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Is there more than One Species in the Genus *Toxoplasma* ??

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A complete life cycle for the ubiquitous protozoan parasite *Toxoplasma* was proposed over 25 years ago. Since that time, despite attempts to make the genus polyspecific, there has been only one species, *Toxoplasma gondii*, consistently recognised in the genus. Recent studies on taxa in genera closely related to *Toxoplasma* such as *Neospora*, *Hammondia*, *Frenkelia*, *Isospora* and *Sarcocystis*, have convincingly showed the need for a reclassification of many of the species in these genera. However, in addition to these genus level studies, over the last 10 years several laboratories have used molecular techniques including isoenzyme electrophoresis, restriction fragment length polymorphism analyses, random amplified polymorphic DNA - polymerase chain reaction, and comparisons of the small subunit ribosomal RNA gene, DNA polymerase alpha intron, and 70 kDa heat shock protein gene nucleotide sequences to investigate the genetic diversity among strains in the species *T. gondii*. Overall, the results of these analyses confirm that the strains in the genus *Toxoplasma* comprise a limited number of clonal lineages, directly correlated with their virulence in mice. The aim of this presentation is to review the molecular research in this area in order to raise the hypothesis that there may be more than one species in the genus *Toxoplasma*, which may contain taxa with distinct and different life cycles.

Keywords : *Toxoplasma*, *Toxoplasma gondii*, Species, Genetic lineages, Virulence, Life cycles

INTRODUCTION

Toxoplasma gondii is an obligately intracellular coccidian parasite of major clinical and veterinary significance because it may cause abortion or congenital disease in humans and domestic animals. It has a global distribution, with roughly 30% of the world's human population infected. The sexual stage of the parasite's life cycle takes place in the intestine of only cats, and humans and other mammals can be infected by ingesting infective oocysts released in cat faeces, or by consuming meat from infected herd animals containing the long-lived tissues cysts [1]. In immunocompetent individuals, infection with *T. gondii* is normally very mild or inapparent in association with the rapidly dividing tachyzoite stage but, following this stage, the parasite converts to the cyst-dwelling, bradyzoite stage and the cysts can be harboured, asymptomatically, within central nervous system, skeletal and heart muscle for life. For a chronically infected individual who develops an immunodeficiency (eg. by immunosuppressive drug therapy or via HIV), the consequences can be dire; up to 40% of AIDS patients are affected by toxoplasmic encephalitis, a disease with a high rate of morbidity and mortality if untreated. Furthermore, if first contracted during pregnancy *T. gondii* can cause stillbirth or foetal abnormalities. Of congenitally infected neonates asymptomatic at birth, 3/4 will develop adverse sequelae such as mental retardation and/or hearing defects later in life and as many as 90% will suffer ocular problems as they grow older (reviewed by [2, 3])

Because of the importance of this coccidian parasite to human and domestic animal medicine, *Toxoplasma* and toxoplasmosis have been the subject of countless research programs over the last 50 years. However, studies on the relationships of the parasite to other coccidian parasites, and of the relationships among the strains of *Toxoplasma* isolated, have only been of major significance in the last decade.

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PHYLOGENY OF THE COCCIDIA

The term coccidia refers to a group of protozoa from the phylum Apicomplexa that corresponds to the suborder Eimeriorina. The scope of the cyst-forming coccidian parasites now encompasses, among others, the genera *Isospora*, *Caryospora*, *Neospora*, *Hammondia*, *Frenkelia*, *Besnoitia*, *Toxoplasma* and *Sarcocystis* (reviewed by [4]). Many species in these genera cause disease in humans and animals and so are of significant medical and veterinary importance. Although this has been recognised since the last century, the biology and taxonomy of the coccidia have been investigated only since the 1960s, and their phylogeny is now being actively investigated [5].

Classification of the coccidia has previously been based on aspects of their host specificities, life-cycles and the morphology of their life-cycle stages (reviewed by [4, 5]). However, it is often unclear what weight should be placed upon these characters as they are limited in their information content. Hence there is controversy surrounding the inter- and intra generic phylogeny of these organisms.

It has been well established that in cases where informative characters are relatively rare or their interpretation is confused, molecular data can provide a robust means of inferring phylogeny [6, 7]. Of particular advantage in resolving a range of controversial phylogenetic proposals has been the use of molecular data generated in our laboratory. For example, the use of 18S ribosomal DNA sequence data has helped us to resolve some controversial phylogenetic proposals [8-12], but has left some relationships unresolved or has identified more ambiguous relationships that require investigation such as the relationship among species of the genera *Sarcocystis*, *Toxoplasma* and *Neospora*. Evidence from 18S ribosomal DNA sequences suggest that the genus *Sarcocystis* may not be monophyletic, but rather paraphyletic [13-15]. In addition, due to the relatively low number of nucleotide changes in the 18S ribosomal DNAs, the relationships among *Sarcocystis muris*, *T. gondii* and *Neospora caninum* have not been definitively resolved [12, 16, 17].

While some clarification of the phylogenetic relationships of the genus *Sarcocystis* has been obtained by using only the phylogenetically informative nucleotide positions that make up the helices in the secondary structure of the 18S ribosomal DNA [18], potential problems associated with the use of a single gene as the only source of data [19] have not been addressed, nor has the problem of scarce informative data been overcome. As Olsen [20] stated "finally there is no substitute for raw data: more information will always yield more reliable phylogenetic inferences."

For these reasons, the phylogenetic relationships of some of the more important members of the cyst-forming coccidia, in terms of veterinary and human health, remain unresolved.

Recently, sequence comparisons of 18S ribosomal RNA genes have highlighted and confirmed the relatively close genetic relationships between *Toxoplasma* and *Hammondia hammondi* [21] between *Toxoplasma* and *Neospora* [12], among *Toxoplasma*, *Sarcocystis* and *Isospora* [22] and between *Sarcocystis* and *Frenkelia* [14, 15] These recent findings, coupled with earlier assessments based on morphology and life cycle [23] mean that the taxa placed into these genera will need to be redefined in the near future. However, even given this necessary redefining of the taxa classified within some of the individual genera, and in fact, even the need for some of the genera, questions over the genetic relationships of taxa currently classified within some of these individual genera still remain unanswered.

GENETIC DIVERSITY IN THE GENUS *TOXOPLASMA*

In 1977, Levine [24] called for a reclassification of the genus *Toxoplasma* to contain seven species, one of which was to be the currently recognised species, *Toxoplasma gondii*. However, this idea gained no significant support, and until recently the genus *Toxoplasma* was considered to contain only the one species, *T. gondii*. Then, over the last 10 years, new technologies in molecular biology have been used to compare the genetic makeup of the strains constituting the current species *T. gondii*. These studies have been instigated largely because the characterised and catalogued strains of *Toxoplasma* can be divided into two broad categories based upon their virulence in mice.

Several laboratories have applied molecular techniques to study the genetic diversity among the strains of *T. gondii*. Darde and colleagues [25-27] have concentrated on comparisons based on isoenzyme electrophoresis. Cristina and co-workers [28, 29], Sibley and colleagues [30-32], Asai and colleagues [33], Parmley and colleagues [34], Brindley and colleagues [35] and Literak and co-workers, [36] have used restriction fragment length polymorphism analyses. Meisel and colleagues [37], Rinder and co-workers [38], Homan and colleagues [39] and my co-workers [40-43] have used gene sequence comparisons. In addition, my laboratory has used random amplified polymorphic DNA polymerase chain reactions for the genetic comparison of *T. gondii* strains [44-46].

Comparisons of the ribosomal RNA gene sequences [41] and internal transcribed spacer 1 sequences [39] of virulent and avirulent *T. gondii* strains found few meaningful differences, indicating a relatively close genetic relationship among the strains of *T. gondii*. In an early restriction fragment length polymorphism study [30], Sibley and Boothroyd found that mouse virulent strains of *T. gondii* were clonal, while the avirulent strains were moderately polymorphic. However, this study used a low number of strains and few loci. Later restriction fragment length polymorphism studies using more strains and larger numbers of loci [31],the results of the studies using isoenzyme electrophoresis [25-27], and the results of random amplified polymorphic DNA polymerase chain reaction analyses [43-46] are in general agreement supporting the hypothesis that the genus *Toxoplasma* can be subdivided into a limited set of genetically distinct lineages of approximately similar genetic divergence. In nature, the strains of *T. gondii* fall into two distinct groups based on virulence for mice [47], and although there are clear differences among strains within the lineages, there are a number of loci that show a nearly complete distinction between virulent parasites and avirulent parasites. These were recently reviewed [48], but since then there have been another two studies reported that highlight the two clonal lineages in *Toxoplasma*.

One of these studies arose from our own studies implicating the 70 kDa heat shock protein in both parasite virulence and immunity to *T. gondii*. Our initial characterisation of heat shock proteins expressed in the virulent RH strain and the avirulent ME49 strain of *T. gondii* focussed on Western blotting of protein from tachyzoites grown in mice. Whilst the 65 kDa heat shock protein was expressed at similar levels in the virulent RH and the avirulent ME49 strain, the 70 kDa heat shock protein was expressed at much higher levels in the RH strain compared with expression in the ME49 strain. Western blot analysis for two other virulent strains and two other avirulent strains resulted in similar findings, confirming differences in 70 kDa heat shock protein expression of virulent and avirulent strains of *T. gondii*. As it was necessary to immunocompromise the mice for cultivation of tachyzoites of only the avirulent strains, we suggested that 70 kDa heat shock protein expression in virulent strains may be induced by one or more factors of the host immune response to *T. gondii*, and that this induced expression of 70 kDa heat shock protein may enable virulent strains to proliferate as acute infections. This hypothesis was supported by the fact that neither virulent nor avirulent *T. gondii* strains expressed 70 kDa heat shock protein in cell culture [49].

We have recently compared 70 kDa heat shock protein gene structure in virulent and avirulent *T. gondii* strains. Primers were designed by alignment of nucleotide sequences of 70 kDa heat shock protein-encoding genes of related parasites available in GenBank and used to polymerase chain reaction amplify genomic DNA of a virulent and an avirulent *T. gondii* strain. As a result, we obtained the entire sequence of the 70 kDa heat shock protein gene from the virulent RH strain and the avirulent ME49 strain for comparison [42].

Copy number studies showed that the 70 kDa heat shock protein gene was single copy in both virulent and avirulent strains. Northern blot analysis of 70 kDa heat shock protein mRNA expression revealed that there is little difference in transcriptional regulation between virulent and avirulent *T. gondii* strains. This suggests that it is unlikely that differences in Heat Shock Factors, Heat Shock Elements or the promotor region of 70 kDa heat shock protein can account for differences in expression of the protein in

virulent and avirulent strains, so it is probably due to post-transcriptional regulation.

Sequence analysis of six virulent and six avirulent strains revealed identity at the amino acid level except for a seven-peptide repeat unit (GGMPGGM) at the 3'- end of the gene. Thus, all avirulent strains have five copies of this repeat unit and all virulent strains have only four copies. Since this is the only notable difference between the 70 kDa heat shock proteins of avirulent and virulent strains, it seems reasonable to conclude that the deletion of this one GGMPGGM repeat unit has some significant impact on expression of the protein. Similar repeat units (mainly of GGMP) are found in 70 kDa heat shock proteins of other protozoa [50, 51] but no specific function has been ascribed to this sequence motif in parasites. GGMP repeats are predicted to adopt secondary structures consisting of a series of turns [52] and our computational analysis predicts that there are sufficient secondary structural differences between 70 kDa heat shock proteins of virulent and avirulent strains for us to believe that there may be differences in protein stability. This could account for the differential expression of 70 kDa heat shock protein observed in virulent and avirulent strains of *T. gondii*.

In a second research program on *Toxoplasma* molecular biology, we have recently cloned and sequenced the gene encoding the DNA polymerase alpha of the avirulent Me49 strain of *T. gondii* [53]. This gene has three introns of 339, 422 and 652 bp in size. The nucleotide sequence of the largest of these, termed, IA, was determined for 10 strains of *T. gondii*: five virulent strains, four avirulent strains, and the mutant S48 strain [40]. The IA intron in all the virulent strains was 654 bp long while the avirulent IA introns was 652 bp long. The four avirulent strains were found to contain an *Nde*I restriction enzyme site, producing two fragments 467 bp and 374 bp, in size. The five virulent stains and the mutant strain did not have this restriction enzyme site. Not only does this restriction fragment length polymorphism provide an ideal way to discriminate avirulent and virulent strains of *T. gondii* without the need to test the strains in vivo, but it further confirms the two clonal lineages in the genus *Toxoplasma*, directly correlated with murine virulence.

TOXOPLASMA SEXUAL LIFE CYCLE

It is traditionally recognised that the sexual life cycle of *T. gondii* in nature is via the intestinal epithelium of the cat [1]. This life cycle was confirmed in the early 1970's (reviewed by [5]). Strains of *T. gondii* are therefore practically divided into two categories based on their virulence for mice and whether or not they can undergo a sexual cycle in the intestinal epithelium of the cat. Avirulent strains are defined as those where the intraperitoneal injection of large doses such as 10^4 tachyzoites into mice cause no significant symptoms, but cysts form in the brains of these chronically infected mice. Virulent strains are defined as those where the intraperitoneal injection of less than 100 tachyzoites causes acute symptoms leading to death of the mice within about a week. Dubey [54] and Pettersen [55] have suggested that the mouse virulence of *T. gondii* strains can be laboratory manipulated, but we do not know if there is a definite change in the parasite's genotype associated with these phenotype changes that are altered by laboratory manipulation.

The host mouse strain can have a marked affect on the virulence of the *Toxoplasma* strain injected [56], the number and virulence of the infecting strain and the route of infection, and factors such as the hormonal and immune status of the host, all play a role in determining the symptoms suffered by the host (reviewed by [2]). However, even when all these factors are taken into account, these is/are some specific genetic characteristic(s) within the parasite itself that defines the parasite as being either mouse virulent or mouse avirulent.

DIFFERENT SPECIES ??

Based on the molecular characterisations of *Toxoplasma* strains described above, it was suggested [57] that it was not possible to conclusively determine whether the virulent and avirulent lineages of *Toxoplasma* corresponded to two groups of closely related clones or sexual cryptic species, although the former hypothesis was more likely. However, the virulent clonal lineage could be defined as a separate species if the members of it were found to correlate with some significant biological or medical characteristics, and scientists working in the area thought it worth-

while [57]. However, Howe and Sibley [31] and Boothroyd [47] recognised the genetic distance between the two lineages, but concluded that because there has been some (albeit infrequent) genetic exchange between the lineages in the wild, they are not true species.

However, there are pragmatic problems with accepting only a rigorous standard for a species definition formulated over 50 years ago, and their has been much debate on the definition of species [58-61]. In fact, the two genetic groups of *Toxoplasma* do seem to fit at least several possible definitions of a species [62]. Of course, evolution is a continuing process, and the research on the lineages of *Toxoplasma* has occurred over an extremely small time period compared with the amount of time that has passed since the two lineages may have diverged. In fact, it is generally acknowledged that the lineages would have split and speciation occurred only recently, it indeed they had speciated [47, 63]. The infrequent sharing of genetic information may mitigate against speciation, but it could be argued that the molecular data are consistent with the hypothesis that the two clonal lineages of *Toxoplasma* are evolving to the point where they will eventually (but not perhaps in our lifetime) become two distinct, reproductively isolated species. Usually this is the level where the discussion stops, because it is seen to be academic theory that has little or nothing to do with the clinical or diagnostic aspects of toxoplasmosis, however, the infrequent sexual recombination between the two lineages means that the least some members of the virulent lineage may have their own asexual life cycle in nature.

An hypothesis to explain a natural life cycle for the virulent lineage can be based on several characteristics of *T. gondii* , some of which appear to be well documented in the scientific literature but have largely been forgotten or overlooked (reviewed in [48]).

The recent identification [64] of the dog as the definitive host of the closely related coccidian *N. caninum*, means that the life cycle of *N. caninum* may not be exclusively by repeated vertical transmission [65, 66], as earlier hypothesised. However, if even only a very small number of isolates of *N. caninum* multiply by repeated vertical transmission, and then even infrequently, it at least raises the possibility that repeated vertical transmission could also be a natural life cycle for virulent *T. gondii* strains ? This means that they would not need to undergo sexual recombination with avirulent *T. gondii* strains, which would define the two lineages as different species. Sexual recombination of avirulent *T. gondii* strains has been found after simultaneous ingestion of two different strains of cysts in the laboratory [67]. However, more such experiments investigating sexual recombination between virulent and avirulent *T. gondii* strains are essential before we can definitively say that the results of the molecular characterisation studies suggesting that the two lineages of *T. gondii* are genetically distinct, consistent with an hypothesis of them being different species, are invalid.

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