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Research paper

Genetic analyses of Chinese isolates of *Toxoplasma gondii* reveal a new genotype with high virulence to murine hosts

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ABSTRACT

A great deal of evidence demonstrates that a strongly clonal population structure of *Toxoplasma gondii* strains exists in humans and animals in North America and Europe, while the strains from South America are genetically separate and more diverse. Potential differences in virulence between different strains mean that an understanding of strain diversity is important to human and animal health. However, to date, only one predominant genotype, ToxoDB#9 (Chinese I), and a few other genotypes, including ToxoDB#205, have been identified in China. By using DNA sequence-based phylogenetic analyses, we have re-evaluated the population structure of *T. gondii* strains collected from China and compared them with other global strains. Based on phylogenetic analysis of restriction fragment length polymorphisms, multilocus sequence typing and intron sequences from *T. gondii*, we propose that the Chinese isolates described as Chinese I are divided into two groups called Chinese I and Chinese III. Our results demonstrate that significant differences were found in mouse mortality caused by some Chinese strains, and also the archetypal I, II, III strains in mice. Furthermore, a comparison of cyst loading in the brains of infected rats showed some Chinese strains to be capable of a high degree of cyst formation. Furthermore we show that genotyping using neutral genetic markers may not be a useful predictor of pathogenic phenotypes.

1. Introduction

Toxoplasma gondii is an important, globally distributed, intracellular parasite and provides a valuable model system to understand the evolution of intracellular pathogens. It not only infect large numbers of warm blooded animals including birds, livestock and humans, but also marine mammals (Dubey, 2010; Montoya and Liesenfeld, 2004). Animals and humans are mainly infected by ingesting food or water contaminated with *T. gondii* oocysts or consuming raw or undercooked meat containing parasite cysts (Dubey and Beattie, 1988). The life cycle of the parasite includes domestic cats and other felids as definitive hosts (Frenkel et al., 1970) while virtually all warm-blooded vertebrates can act as intermediate hosts (Dubey and Beattie, 1988). In the intermediate hosts, *T. gondii* undergoes asexual reproduction as either tachyzoites, during acute infection, or bradyzoites (cysts) during chronic infection. In the definitive host it goes through sexual reproduction to produce a high output (many millions daily) of the highly infective oocyst stage.

However, despite the sexual reproductive phase in the life cycle, initially only a few genotypes were recognized in *T. gondii* and were referred to as type I, II, and III. These archetypal types, all together, accounted for 95% of the strains isolated in North America and Europe (Ajzenberg et al., 2002; Howe and Sibley, 1995). In addition, these strains (type I, II, and III) also predominate in chickens from Africa, where a higher prevalence of type II and III were found (Velmurugan et al., 2008). Intriguingly, although the differences at the genomic level among the three main lineages are less than 1%, the virulence phenotypes in mice can differ markedly. Typically, type I strains are uniformly lethal ($LD_{100} = 1$) to mice; in contrast, types II and III strains are less virulent ($LD_{50} \geq 10^5$) (Howe and Sibley, 1995; Khan et al., 2009; Sibley and Boothroyd, 1992). These archetypal strains are typically identified by techniques such as restriction fragment length polymorphism (RFLP) (Pena et al., 2008) or microsatellite analysis (Lehmann et al., 2006). These techniques have been widely used for genotyping a broad range of organisms (Anderson et al., 2000; Cameron et al., 1988; Hide and Tait, 2009; Widmer et al., 2004; Severson et al.,

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1995). In addition, use of these methods have identified other minor subpopulations of *T. gondii*, e.g. the Africa 1 and Africa 3 strains, as well as the major types I, II and III in Africa (Mercier et al., 2010; Rajendran et al., 2012). Using the same approaches, a wide diversity of genotypes have been observed in South America and probably reflects frequent sexual recombination (Khan et al., 2006; Lehmann et al., 2006; Pena et al., 2008; Su et al., 2012). To date, *T. gondii* strains have been categorized into at least six major clades and over one hundred haplotypes (Lehmann et al., 2006; Su et al., 2012). However, data from China has revealed only a few genotypes of *T. gondii* from human and animal hosts, including ToxoDB#205 (designated as Chinese II in this paper) and predominantly Chinese I (ToxoDB#9) (> 73% based on over 60 isolates) (Chen et al., 2011; Dubey et al., 2007; Qian et al., 2012; Wang et al., 2013; Zhou et al., 2009). This dominance of Chinese I occurs across a broad geographical regions (over 640,000 km^2).

Intriguingly, previous studies have demonstrated that the Chinese I isolates vary in their virulence to mice (Chen et al., 2011; Qian et al., 2012; Wang et al., 2013). Some isolates consistently display high virulence, while others show low virulence. This suggests that they could be mutants or possibly distinct strains that have not yet been resolved using traditional approaches. In order to better understand the correlation between virulence and genotype of these Chinese *T. gondii* isolates, a more sensitive and accurate method is needed. Multilocus sequence typing (MLST) is an approach which could create a better dataset by revealing a greater range of possible DNA sequence polymorphisms including SNPs, insertions and deletions among strains (Su et al., 2012). Therefore, here, we re-examined the genotyping of an expanded set of *T. gondii* isolates using classical RFLP as well as MLST and performed further phylogenetic and population genetic analyses to re-evaluate the population structure of isolates in China.

2. Materials and methods

2.1. Animals

Female Swiss Webster mice and female Sprague-Dawley (SD) rats were purchased from the Experimental Animal Center of Sun Yat-Sen University. Animals at age 8–10 weeks (mice, weighing 20–25 g) and 4 weeks (rats, weighing approximately 60 g) were used for experiments. They were routinely maintained in a special pathogen free room with free access to food and water. Protocols for the use of animals were approved by the Institutional Review Board for Animal Care at Sun Yat-Sen University.

2.2. Toxoplasma gondii isolates

A total of 18 Chinese *T. gondii* isolates were included for analysis in this study alongside a bank of reference strains (Table 1). The Chinese *T. gondii* isolates have been genotyped previously (Chen et al., 2011; Qian et al., 2012; Wang et al., 2013). Tachyzoites of *T. gondii* isolates were maintained in mice or cryopreserved in liquid nitrogen. At a late stage of animal infection (3–5 days post-inoculation), animals were euthanized. Ascites were collected by injection of ice cold D-Hanks solution. Two steps of differential centrifugation at 4 °C (50 × *g* for 8 min and 1500 × *g* for 10 min) were applied to remove host macrophages and pelleted tachyzoites (Li et al., 2012; Zhao et al., 2013). Additionally a brief trypsin digestion with 0.25% trypsin and 0.02% EDTA were used to digest residual macrophages, as modified from Derouin et al., 1987 and Wu et al., 2012. *T. gondii* tissue cysts were obtained from the brains of orally infected mice and prepared as previously described (Brinkmann et al., 1987; Letscher-Bru et al., 2003).

2.3. DNA extraction, amplification and sequencing

Genomic DNA was extracted from haploid stages (tachyzoites or bradyzoites within cysts) of *T. gondii* isolates using the Axygen DNA Kit (Axygen, USA). Primers (Table 2) were designed for each MLST marker based on the published sequences in ToxoDB (http://www.toxodb.org/toxo/) and using Primer 3 software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and primers for each RFLP marker were based on previous publications (Grigg et al., 2001; Khan et al., 2005; Su et al., 2006).

PCR amplification was carried out on eight introns using 5 housekeeping genes including the *UPRT*, *MIC*, *BTUB*, *HP* and *EF* genes, exons from rhoptry protein 18 (*ROP 18*) and dense granule proteins (*GRA6* and *GRA7*), and RFLP markers *SAG1*, *SAG3*, *BTUB*, *GRA6*, *c22-8*, *c29-2*, *L358*, *PK1*, *N-SAG2* and *Apico* as described previously (Khan et al., 2007, 2009, 2011). In brief, the target DNA sequences were used for the first amplification with PCR primers for each marker separately and PCR amplified products (1 μl) were then independently used for nested PCR amplification with sequencing primers. Amplicons were purified using the UNIQ-10 Spi Column PCR Product Purification Kit (Sangon, China) and subjected to commercial DNA sequencing (BGI, China) with the sequencing primers.

Nucleotide sequence data reported in this paper are available in the GenBank databases under the accession numbers: KY618681-KY618707; KY628060-KY628212.

2.4. DNA polymorphism analysis

Sequences of classical RFLP typing fragments from representive *T. gondii* strains were downloaded from ToxoDB (http://www.toxodb.org/toxo/). The restriction sites and resultant fragments obtained after digestion were predicted by NEB Cutter (http://nc2.neb.com/NEBcutter2/index.php). The actual electrophoresis patterns were obtained from the literature (Su et al., 2010) where fragments of similar molecular weight or small fragments (< 40 bp) could not be distinguished.

The similarity coefficient (SC) between samples was calculated using the formula $SC_{XY} = 2n_{XY}/(n_X + n_Y)$ (Nei and Li, 1979), where SC_{XY} represents the similarity coefficient of taxa X and Y, n_{XY} is the number of common fragments for taxa X and Y, and n_X or n_Y is the number of specific fragments for taxon X or Y, respectively. The genetic distance (GD_{XY}) was generated using the formula $GD_{XY} = 1 - SC_{XY}$. SC and GD were calculated using data from each RFLP gene and combined later both as a simple average and as a weighted mean of all genes (by number of fragments). These were denoted $SC_{average}$ and $GD_{average}$ and $SC_{weighted}$ and $GD_{weighted}$ respectively.

Nucleotide sequences were obtained from PCR-RFLP or MLST from our results or taken from ToxoDB (http://www.toxodb.org/toxo/) and were compiled and aligned with Clustal X 1.83 (Thompson et al., 1997) using default parameters and further manual verification. A substitution model and the gamma distribution shape parameter for the rate of heterogeneity among sites were determined using Modeltest 3.07 (Posada and Crandall, 1998) based on Hierarchical Likelihood Ratio Tests (hLRTs).

The p-distance model was selected for the phylogenetic analysis. Genetic relationships among haplotypes were reconstructed using the neighbour-joining (NJ) and maximum parsimony (MP) methods implemented in MEGA 4.0 (Saitou and Nei, 1987; Tamura et al., 2007) and bootstrap values were estimated with 1000 replicate searches to evaluate support for the NJ/MP trees. Pairwise genetic differentiation between T. gondii genetic types was estimated using the fixation index (F_{ST}), and statistical significances were tested with 10,000 permutations and F_{ST} calculations was performed in Arlequin 3.5 (Excoffier and Lischer, 2010).

In addition, genealogical relationships were examined by constructing haplotype networks in Network 5.0.0.0 using the median – joining network approach (Bandelt et al., 1999) with Maximum Parsimony (MP) calculation (Polzin and Daneshmand, 2003).