocked with 2% skim milk in PBS for 1 h and washed 3 times with PBS-T. The nitrocellulose strips were incubated overnight at 4 °C with serum samples diluted 1 : 1200 in PBS-T and, after washing, developed by incubation with the donkey anti-sheep IgG peroxidase conjugate diluted 1 : 2000 in PBS-T. The immunocomplexes were detected using 60 mg of 4-chloro-1-naphthol (Sigma-Aldrich) in 10 ml of methanol added to 10 ml of PBS plus 100 µl of 30% H_2O_2. Samples were considered positive when at least 3 bands were detected in the nitrocellulose strip.

IgG avidity

In order to distinguish recent (acute) from long-standing (chronic) infections an IgG avidity ELISA was employed. The detailed avidity ELISA procedure followed was described by Suárez-Aranda *et al*. (2000). Briefly, polystyrene plates were sensitized and washed as described for the conventional ELISA. Sera were serially diluted 2-fold, starting at 1 : 400, and loaded into plate wells. After 1 h of incubation, plates were washed 3 times with PBS-T, and incubated for 30 min at 37 °C with PBS-T alone or with PBS-T containing 6 M urea (Promega Corp., Madison, WI, USA). After 2 washes with PBS, the plates were incubated with 100 µl/well of a donkey anti-sheep IgG peroxidase conjugate (Sigma) diluted 1 : 10 000 in PBS-T overnight at 4 °C. The reaction was revealed and stopped as described in the ELISA section. IgG avidity index was determined as the absorbance fraction (percent) resistant to urea. Avidity values $\geqslant$0·5 ($\geqslant$50%) indicated chronic infection, whereas values $<$0·5 suggested a more recent infection.

Table 1. Diagnostic performance of the two tests used and concordance versus reference test

	Kit*	
	vs Home	*vs* IB
Number of samples	103	70
Observed proportion of agreement (%)	91·3	91·4
Expected proportion of agreement	0·69	0·66
Kappa	0·72	0·75
Diagnostic sensitivity	0·92	0·96
Diagnostic specificity	0·88	0·75
Apparent prevalence (%)	78·8	80·0
Corrected estimate of the true prevalence (%)	83·7	77·1
Positive predictive value	0·98	0·93
Negative predictive value	0·68	0·86

* Kit, Kit *Institut Pourquier*®; Home, home-made ELISA; IB, immunoblot.

Statistical analysis

All data were analysed using the SPSS 12.0 software (SPSS Inc., Chicago, IL, USA). The correlation between hmELISA and *IP* kit, as well as between values of the first versus the second sample, was determined by linear regression analysis, with 95% confidence limits. The frequency of *T. gondii* antibodies was calculated and positive serum samples were confirmed by IB. The diagnostic parameters (sensitivity, specificity and positive and negative predictive values) were calculated taking the *IP* kit as the reference test with the formula described by Conraths and Schares (2006).

RESULTS

In order to use the hmELISA and IB in this and future studies, their diagnostic performances were compared with that of the *IP* kit (Table 1). More than 91% of the samples had the same result by the two methods compared with the *IP* kit. The concordance was similar for both immunoassays (kappa = 0·72 and 0·75, respectively). The highest sensitivity was obtained with IB, while specificity was maximal using hmELISA (Table 1). The correlation between absorbance values of hmELISA and *IP* kit was 0·87, $P<0·0001$.

Fifty-six of the original sheep were sampled 10 months later and then tested again. The correlations between the absorbance values of the first and second

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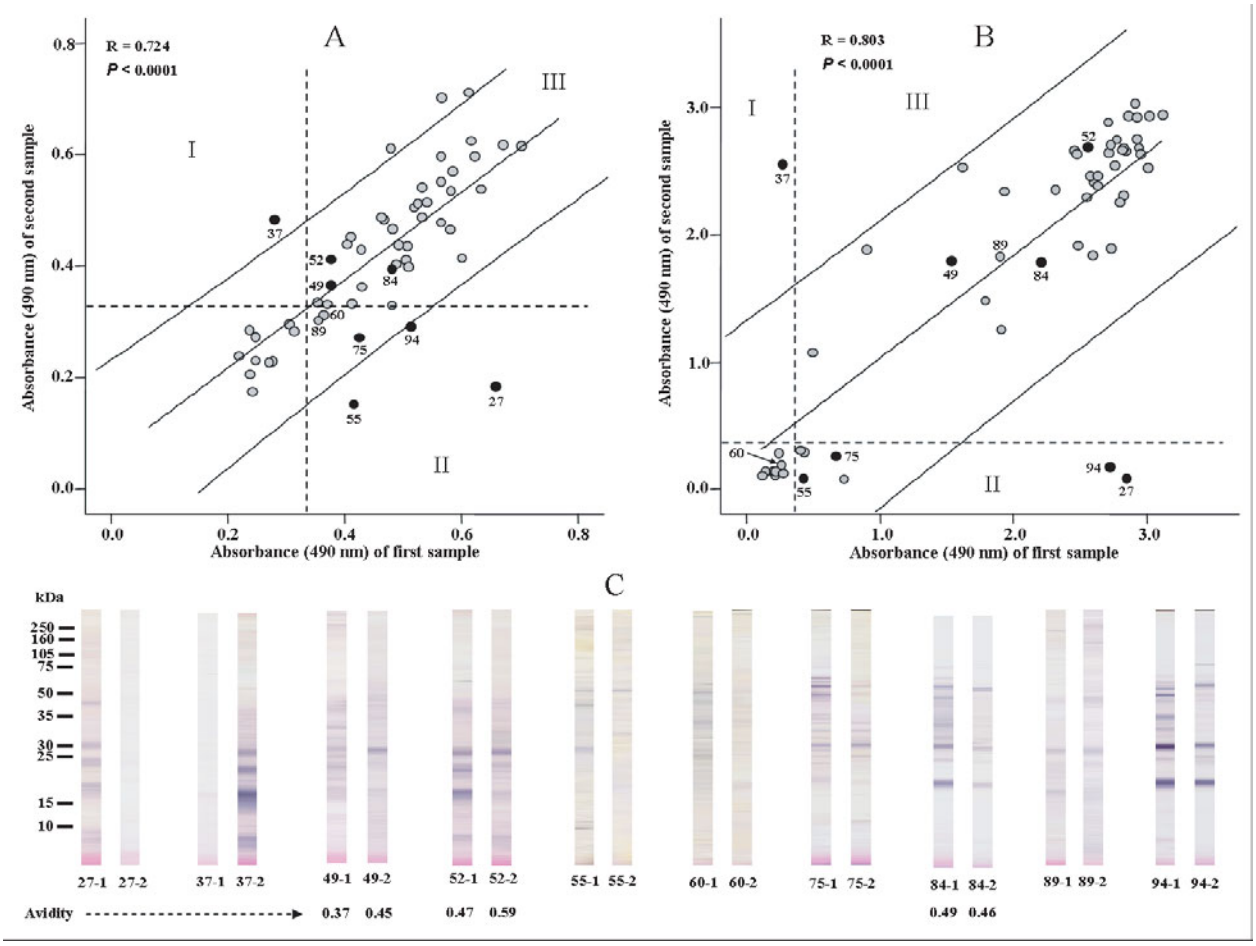

Fig. 2. Correlation of the ELISA absorbance values of anti-*Toxoplasma gondii* between first and second samples using home-made (A) and commercial (B) ELISAs and comparison of some cases in immunoblot (C). Dashed lines represent cut-off values for the first and second sample. Strips without avidity data presented high values (>0·5). R = Pearson correlation.

tests for each animal are shown in Fig. 2. They were high (0·7 and 0·8), although there were some exceptions: using both hmELISA and *IP* kit 4 animals became negative in the second sample (Fig. 2A and B, regions II) and 1 case was negative the first time and strongly positive 10 months later (Fig. 2A and B, regions I). In order to further analyse these changes, comparisons by immunoblot were also performed (Fig. 2C). The first observation was a heterogeneous antigenic pattern among animals even in the first sample, with at least 8 antigen bands detected in positive serum samples ranging from 10 to 250 kDa. A seroconversion was confirmed for sample 37 in region I; also, the sera from regions II in Fig. 2A and B presented immuno-reactivity in IB.

Thirty-six sera were positive on both occasions, so IgG avidity was tested in these pairs. Thirty-three sheep (91·7%) presented antibodies of high avidity in both samples, while slight increases in avidity were found in 2 (samples 49 and 52) and conservation of low avidity was observed in sheep 84 (Fig. 2C).

The frequency of anti-*T. gondii* antibodies ranged from 77 to 84% depending on the technique employed. In any case, the frequency found was much higher than the prevalence reported for other regions of the country, where sheep toxoplasmosis has been investigated (Fig. 1).

DISCUSSION

Herbivorous animals may be used as sentinels to determine the burden of soil- or water-transmitted infectious agents. *Toxoplasma gondii* oocysts may live for months in humid/warm weather conditions. In this study we had two main objectives, i.e. to standardize and evaluate an indirect ELISA and immunoblot for diagnosis of *T. gondii* infection in sheep, and to test them under field conditions, and by these means evaluate *T. gondii* infection frequency in a region of Mexico with presumed high transmission risk.

Previous studies have demonstrated that ELISA is a sensitive technique to diagnose this protozoan infection in sheep (van der Puije *et al.* 2000; Sawadogo *et al.* 2005). Although *T. gondii* is closely related to *Sarcocystis* species and other apicomplexans, cross-reactivity has not been considered a major issue (Ortega-Mora *et al.* 1992; Paré *et al.* 1995; Dubey

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and Lindsay, 2006). The exception is *Neospora* sp. (Harkins *et al*. 1998), but this parasitosis is considerably less frequent than toxoplasmosis in sheep (Figliuolo *et al*. 2004; Masala *et al*. 2007; Romanelli *et al*. 2007). Thus, the frequency of seropositivity in the present study is most probably due to *T. gondii* infection. Besides, the techniques standardized presented adequate concordance between them and with the *IP* kit, supporting true diagnosis in most animals.

Immunoblot has been applied to follow the immune response after experimental infection; a heterogeneous pattern with predominance of a ~30 kDa band has been shown (Wastling *et al*. 1994, 1995; Harkins *et al*. 1998). To our knowledge there are no reports concerning *T. gondii* antibody searches in naturally infected sheep by immunoblot, and therefore our study was important since it gave interesting qualitative information. A quite heterogenic pattern was observed among animals, which can be explained by some phenomena, one of them being variability in infection time. In this regard, the animals studied were 1–4 years old and the results on avidity changes supported chronic infections in most of them. Only 3 sheep had evidence of recent infection by either antibody maturation (2) or seroconversion (1), and 4 seropositive sheep seemed to become negative 10 months later, both in hmELISA and IB. It is universally believed that an individual infected by *T. gondii* will harbour tissue cysts for several years or even life-long (Tenter *et al*. 2000). However, it is possible that these sheep were able to eliminate the *T. gondii* infection, depending on the type or number of parasites that infected them; the ultimate destiny of the tissue cyst is not completely known (Dubey, 2004). Another possible explanation could be an apparent disappearance of the antibody followed by later reappearance as occurs in *Neospora caninum* infection in pregnant cows (Cox *et al*. 1998). The heterogeneity of the IB patterns could also be due to variability in host immune responses or parasite genetic types. Since domestic and wild felines (margay – *Leopardus wiedii*-, ocelot – *Leopardus pardalis*-, bobcat – *Lynx rufus*- and probably also a melanistic jaguar – *Phantera onca*-) live in this zone, the later possibility would be interesting to analyse.

In the present study, a minimum 77% frequency of *T. gondii* infection in adult sheep was found. A broad range of prevalence values have been found in various countries, from 24·5% in Iran to 84·5% in Serbia (Hashemi-Fesharki, 1996; Klun *et al*. 2006). Higher rates of anti-*T. gondii* antibodies in warm/moist compared to cold/dry areas is attributed to the long viability of *T. gondii* oocysts in the former (Fleck, 1972; Fayer, 1981). This may explain why transmission of the parasite is occurring frequently in the region studied, because it presents favourable climatic conditions, and sheep are probably at high risk of exposure to *T. gondii* oocysts released by wild and domestic felines onto the pasture they are fed on. In one animal seroconversion was observed. Considering this a true case, a preliminary incidence of 2·1% ($CI_{95\%}$ 0·6–4·8%) per year was calculated, supporting a high transmission rate in this zone. Sheep meat is considered an important source of *T. gondii* infection and is among the main risk factors during pregnancy in various European countries (Tenter *et al*. 2000; Dumètre *et al*. 2006). Accordingly, the region of the present study is among those with the highest seroprevalence found for humans several years ago (Velasco-Castrejón *et al*. 1992).

In conclusion, at least 77% of the sheep from this region were seropositive against *T. gondii*. Due to economic losses and the risk for transmission to humans, these results represent valuable information to map the *T. gondii* burden in different parts of Mexico.

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