

1 Title page

2 Toxoplasmosis in female high school students, pregnant women and ruminants in
3 Cyprus

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Abstract

Background The protozoan parasite *Toxoplasma gondii* is important to human and animal health worldwide. This is the first study of prevalence of infection with *T. gondii* and associated risk factors in human populations and small ruminants in Cyprus. **Methods** A random sample of 18 schools out of 46 participated: 1056 girls aged 16 to 18 years completed a questionnaire and were serologically tested for *Toxoplasma* between 2008 and 2011 (response rate 30%). In addition, infection with *T. gondii* laboratory results of 23,076 pregnant women tested between 2009 and 2014 were obtained from hospital records. Finally, 163 (out of 3123) farms were randomly sampled and blood samples from 515 sheep and 581 goats were obtained. **Results** Estimated seropositivity prevalence in female students was 6.5% (95% CI 4.3% to 8.7%) and 18% (95% CI 17% to 19%) in pregnant women. Overall, 40.1% of the ruminants tested were seropositive (95% CI 37.2% to 43.0%). Seropositivity differed according to geographical region in all three groups. **Conclusions** Further studies are needed to investigate the differences between regions that lead to differing prevalence levels and patterns between ruminants and humans so that health education policies can be developed to help prevent infection and reduce environmental contamination.

Key words: CYPRUS, FEMALE STUDENTS, GOATS, HIGH SCHOOL, SHEEP, TOXOPLASMOSIS

Introduction

Toxoplasma gondii (*T. gondii*) can infect practically all warm-blooded animals and it is estimated that, on average, 30% of the world's human population hosts this protozoan parasite.¹ However, seroprevalences vary widely between countries (from 0 to 80%) and often within a given country or even between different communities in the same region.² These differences depend on anthropogenic factors such as dietary habits (methods of cooking meat, kinds of meat consumed, vegetable washing, hand washing, etc); economic, social, cultural habits; quality of water; sanitation coverage.¹ Transmission of this zoonotic pathogen to humans takes place by the ingestion of tissue cysts³ (found in infected meat) or sporulated oocysts (shed in the environment by infected cats within their feces contaminating soil, water, and food products); transplacentally as well as by organ transplantation from infected donors. Two groups of people are principally at risk of developing clinical toxoplasmosis: embryos, whose mothers become infected during gestation which may result in abortion or congenital disease and immunosuppressed patients (mostly with HIV infection) who may develop lethal toxoplasmic encephalitis.⁴ In addition, this parasite may cause ocular toxoplasmosis in immunocompetent patients and it is of veterinary importance worldwide as it causes economic losses due to abortions in small ruminants like sheep⁵ and goats⁶ and in horses.⁷

In a seroepidemiological study concerning infection with *T. gondii* in Crete, an island with similar anthropogenic, climatic and geological characteristics as Cyprus, seroprevalence in pregnant women was found to be 29.5%.⁸ In Cyprus, although congenital toxoplasmosis is a notifiable disease since 2004⁹ no cases have being reported¹⁰. However, Spiramycin, a drug administered only to pregnant women suspected of acute *Toxoplasma* infection in the Republic (which is available

centrally by the Pharmaceutical services of the Ministry of Health to doctors working in the public as well as the private sector) has been administered to six pregnant women suspected of acute toxoplasmosis in Nicosia, three in Larnaca and two in Limassol hospitals in 2015; and between 2010 and 2015 at least four infants received treatment for suspected congenital toxoplasmosis at the Limassol hospital.¹¹ These data indicate that the problem is underreported in the island.

The only seroepidemiological study in the island regarding this zoonosis was carried out on rodents and showed 27.9% of the animals tested to be seropositive against this parasite.¹² At the same time, one *Toxoplasma* strain, isolated from a child with lymphadenopathy, was typed as type III,¹³ a low-virulence genotype.¹⁴

The primary objective of this study is to estimate the prevalence of human infection with *T. gondii* in Cyprus. A secondary aim is to investigate potential risk factors in school girls. To achieve this, we aimed at: serologically testing girls 16-18 years old to determine seropositivity prevalence in this population and informing each girl examined of what she needs to do before pregnancy (according to her serological results) for a safe, regarding *Toxoplasma*, gestation; to compare the results of our serological investigation to those of pregnant women (obtained from the hospitals' microbiology laboratories, tested at the time of our investigation); to serologically test sheep and goats and relate the results to the human data in order to identify the risk of infection through meat consumption; to relate these results to those of the epidemiological study conducted in the island, in 2000-2003, on rodents.¹² The results obtained could be used to develop policies to safeguard public health against *Toxoplasma* infection.

Materials and methods

Study location and sample

The study was undertaken in the government controlled part of the island of Cyprus, covering 5,896 Km², comprised of five prefectures (Figure 1). This area has 46 High Schools (Lyceums) with 21,171 students aged between 16 and 18 years (56% female).¹⁵ A pilot study was carried out in order to investigate the seropositivity prevalence and thus to ascertain the sample size required for the present study. A hundred students from three different Lyceums participated: the proportion of seropositive students was 6%. Of the 11,855 female students 3,556 (30%) had selected 'Special Biology' as option for their studies. The aim was to test serologically a random, geographically representative, sample of these girls. Allowing for an error of 4 percentage units, an intracluster correlation of 0.15, a design effect of 9.9 and a finite sample correction factor of 0.42, an adequate sample size was calculated to be approximately 830 students, that is 17 schools assuming 50 participants per school.^{16,17} A random sample of 18 Lyceums was taken. In total, 1,056 girls, from the five prefectures, were tested for *Toxoplasma* between 2008 and 2011. The response rate was approximately 30%.

Procedure

The headmaster/mistress of the Lyceum was informed about the project. A lecture was given to the children (aged 16-18) of each school to introduce them to the parasite *Toxoplasma*. In collaboration with each school's parents' association, an evening presentation for the parents on toxoplasmosis and the project was organized. A consent form was given to the girls who showed interest in taking part in the study to be completed at home with their parents, who were also required to sign the form.

Two medical doctors and three nurses carried out blood sample collection (one sample with and one without anticoagulant, EDTA) and a questionnaire was completed by each girl. A code was given to each questionnaire and to the relative blood sample so that samples could be processed anonymously.

The questionnaire consisted of 20 items: seven related to demographic information (school of attendance, class, prefecture, the name of the student, age, nationality, present postal address and address up to one year before if she had changed residence) and thirteen related to factors believed a priori to be important for *Toxoplasma* acquisition (listed in Tables 1, 2 and 3).

The blood samples were kept in a cold box until transferred to the laboratory where the serum was collected and kept at -20°C until tested by serology whilst the sample with EDTA was kept at -80°C for performing PCR if serology revealed acute infection.

A letter was sent, by post, to the parents informing them of the serological results of their daughter and the relevant recommendations, according to whether they were sero-positive/negative and what actions to take for a safe, from *Toxoplasma*, pregnancy in the future. The results of this investigation were made available to the ministry of Health and Education.

Serological investigation of girls

Serum samples from 1,056 girls were tested (Table 1) for IgM and IgG antibodies against *Toxoplasma* using ELISA tests (NovaLisa™, NovaTec Immunodiagnostica GMBH, Germany) according to the manufacturer's instructions (IgM positive > 1.1; sensitivity 95.8%, specificity > 98%. IgG positive > 35; sensitivity 96.6%, specificity > 98.2%). For all sera exhibiting a high IgG titre or any IgM titre, the IgG Avidity test

was performed to establish recent infection (NovaLisa™, low avidity < 40; agreement with acute and past infection 96.5%). In all ELISA tests, for each batch of 90 sera, positive and negative controls were run.

Pregnant women, serological results

The serological results of 17,631 pregnant women tested in 2009-2011, as well as of 5,445 pregnant women tested in 2012-2014 (Table 1) were obtained from the 4 hospitals' microbiology laboratories (which perform the test for the population in the five prefectures). Initially, samples were obtained for the same time period as the prospective schoolgirl study. As we were informed by the authorities that 12,000 Pontiac Greek refugees, mostly young couples, had recently settled in Paphos (and left Cyprus in 2013 due to the economic crisis), we decided to acquire serological results for pregnant women for all regions for the 2013-2014 time period. The total population of Paphos prefecture at the time was 88,000 inhabitants (13.6% were Pontiac Greeks). The results were made available with codes (anonymously). The serological tests performed were: ELISA (Architect Abbott; IgM positive > 0.6; relative sensitivity 89.9%, relative specificity 99.8%. IgG positive > 0.3; relative sensitivity 97.5%, relative specificity 99.1%).

Seroepidemiological study of sheep and goats

There are 3,123 farms with sheep and goats scattered throughout the island, with approximately 536,727 animals. All data on the livestock population is kept in a national livestock database of the Ministry of Agriculture. A random sample of 163 farms was selected; blood and epidemiological information was collected by veterinarians from 515 sheep and 581 goats, selected randomly from these farms, in

2013-2014; 192-241 animals from each prefecture. The blood samples (without EDTA) were kept in a cool box until transferred to the laboratory. The sera were tested for IgG antibodies against *T. gondii* by ELISA (CHEKIT-Toxotest ELISA Test kit, IDEXX Laboratories: positive \geq 100%). For each batch of 90 sera, positive and negative controls were run.

Statistical analysis and mapping of the results

Seroprevalence in female students was estimated overall, for each prefecture and for each school. A confidence interval for the overall prevalence was calculated adjusting for the clustered sampling procedure (using the sampling fraction 18/42) as described in the FAO United Nations depository.¹⁸ Univariate associations between variables were undertaken using a corrected, weighted Pearson chi-square statistic to account for the clustered design. Complete case analysis was used for the risk factor investigation: 854 (81%) of the 1056 schoolgirls had complete records. Both univariable and multivariable logistic regression models were fitted, accounting for clustering by defining the school variable as the primary sampling unit. Only variables that did not display multicollinearity and did not have seropositivity frequencies less than five in each category were included in the multivariable model. These were: region, age, country/town house, pets and smoked meat consumption. A 5% significance level was selected. Stata 11 was used (applying the `svyset` command prior to analyses to account for clustering by school).

The serological and geographical data of the students, pregnant women, sheep and goats as well as rodents (data from Psaroulaki et al., 2010) were mapped using GIS (ArcGIS 10). Pie charts showing the seropositivity proportions for each prefecture were created and superimposed. The 104 villages and towns in which the

tested girls lived in were also shown to indicate the coverage of the area investigated (Figure 1).

Results

Of the 1,056 girls tested in total, 69 (6.5%, 95% CI 4.1% to 10.4%) were seropositive. Eight students had IgM antibodies (0.8%: 4 from Paphos prefecture, 3 from Nicosia and 1 from Limassol) and two had both IgM and IgG antibodies. The IgG avidity test, in all IgM positive and in 11 girls with high IgG titre, was high (54.9 to 91.4) indicating that the infection occurred more than 4 months before the time of sample collection. The prefecture with the highest seropositivity against *Toxoplasma* in girls was Paphos (15.5%) (Table 1, Figure 1).

The odds of a female student acquiring the parasite, was found to differ significantly according to the region she lived (Table 1, $p < 0.0001$), with the odds being lower in the regions of Ammochostos, Larnaca and Nicosia compared to Paphos (ORs 0.10, 0.15 and 0.28 with p-values 0.004, 0.011 and 0.021 respectively). Paphos prefecture had the highest proportion of seropositive girls, with overall seropositivity 15.5% (the seropositivity ranging from 8.4% to 26.9% depending on the lyceum); Nicosia prefecture 5.8% (4%-12.9%); Limassol 5.4% (2%-7.1%); Larnaca and Ammochostos had the lowest seropositivity 2.5% and 1.5% respectively (Figure 1). Pyrgos Lyceum, Paphos prefecture, had the highest seropositivity rate and the Paralimni Lyceum, Ammochostos prefecture, the lowest (Figure 1).

The percentage of seropositive students is presented by demographic characteristic and dietary habit in Tables 2 and 3 respectively. Using univariate logistic regression models, adjusting for clustering by school, eating pork salami and

eating greens were found to be associated with *T. gondii* seropositivity, to a statistically significant extent. The odds of being seropositive were estimated to be almost double in pork salami consumers compared to those who did not consume salami (OR 1.9, 95% CI 1.1 to 3.3, $p = 0.023$) and in consumers of raw not well washed vegetables 1.8 times that of non-consumers (OR 1.8, 95% CI 1.04 to 3.2, $p = 0.037$). Bonferroni-adjustments for multiple comparisons, however, resulted in neither of these risk factors remaining statistically significant. At multivariable analysis only region of residence was statistically significant.

Of the 17,631 pregnant women tested in 2009-2011, 3,065 were IgG and 107 IgM positive. Overall, 18% of the pregnant women were seropositive, with Larnaca and Limassol prefectures showing the highest seropositivity (22% and 21.2%, respectively) (Table 1). The 2012-14 results were similar to the 2009-2011 results for all prefectures (Table 1) except for Paphos which reached 20.9% for the more recent time period (798 women tested).

Overall, 40.1% of goats and sheep were seropositive, with Ammochostos presenting the highest overall seroprevalence (49.8%) (Table 4). The risk of seropositivity was found to differ significantly according to the geographical origin of sheep and goats ($p < 0.0001$), with an increased risk for Nicosia prefecture (Figure 1). Seroprevalence in sheep was higher compared to that of goats ($p = 0.002$)

Discussion

The position of European countries varies regarding the measures taken for congenital toxoplasmosis prevention. At least five countries recommend a systematic screening of pregnant women whilst 21 countries (Cyprus among them) do not.^{9,19} Gynecologists in Cyprus usually test for infection with *T. gondii* by serology once

during pregnancy and there is no common practice regarding the management of pregnant women with positive serological results. So, often, clinicians are faced with the dilemma of administering potentially toxic drugs or pregnancy termination based mainly on serological findings. In a study conducted in Crete, Greece, only 0.2% of the pregnant women had their first serological screening for *T. gondii* before pregnancy and 45% after the first trimester.⁸ Of these, 29.5% were seropositive making it difficult for the doctor to place the time of infection; essential information on which the handling the pregnancy for a safe outcome is based.

Raising awareness of the way *Toxoplasma* infects people and animals can reduce seroprevalence in a country.^{20,21} In Cyprus, health education can prove very important in avoiding infection since a proportion of the female population appears to seroconvert between the crucial ages of 19 and 38 years. Overall, seropositivity among girls (16-18 years old) was 6.5% (Table 1). This seropositivity depended on the geographical origin of the girls ($p < 0.0001$) with Paphos having, by far, the highest seropositivity (15.5%), whilst in the other prefectures seropositivity did not reach 6%.

As expected, seropositivity in pregnant women was higher than that of schoolgirls, in all prefectures. The highest seropositivity in pregnant women was found in Larnaca prefecture (22.0%) with Limassol following closely (21.2%) (Table 1). In both these prefectures rodent seropositivity, examined 7-10 years earlier, was the highest for *Toxoplasma* (45.7% and 29.5%, respectively)¹² (Table 4).

Paphos prefecture, with the highest girl seropositivity, presented nearly the same seropositivity for pregnant women (15%). The second analysis of serological data, provided by the hospitals for the years 2013-2014, showed 20.9% seropositivity in pregnant women in this prefecture (Table 1). We assume that the low seropositivity

obtained in the 2009-2011 analysis was due to the non indigenous population of Pontiac Greek refugees, mostly young couples, (making up 13.6% of the population in the area of Paphos at that time).

In Europe, while soil contact is shown to represent 6 to 17% of the seropositive cases, meat consumption appears to be responsible for 30 to 63%.^{22,23} Sheep, rather than pigs, is the main source of infected meat in Southern European countries with reported rates of seropositivity for goats varying from 4 to 77%.^{24,25} In Cyprus, sheep and goat farming is mainly intensive but many farmers allow their animals in open fields for grazing. Farms for raising animals are found in the whole island. Overall, the farms are both dairy and meat farming with no important differences among them concerning biosecurity level. A better understanding of the farming practices in Cyprus is needed in order to draw conclusions on their role in *Toxoplasma* epidemiology in the island.

The weather in Cyprus is mild (Mediterranean) with relatively warm and dry summers and rainy and mild winters. *T. gondii* oocysts can survive and remain viable in soil for 12–18 months under such climate conditions.^{26,27} Overall, there are no extreme differences in climate (temperature and humidity) in the five prefectures and so they are not likely to result in differential levels of *Toxoplasma* oocyst survival. It appears that *Toxoplasma* is circulating in the environment and conditions favor its geographical spread through cats and rodents, reaching humans and animals for consumption. Controlling cat and rodent populations, therefore, may help reduce the problem.

Our study had a number of limitations which may affect the generalizability of the results. The retrospective nature of the data collection for pregnant women may have resulted in a level of sampling bias being introduced. In addition, it was not

possible to account for age in the analyses. It is known, however, that the average age of women at pregnancy for the whole of Cyprus in the years 2007-2013 was 29.1 years (28.4-29.7 depending on the prefecture) (Health Monitoring Unit, Ministry of Health of the Republic of Cyprus). Another limitation is the use of different ELISA tests for the different population groups. Finally, it should be clarified that the main aim of the study was to examine seropositivity and possible associated risk factors in schoolgirls; only crude seropositivity estimates were obtained for the pregnant women and animal populations.

Conclusions

Our study found that seropositivity against *Toxoplasma* in Lyceum students is associated with region of residence. The percentage of seropositive females is higher in pregnant females and warrants the development of health education measures. These measures could be aimed at the student population and could provide the necessary information: hygienic measures required in relation to external activities and the correct processing of food. Parallel to this, offering serological testing for infection with *T. gondii* before pregnancy will reduce the risk of congenital toxoplasmosis.

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Authors' contribution:

All authors listed on the manuscript have contributed significantly to the experimental design and/or its implementation and/ or the analysis and interpretation of the results presented and the writing of the manuscript. MA conceived the study; ML, VC, JM and MA designed the study protocol; ML, VC, CK and GM carried out the laboratory tests and assessment; JM, MK and MA did the analysis and interpretation of the data; MA drafted the manuscript; ML, VC, JM and MK critically revised the manuscript for intellectual content. All authors read and approved the final manuscript. MA and JM are guarantors of the paper.

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Ethical approval: The Bioethics committee of Cyprus, after examining the protocol, gave permission for this investigation to be carried out (permission codes EEBK/ΕΠ/2007/2B). The Ministry of Education was informed about this project and their permission and assistance was acquired (7.19.46.7/9). The Ministry of Health was informed about this project and their approval was obtained. Informed, written consent was acquired from all girls taking part in the study and their parents. The team carrying out the project consisted of two medical doctors, one biologist, one veterinarian and three nurses.

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