The rural–urban effect on spatial genetic structure of type II *Toxoplasma gondii* strains involved in human congenital toxoplasmosis, France, 2002–2009

Daniel Ajzenberg \textsuperscript{a,b,⁎}, Frédéric Collinet \textsuperscript{a}, Dominique Aubert \textsuperscript{c,d}, Isabelle Villena \textsuperscript{c,d}, Marie-Laure Dardé \textsuperscript{a,b}, French ToxoBs network group \textsuperscript{e,1}, Sébastien Devillard \textsuperscript{f}

\textsuperscript{a} INSERM, UMR_S 1094, Université de Limoges, Limoges, France
\textsuperscript{b} Centre National de Référence (CNR) Toxoplasmose/Toxoplasma Biological Resource Center (BRC), Centre Hospitalier Universitaire Dupuytren, Limoges, France
\textsuperscript{c} EA3800, SFR Cap-Santé, Université de Reims Champagne-Ardenne, Reims, France
\textsuperscript{d} Centre National de Référence (CNR) Toxoplasmose/Toxoplasma Biological Resource Center (BRC), Centre Hospitalier Universitaire Hôpital Maison Blanche, Reims, France
\textsuperscript{e} Members of the French ToxoBs network group who contributed data are listed in the appendix
\textsuperscript{f} CNRS, UMR5558, Université de Lyon 1, Villeurbanne, France

1 Appendix: French ToxoBs network group

**Abstract**

Congenital toxoplasmosis involves *Toxoplasma gondii* type II strains in 95% of cases in France. We used spatial principal component analysis (sPCA) and 15 microsatellite markers to investigate the spatial genetic structure of type II strains involved in 240 cases of congenital toxoplasmosis in France from 2002 through 2009. Mailing addresses of patients were geo-referenced a posteriori in decimal degrees and categorized into urban or rural areas of residence. No spatial genetic structure was found for type II strains that infected mothers who were living in urban areas, but a global spatial genetic structure was found for those that infected mothers who were living in a rural environment. Our results suggest that sources of infection by *T. gondii* are different in rural and urban areas in France, and advocate for targeted messages in the prevention of toxoplasmosis according to the type of residence of susceptible people.

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1. Introduction

*Toxoplasma gondii* is considered to be one of the most successful parasites because this protozoan is virtually able to invade any nucleated cell of any warm-blooded animal at all latitudes. This parasite is efficiently transmitted by ingestion of oocysts shed by felids in the environment or cysts present in tissues of mammalians and birds. Toxoplasmosis is considered a benign disease except in fetuses infected during early pregnancy and in severely immunocompromised patients because they are at risk of severe brain and eye damage, and in tropical South America where the prevalence of ocular disease is high and where life-threatening cases of pulmonary toxoplasmosis may occur in otherwise healthy people (Carme et al., 2009; de-la-Torre et al., 2013). It is estimated that 25% of the human population is currently chronically infected by *T. gondii*. In fact, seroprevalence in the general population can tremendously vary with geography and also with time. Less than 10% of people born in the USA have antibodies against *T. gondii* whereas in certain tropical areas of South America and Africa, the prevalence is as high as 80% (Krueger et al., 2014). Big variations of prevalence can also be observed at a country scale. For example in France, the median prevalence among women of childbearing age was 43.8% in 2003 but the prevalence varied substantially by French region, being lowest in North-eastern France (29.5%) and highest in the greater Paris area (52.7%) (Berger et al., 2009). Seroprevalence varies also with time as shown in France with a decrease in prevalence from 83% in 1965 to 37% in 2010 (Nogareda et al., 2014). These data indicate that the epidemiology of
human toxoplasmosis is complex and involves many different aspects including intensity of *T. gondii* circulation in the environment, geoclimatic variables such as temperatures, precipitations or altitude, and human factors such as personal hygiene, cultural and food habits, socio-economic status, level of education and residence in a rural or urban area.

The population structure of this cosmopolitan parasite is more complex than initially thought and has distinct geographic patterns. The hotspot of *T. gondii* genetic diversity seems to be tropical South America, especially in the Amazonian forest, where a combination of a large gene pool with frequent genetic exchanges have generated a wide variety of so-called atypical genotypes (Ajzenberg et al., 2004). Elsewhere, and especially in Europe, *T. gondii* displays a striking clonal population structure. Based on genotyping data obtained from domestic and wild animals in France, it is estimated that 99% of *T. gondii* strains that circulate in the French environment belong to type II (Aubert et al., 2010; Halos et al., 2010). Type III strains are much less frequent in animals in France than in animals from other areas such as North America or some regions of Africa (Shwab et al., 2014). To date, type I and atypical strains have never been isolated from any animal species in France. As a consequence, isolating a non-type II strain in a patient in France raises the possibility that this patient had been infected with an imported strain during a journey abroad or because he had eaten imported food, as already shown in some reports (Ajzenberg et al., 2009; Pomares et al., 2011).

The aim of this study was to better understand the epidemiology of human toxoplasmosis in rural versus urban areas by investigating the spatial genetic structure of *T. gondii* strains in both environments at a country scale in France. If people who live in rural areas are predominantly infected by local food produced in their region of residence, then a significant spatial genetic structure of *T. gondii* strains should be observed in rural areas. Conversely, if we consider that people living in urban areas are more likely to get *T. gondii* infection from food purchased from supermarkets, no spatial genetic structure should be observed in urban areas because goods distributed in supermarkets come from many different suppliers all over the French territory. To test this hypothesis, we used strains isolated in France from congenital cases because these strains are predominant in the collection of human strains at the French national reference center of toxoplasmosis and because women are infected during a short period of time during pregnancy, which minimizes the likelihood of *T. gondii* infection outside their residency area.

### 2. Material and methods

#### 2.1. *T. gondii* strains

From 1992 to 2009, the French national reference center of toxoplasmosis genotyped 325 *T. gondii* strains responsible for congenital toxoplasmosis in 16 French teaching hospitals. These strains had been isolated in mice after inoculation of amniotic fluids, placenta, or blood cord samples for evidencing congenital toxoplasmosis in children or fetuses from mothers infected by *T. gondii* during pregnancy in this period. All these strains were genotyped with 15 microsatellite markers as previously described (Ajzenberg et al., 2010). Of these 325 strains, 305 (94%) were type II, 10 (3%) were type III, and 10 (3%) could not be classified into one of the three major clonal types and therefore considered as atypical.

For the analysis, we restricted the sample to the strains isolated during a shorter period of time, from 2002 through 2009, in order to minimize temporal effect on genetic variability. We also excluded the type III and atypical strains in order to minimize the genetic variability due to imported strains from non-European countries. In total, 240 type II strains isolated from 240 cases of congenital toxoplasmosis in France from 2002 through 2009 were included in the analysis (supplementary material).

#### 2.2. Geo-referencing *T. gondii* strains in France

The 240 *T. gondii* strains were geographically associated to the place of residence of the corresponding mothers in whom these strains had been collected. The mailing addresses (number, street and commune names) of the 240 patients were geo-referenced *a posteriori* in decimal degrees using the web portal of the French National Institute of Geography (IGN, http://www.geopostal.fr). The spatial distribution in France of the 240 strains is displayed in Fig. 1. Some gaps can be identified in this map. The big gap in the center of the country is mainly due to the fact that this area (called Massif Central) is the least densely populated area in France. Other gaps in certain densely populated areas are explained by the poor availability of strains from these areas at the French national reference center of toxoplasmosis.

#### 2.3. Classification of *T. gondii* strains into rural and urban

In order to investigate the relative degree of spatial genetic structure of *T. gondii* strains isolated from women living in a rural environment versus those living in an urban environment, the geographic coordinates of the 240 strains were classified as rural or urban based on two different classifications.

##### 2.3.1. TUU classification

A detailed classification of the 36,569 French communes within rural versus urban socio-economic environments was delivered in 1999 by the French National Institute for Statistics and Economic Studies (INSEE, available at http://www.insee.fr/en/default.asp) and updated in 2009. This classification is based on urban unit sizes ("Taille d’Unité Urbaine" or TUU in French) and includes several criteria such as population size, socio-economic activity within the commune, or frequency of travel between place of residence and work. Based on the TUU classification, the rural communes are therefore those that are not urban. The TUU classification allowed us to classify $n = 85$ *T. gondii* strains as rural and $n = 155$ *T. gondii* strains as urban (supplementary material).

##### 2.3.2. GE classification

The TUU classification can be completed by another one that classifies urban communes as those that are not rural. We used the Google Earth (GE) application (http://earth.google.com/) to display a satellite windows (10 km × 10 km) centered on the mailing addresses of the 240 patients. When no commune of more than 5000 inhabitants was found at less than five kilometers of a given mailing address, the *T. gondii* strain isolated from this given patient was classified as rural. A strain was thus classified as urban when the previous condition was not met. Overall, it allowed us to classify $n = 98$ *T. gondii* strains as rural and $n = 142$ *T. gondii* strains as urban (Fig. 1 and supplementary material). Matching between the TUU and GE classifications was very good since 79 out of the 85 rural *T. gondii* strains that were defined with the TUU classification were also rural with the GE classification (observed Cohen’s unweighted Kappa coefficient $= 0.78$).

#### 2.4. Genetic and genotypic diversity within rural and urban populations

Genetic polymorphism was measured by allelic richness (A) per locus and sample and by Nei’s unbiased genetic diversity within subsamples (Hs) (Nei and Chesser, 1983). Allelic richness was corrected for unequal sample size using the rarefaction method (El Mousadik and Petit, 1996). The A and Hs values were calculated with the FSTAT software version 2.9.4 (Goudet, 1995). Genotypic diversity was calculated from the number of multilocus genotypes on the total number of strains for both rural and urban populations. Linkage disequilibrium (LD) between pairs of loci was assessed with a randomization test performed in FSTAT. The statistics used was the log likelihood ratio G summed over all subpopulations. Because this procedure was repeated on all pairs of polymorphic loci, we applied the sequential Bonferroni...
correction (Holm, 1979) to the p values. LD was calculated for both the urban and the rural populations. To test for genetic isolation by geographical distance (IBD) within both rural and urban populations, Mantel’s tests (Mantel, 1967) (n = 9999 permutations) were performed for matrix correspondence to compare pairwise geographical (km) and genetic distances based on the Cavalli-Sforza and Edwards chord-distance estimator Dc (Cavalli-Sforza and Edwards, 1967) among T. gondii strains using R 2.10.1 software (R Development Core Team, 2009). Dc was computed using Populations 1.2.30 (1999, Olivier Langella, CNRS UPR9034, http://bioinformatics.org/_tryphon/populations/).

2.5. Spatial genetic structure of T. gondii strains

We used spatial principal component analysis (sPCA), implemented in the package adegenet for the software R (R Development Core team, 2013), to investigate the spatial genetic structure of T. gondii strains isolated from congenital cases in France (Jombart, 2008; Jombart et al., 2008). The sPCA seeks principal components that optimize the product of the variance and Moran’s Index (Cliff and Ord, 1981; Moran, 1948), ensuring that both the genetic diversity and the spatial patterns are taken into account. The sPCA is particularly appropriate to our study because the spatial distribution of T. gondii strains is continuous in our sampling. The sPCA identifies global structures that correspond to a positive spatial auto-correlation between genotypes, indicating the occurrence of patches or clines in the spatial distribution of genetic diversity (Jombart et al., 2008). As in other multivariate analyses, such global structures are typically detected graphically by an abrupt decrease in positive eigenvalues (Jombart et al., 2009). The assessment of the strength of the inferred global structure is facilitated by a non-parametric randomized Monte-Carlo test (Jombart et al., 2008). The sPCA and non-parametric randomized Monte-Carlo tests were performed for each type of T. gondii strain (rural vs. urban) using successively the GE and the TUU rural/urban classification.

The sPCA needs a matrix of proximities between T. gondii strains to be incorporated explicitly in the analysis for the calculation of the Moran’s Index. As seen in Fig. 1, T. gondii rural strains are more evenly distributed than the more aggregated urban T. gondii strains. This difference in spatial distribution of rural versus urban strains implied to use different proximity matrices, for both urban and rural strains, to ensure that our results were not influenced by the proximity matrix type. First, we used a binary connection matrix M (e.g., with value equal to 1 if two urban T. gondii strains were neighboring or equal to 0 if they were not). Neighborhood was defined by building a connection network (also called neighboring graph) following Delaunay’s triangulation (Upton and Fingleton, 1985). The Delaunay’s graphs were modified by removing unrealistic connections crossing the sea (e.g., from northwest to southwest of France). Secondly, we used the matrix of the inverse of Euclidean distances between each pair of urban T. gondii strains. Such a proximity matrix implies that the strength of the spatial auto-correlation between two T. gondii strains is linearly related to the distance separating these two T. gondii strains. This distance-based neighborhood is appropriate to aggregated or irregular distributions (Gabriel and Sokal, 1969) such as the one observed in urban T. gondii strains whereas Delaunay’s triangulation would lead to unrealistic connection in that case. All p values < 0.05 were considered significant.

3. Results

3.1. Genetic diversity

Allelic richness (A) and genetic diversity (Hs) values per locus and population were reported in Table 1. Among the 15 microsatellite markers, only 7 (M48, M102, N60, N82, AA, N61, and N83) showed a
high polymorphism (Table 1). These 7 markers are called fingerprinting markers. The mean calculations were based on the 7 fingerprinting markers data only.

3.2. Spatial genetic structure of T. gondii strains

No spatial genetic structure was found by the sPCA for the urban strains whatever the rural/urban classification and the proximity matrix used. All p values for global test on urban strains were non-significant (Table 2). On the contrary and in agreement with our prediction, an overall spatial genetic structure was found in rural strains since p values for global test reached statistical significance in two out of the four tests (Table 2). That significance level was reached for both the GE and TUU classification by using distance-based and Delaunay proximity matrix, respectively. This result suggests that the global spatial structure for rural strains is not an artefact of a particular classification or proximity matrix. The spatial structure of rural strains according to TUU classification and the Delaunay proximity matrix is displayed in Fig. 2A. Of note, this graphical representation of rural strains matched the one displayed for the GE rural/urban classification with the distance-based proximity matrix (result not shown). This global structure separated mainly the northwestern part of France from the rest of the country. Grey squares corresponding to negative sPCA scores for strains were mainly observed in three administrative regions of the Northwestern France (Bretagne, Pays de la Loire, and Normandie) whereas black squares corresponding to positive sPCA scores were in the rest of the country for most of them. For comparison and despite no spatial genetic structure was found in urban strains, Fig. 2B displayed a graphical representation of the sPCA scores obtained for the urban strains with the GE rural/urban classification and the Delaunay proximity matrix, i.e. the combination of classification and proximity matrix that led to the lowest p-value for urban strains (Table 2).

4. Discussion

Based on bivariate or multivariate analysis with calculation of the odds ratios, most epidemiological studies try to identify the variables associated with an increased risk of toxoplasmosis by interviewing a target population (mostly pregnant women) with standardized questionnaires in a given geographic area (Cook et al., 2000; Jones et al., 2009). The results are interesting and often useful for making preventive strategies and public health policy decisions, but have serious limitations due to the subjective answers given in interviews. Because people may be unaware of their exposures or may have difficulty recalling specific exposure that occurred, it is considered that interviews with questionnaires are not able to explain roughly 50% of the risk for T. gondii infections in these studies (Cook et al., 2000; Jones et al., 2009). When a more objective test is applied, unexpected results may arise. For example, a new blood test detecting a specific antibody to an 11-kDa oocyst protein showed that oocyst-induced infections were largely underestimated in the USA and that about 50% of women infected with oocysts did not identify significant risk factors for oocysts infection (Boyer et al., 2011). It is therefore important to address the epidemiological issues of toxoplasmosis by using different approaches than the classical interviews with questionnaires. Our work is original because it aimed to understand the epidemiology of T. gondii infection in rural versus urban areas by using spatial principal component analysis (sPCA). Restriction fragment length polymorphism markers or intron sequencing are unable to detect enough polymorphism among closely-related strains such as type II strains, but this is possible with highly polymorphic microsatellite markers such as those used in this study (Ajzenberg et al., 2010). Among the 240 type II strains genotyped with 15 microsatellite markers in this study, 222 different multilocus genotypes were identified with 211 unique genotypes and only 11 genotypes common to at least 2 strains. Based on these data, the Simpson’s Index of Diversity 1 — D (Ajzenberg et al., 2002) is 0.999 which is almost maximal and shows the very high discriminatory power of the microsatellite markers used in this study.

There was no spatial genetic structure for type II strains that infected pregnant women who are living in urban areas in France. This means that people living in an urbanized area may be infected by local strains but also by strains from different geographical regions. These conditions are met in supermarkets and restaurants where food has multiple origins from all over the French territory and also from foreign areas.
Role of funding source and conflict of interest

There was no funding source and the authors have no conflict of interest related to this article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.meegid.2015.08.025.

References


