Research Article

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Estimation of *Toxoplasma gondii* seroprevalence in sheep in Sidi Bel Abbes, Algeria

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Abstract: Sheep are considered as an important reservoir of human *Toxoplasma gondii* infections, although more recent data on the prevalence of *T. gondii* in sheep in Algeria are lacking. This study aimed to investigate the seroprevalence of *T. gondii* in sheep to obtain a better insight into the importance of sheep as reservoirs of human infection. A commercial ELISA kit, which detects antibodies against *T. gondii*, was utilized to test 269 sera collected from yearling sheep in the municipal slaughterhouse of Sidi-Bel-Abbes city between October 2020 and February 2021. Simultaneously, we assessed the cut-off as recommended by the manufacturer (S/P>50%) and the bootstrap statistical model to estimate the optimal cut-off value (OD = 0.12). The overall seroprevalence was estimated at 34.2% (92/269) using the ELISA kit cut-off and 50.5% (136/269) using the bootstrapped cut-off, being significantly higher compared to sheep from other regions in Algeria. In conclusion, the seroprevalence of *T. gondii* in sheep was elevated, constituting, therefore, a major public health concern, as sheep meat could be a significant source of *T. gondii* infection for human consumers. Further studies are required to estimate the impact of abortion among livestock animals caused by *T. gondii* infection such as sheep, where it could lead to considerable economic losses.

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1 Introduction

Toxoplasmosis represents one of the most widespread zoonoses caused by *T. gondii* parasite, an intracellular protozoan with a worldwide distribution [1]. This disease infects all warm-blooded animals, including humans. Transmission to humans may occur vertically (during pregnancy) or by ingestion of bradyzoites tissue cysts present in undercooked meat or via the ingestion of food and water contaminated with oocysts shed into the environment through cats.

In humans, infections are mainly asymptomatic [2]. This infection ranges in severity from asymptomatic to lethal. The most frequent clinical form of toxoplasmosis is congenital form once primary acquired infection occurs during pregnancy. Moreover, this infection has severe consequences in immunocompromised individuals [3] as it was reported that genetic profiles of *T. gondii*, especially atypical strains, maybe strongly pathogenic [4].

A closer look at the literature on *T. gondii* in Algeria reveals a number of gaps and shortcomings in the knowledge on its geographic distribution and its genetic profile. Seroprevalence studies of *T. gondii* in humans and/or animals are likely to provide a better explanation to the distribution and the transmission of *T. gondii* in order to prevent infection in humans and minimize complications in pregnant women, but also to prevent the infection of livestock animals, and to estimate the costs due to toxoplasmosis in livestock [5, 6].

As chickens in Algeria are not suitable as sentinel animals for *T. gondii* infection, due to intensive farming model, extensively farmed sheep were selected as a species indicative of the presence of *T. gondii* in the environment. The limited number of studies on sheep in

Algeria reported seroprevalence in the range of 8.3-35.4% with an average of 22.6% [7-10].

In sheep, as in other animal species, a problem of comparing different seroprevalence studies on *T. gondii* infection remains the wide range of serological assays used [11], and the absence of a gold standard test leads us to interesting challenges. The literature describes that IFA (indirect fluorescent antibody assay), ELISA, indirect hemagglutination assay, or MAT (modified agglutination test) have already been used [12], while the dye-test, considered as a gold standard in human, is seldom used in sheep [13]. Some studies have even compared different serological tests, for example, authors [14] found a better agreement between MAT and ELISA with the cut-offs at 1:100 and S/P% = 100% respectively, however, these cut-offs have never been used or validated. This clearly constitutes a major concern, such as the standardization of commercially available ELISA kits, and therefore, the evidence of the fact that there is a real problem concerning false positive or false negative samples, especially when the cut-off is not validated.

Modified agglutination test, as described by Desmonts and Dubey [15], was used with a cut-off at 1:20 [16], as has been used at 1:40 [17,18] and at 1:64 [19]. The same observation for the test kits marketed ELISA, which was used at different cut-offs, as recommended by manufacturers. Thus, the determination of an appropriate positivity cut-off seems to be necessary to have an accurate seroprevalence result. With the lack of a gold standard in sheep, the determination of the cut-off can be performed by comparison between two different tests, considering one as a reference for sensitivity and specificity calculations using the ROC curve model [20]. Also, a sheep serum known to be positive by one of the serological tests is usually used in serial dilutions to statistically estimate the cut-off for another test. In the current study, we used positive sera in serial dilutions to estimate the cut-off. We did the same with the positive control included in the commercial ELISA kit to compare with the positive sera result and see if the ELISA kit positive control can be used as an alternative to the positive sera.

Besides, the importance of studying this parasite in animals intended for human consumption, relates to the risk of toxoplasmic infection due to the probable presence of infectious tissue cysts of bradyzoites in the meat. These cysts being able to remain quiescent for a very long time [21], are considered as the terminal life-cycle stage in the intermediate host.

In this context, our main objective was to detect *T. gondii* antibodies in sheep, which are economically affordable and are the source of meat primarily consumed by the population.

2 Materials and Methods

2.1 Study area

The study was carried out in Sidi-Bel-Abbes, a north-western province in Algeria covering 9,150 km², with a population of up to 730,000 sheep according to the local agricultural department data. The study area is characterized by a hot and dry summer and a cold winter with moderate rainfall (mean annual temperature and rainfall of 15.7°C and 400 mm, respectively) and an average altitude of 450 m.

2.2 Origin of samples

The sample size required for this study was determined using the WHO (World Health Organisation) manual for the statistical determination of sample size [22]. Based on a 95% confidence interval level, a precision of 0.05, and an expected toxoplasmosis seroprevalence of 22.6% corresponding to the mean seroprevalence observed in Algeria, the minimum sample size required was estimated to 269 sheep.

Blood samples, from 269 yearling sheep, were collected between October 2020 and February 2021 in the municipal slaughterhouse of Sidi-Bel-Abbes. All sheep included in the current study were male, aged from one to two years, and were from five different regions in Sidi-Bel-Abbes (Fig. 1). Blood samples were collected from the jugular vein of animals and immediately centrifuged at 3500 rpm for 10 minutes, and the sera were then stored at -20°C for serological analyses.

Ethical approval: The research related to animals' use has been complied with all the relevant national regulations and institutional policies for the care and use of animals.

Fig. 1: Geographical localization of 269 sheep samples across five regions in Sidi Bel Abbes province.

2.3 Enzyme-linked immunosorbent assay (ELISA)

T. gondii specific IgG antibodies were detected by using the commercial multi-species ID Screen® Toxoplasmosis Indirect kit (IDVet, Montpellier, France). The test procedure was performed according to the manual provided by the manufacturer. The optical density (OD) was read at 450 nm using a microplate spectrophotometer TECAN™ Sunrise™ and the OD values were collected by the manufacturer Xfluo-4 software.

2.4 Cut-off

Usually, the cut-off used for this commercial kit is validated by verification of the positive control which must be superior to [OD > 0.350], and the S/P% (ratio between positive control OD value and sample OD value) percentage calculation ($S/P\% = \frac{OD_{\text{sample}}}{OD_{\text{positive control}}} \times 100$) determines the negativity of samples if ($S/P\% < 40\%$), doubtful if ($40 < S/P\% < 50\%$) or positive if ($S/P\% > 50\%$). Additionally, we assessed the cut-off using the positive sera possessing the higher OD value from samples of the current study in serial dilutions at 1:10, 1:50, 1:200, 1:400, 1:600, 1:800, 1:1000, and 1:2000, respectively to reach negativity and statistically estimate the cut-off by excluding background noise which can be caused by insufficient washing or non-specific interactions. This alternative was previously used in another study in France to which one of the authors (AA) using the identical ELISA kit (IDvet, Montpellier, France). The most consistent cut-off was estimated at [OD = 0.1145] according to the sensitivity and specificity calculations and the ROC (receiver operating characteristic) curve to evaluate both serological tests (MAT and ELISA). Simultaneously, authors assessed the cut-off using a serial dilution of positive horse sera, kindly provided by Pr Dubey (USDA, Beltsville, USA), showing that statistical analysis aiming to exclude background noise OD values revealed a very consistent result with the cut-off estimated by ROC Curve analysis (OD = 0.1145) [23]. According to the calculation of the ratio, between positive control OD value and samples OD values, set by the manufacturer, the recommended cut-off value should not be lower than [OD = 0.23] which is significantly higher than the cut-off estimated statistically by bootstrapped values from the positive sera serial dilutions in the current study. In addition, the same bootstrap analysis was applied on the positive control serial dilutions (1:10, 1:50, 1:200, 1:400, 1:600, 1:800, 1:1000, and 1:2000) to confirm the OD values indicating background noise of the ELISA test. A negative

serum (sample No.61) displaying the lower OD value (OD = 0.035) was also assessed in serial dilutions at (1:50, 1:100, 1:200, 1:400) to check the variability of OD values at the background noise and confirm their negativity.

2.5 Statistical analysis

The cut-off was assessed by testing several dilutions of the positive control sera and the positive control of the commercial ELISA kit. Compositional OD relationship between OD values and dilution levels was investigated by Spearman's correlation test. In order to identify the real value of positivity, an adjustment of the results was performed by the Bootstrapping method [24] where results were analyzed by the multiple linear regression using R program (version 3.4.1).

3 Results

3.1 Cut-off determination

The serological test of the commercial ELISA kit used in this study is validated according to the manufacturer's instructions if the mean value of the Positive Control OD (OD_{pc}) is greater than 0.350 (positive control in our study was OD = 0.455). Depending on the OD value of the positive control, the OD of equivocal samples ranged from [OD = 0.18] to [OD = 0.23], theoretically indicating the commercial ELISA kit cut-off.

Statistical analysis of the positive sera and positive control serial dilutions, using multiple linear regression, allowed us to assess the cut-off with the exclusion of the background noise using the robustness analysis performed by bootstrap (CI95). The results showed a mean cut-off that ranged from [OD = 0.076] and [OD = 0.123] for the positive sera and from [OD = 0.107] and [OD = 0.115] for the positive control of the commercial ELISA kit, with a value of [OD = 0.12] being satisfactory in order to minimize false-negative results. Furthermore, the adjusted cut-off was highly consistent with the cut-off estimated in a previous study using the ROC curve model for the identical commercial ELISA kit [23].

The bootstrap analysis of OD values estimating the cut-off from the positive control serial dilutions showed a very consistent result with the cut-off estimated using the positive sera (Fig. 2).

The negative sera used in serial dilutions showed predictable results where all OD values were lower than the estimated cut-off (OD = 0.12). Results from serial dilutions of the positive sera, the positive control, and the negative control are displayed in Fig. 3.

Fig. 2: Statistical analysis for cut-off determination: **A.** Bootstrap analysis on two R^2 mathematical models according to serial positive sera dilutions. **B.** Bootstrap analysis on two R^2 mathematical models according to serial kit positive control dilutions.

Fig. 2: OD values from serial dilutions of positive sera, negative sera and positive control.

3.2 Seroprevalence

For all sheep tested ($n=269$) the prevalence of *T. gondii* was estimated to be at 34.2% (92/269) using the ELISA kit cut-off, and 50.5% (136/269) according to the adjusted cut-off value ($OD = 0.12$). Table 1 displays the seroprevalence variability rates recorded over the five regions of sampling using the adjusted cut-off value and the ID screen® commercial ELISA kit cut-off ranging-value.

4 Discussion

The seroprevalence finding obtained in our investigation showed two different values due to the two different cut-offs used, with both values indicating a high seroprevalence.

Sheep were used as they are the better sentinel animal species for *T. gondii* infection in Algeria. They are always reared in an extensive and semi-intensive mode, where they have sufficient time for being naturally infected with *T. gondii* oocysts from the environment.

Table 1. Seroprevalence of *T. gondii* in sheep in the region of Sidi Bel Abbas, Algeria.

		Cut-off adjusted OD = 0.12		Cut-off ID screen® OD = [0.18-0.23]	
		samples	prevalence	samples	prevalence
Ras el ma	+	31	46.2%	21	31.3%
	-	36		46	
Oued Sefioun	+	25	50%	18	36%
	-	25		32	
Marhoum	+	45	56.9%	32	40.5%
	-	34		47	
Tenira	+	23	51.1%	13	28.8%
	-	22		32	
Tilmouni	+	12	42.8%	8	28.5%
	-	16		20	
Total		269	50.5%	269	34.2%

The examined sheep shared the same gender and age, therefore precluding the analyses of these factors. The seroprevalence, determined in the current study, maybe a realistic indication of the environment contamination caused by *T. gondii* oocysts, since the sheep are reared extensively for the first seven months, before transitioning to semi-intensive farming for fattening purposes. Seroprevalence in extensive management is known to be higher than in intensive or semi-intensive ones due to exposure to cats secreting oocysts or to contaminated stagnant puddles [25-27].

We evaluated the seroprevalence of *T. gondii* in yearling sheep using the serological ELISA test from 269 samples. Because we used an adjusted cut-off ($OD=0.12$), the seroprevalence was estimated at 50.5% (136/269). That was an expected result since the cut-off in our study was lowered, therefore increasing the prevalence from 34.2 to 50.5%. Nevertheless, the seroprevalence of 34.2% remains higher than in other studies [7-10]. This variability may logically reflect the presence of *T. gondii* in the region of Sidi Bel Abbas, located in the northwest of Algeria, and where no studies have been carried out. It may have biological and epidemiological explanations, especially regarding the environmental abundance of oocysts in the soil, hygiene standards in farms, and the choice of the serological test or the cut-off used [21].

Modified agglutination test (MAT) constitutes the major recommended test for *T. gondii* infection of most animals [28,29], since this test possesses the highest sensitivity among all serological tests [30]. Moreover, test performances may vary when applied to various animal

species [17]. ELISA represents a suitable alternative regarding its perfect concordance with the MAT in sheep toxoplasmosis diagnosis [31]. However, seroprevalence is not strictly comparable because of the use of different serological tests and different cut-off values to determine seropositivity. As reviewed by Dubey [6], several authors used 1:16 as a cut-off in the IFA for sheep sera, while others used 1:64 [32], or even 1:200 and 1:256 [33,34].

Determination of the cut-off value is crucial to better estimate the seroprevalence, although an unsound and commonly reported practice constitutes the assessment of *T. gondii* seroprevalence by using tests with arbitrary cut-offs and without any data on the sensitivity, specificity, and agreement with other tests [23].

The molecular diagnostic can be considered as a gold standard test, indicating the detection of toxoplasmic DNA as evidence of the animal infection. The previous French study compared ELISA and MAT serological tests on horse meat juices using MC-PCR as the reference test. However, the absence of detection of *T. gondii* DNA does not necessarily mean that the animal was not infected by *T. gondii*, because the distribution of tissue cysts may be low and heterogeneous in muscle samples [35]. This may explain why the molecular diagnostic could be negative even if the animal is infected, leading logically to discrepancies between molecular and serological tests.

The cut-off ranging-value of the ID Screen® commercial ELISA kit used in this study is based on the S/P% percentage calculation. It depends only on the OD value of the positive control, which may logically be different each time when using this commercial kit. This cut-off is supposed to be used for numerous animal species (ID Screen® Toxoplasmosis Indirect Multi-species ELISA), however, the cut-off of the same ELISA commercial kit was adjusted using ROC curves in horse meat samples suggesting that the cut-off needs to be assessed by the estimation of sensitivity and specificity of the serological test [23]. For this reason, and due to the non-availability of further serological test that can be considered as gold-standard in this study, we merely conducted a serial dilution of positive sera displaying the higher OD value (sample n°159, OD = 0.898, S/P%=197) aiming to exclude the background noise when reading the OD values suggesting the coefficient of determination of the two R^2 mathematical models to be at 0.35 and 0.50 respectively. This means that the equation of the line regression determines a portion that does not exceed half of the distribution of points. The two mathematical models used do not explain the distribution of points. To extract the cut-off with the two R^2 models, a robustness analysis was performed by bootstrap. For a bootstrap

confidence interval of 95%, we recognise that the mean cut-off is ranging from [OD=0.076] and [OD=0.123]. Further adjustment, [OD=0.12] was determined as the approximate value of the representative cut-off for our samples (Fig.2). The same bootstrap statistical analysis was applied on results from the commercial kit positive control showing a concordant cut-off OD value. This hypothesis may explain the low seroprevalences of *T. gondii* previously reported in sheep in Algeria as the authors used only the calculation of the S/P% percentage as recommended by the manufacturer. The assessment of the cut-off may increase the positive samples, and therefore, seroprevalence may be higher. The definitive prevalence may only be determined by correlation with bioassay isolations or other direct diagnostic methods.

Low or very close IgG concentrations to the cut-off value frequently leads to interpretation issues as equivocal results. The cut-off recommended by the manufacturer of the commercial ELISA kit arises from the validation of the serological test on pigs, where almost all of their negative samples had S/P% lower than 22% which visibly are negative and in agreement with the IFA test (https://www.id-vet.com/pdfs/newsletter1/dp_toxos_swine12.pdf). Thus, there were no equivocal samples that may help to adjust the cut-off. According to the manufacturer, the commercial ELISA kit was also used to detect antibodies against *T. gondii* in sheep (https://www.id-vet.com/pdfs/newsletter1/poster_toxos_dublin13.pdf), but, this study is not sufficiently informative, and numerous points need to be clarified, such as sample size calculation, the unavailability of the results for the most of the positive samples, including weakly positive sera, and the cause of the important disagreement between the two serological tests (ELISA and IFA). Therefore, the commercial ELISA kit may be more sensitive than IFA, but not necessarily very specific because of the cut-off that may be overestimated.

The cut-off, estimated in percentage S/P has a wide range among several commercial ELISA kits for *T. gondii*, especially in kits intended for animal diagnostics. For example, the multi-species ID Screen® Toxoplasmosis Indirect kit (Catalog No. TOXOS-MS-2P) from IDvet defines the cut-off at [S/P=49.99%], while PrioCHECK® Toxoplasma Ab (Catalog No. 7610240) from Applied biosystems defines the cut-off at [S/P=20%] and pigtype® Toxoplasma Ab (Catalog No. 273401) from Qiagen defines the cut-off at [S/P=30%] showing a better agreement ($\kappa = 0.92$) with the reference test MAT, compared to ID Screen® Toxoplasmosis Indirect kit ($\kappa=0.83$) in an old study comparing the performance of four commercial ELISA kits in detecting *T. gondii* antibodies in pigs [36].

Moreover, another study suggested that a lower cut-off of PlateliaToxo IgG kit, one of the most widely used tests for human anti-toxoplasma antibody detection [37], should be reduced from 9 IU/mL, as recommended by the manufacturers, to 4.4 IU/mL to meet the definition of positivity, while positive predictive value (PPV) estimated at 99.0% using the lower cut-off (4.4 IU/mL) suggested the elimination of the risk of the false positive. [38,39].

Our study demonstrates that the risk level of *T. Gondii* infection is high and should not be neglected because of the frequent sheep meat consumption in the Algerian population. It should be mentioned that, so far, there is almost a lack of data on the *T. gondii* population structure in Algeria. The only one isolate from a case of congenital toxoplasmosis was genotyped as type II [40]. Further studies are needed to assess the risk of toxoplasmosis for consumers and to estimate the extent of *T. gondii* induced abortions among livestock animals where it could be responsible for considerable economic losses.

Conflict of interest: Authors state no conflict of interest.

Data Availability Statement: The datasets generated during and analysed during the current study are available from the corresponding author on reasonable request.

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