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Mini Review

Animals are key to human toxoplasmosis

Dirk Schlüter[a,b,*], Walter Däubener[c], Gereon Schares[d], Uwe Groß[e], Uwe Pleyer[f], Carsten Lüder[e]

[a] Institute of Medical Microbiology, Infection Control and Prevention, Otto-von-Guericke-University, Magdeburg, Germany
[b] Helmholtz Centre for Infection Research, Braunschweig, Germany
[c] Institute of Medical Microbiology and Hospital Hygiene, Heinrich-Heine-University, Düsseldorf, Germany
[d] Institute of Epidemiology, Friedrich-Loeffler-Institute, Greifswald-Insel Riems, Germany
[e] Institute for Medical Microbiology, University Medical Center, Göttingen, Germany
[f] Eye Clinic, Charité Universitätsmedizin, Berlin, Germany

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ABSTRACT

Toxoplasma gondii is an extremely sucessfull protozoal parasite which infects almost all mamalian species including humans. Approximately 30% of the human population worldwide is chronically infected with *T. gondii*. In general, human infection is asymptomatic but the parasite may induce severe disease in fetuses and immunocompromised patients. In addition, *T. gondii* may cause sight-threatening posterior uveitis in immunocompetent patients. Apart from few exceptions, humans acquire *T. gondii* from animals. Both, the oral uptake of *T. gondii* oocysts released by specific hosts, i.e. felidae, and of cysts persisting in muscle cells of animals result in human toxoplasmosis. In the present review, we discuss recent new data on the cell biology of *T. gondii* and parasite diversity in animals. In addition, we focus on the impact of these various parasite strains and their different virulence on the clinical outcome of human congenital toxoplasmosis and *T. gondii* uveitis.

Introduction

Toxoplasma (*T.*) *gondii* is an extremely successful obligate intracellular protozoan parasite (phylum apicomplexa, subclass coccidia), which infects almost all species of mammals and birds on all continents. Although most infections are clinically asymptomatic, the parasite can induce severe disease in selected human and animal populations. *T. gondii* cell biology and parasite/host interaction are widely studied and have led to the recent discovery of different *Toxoplasma* strains and genotypes, important new insights into the life style of intracellular pathogens and a better understanding of the immunological control of intracellular persisting pathogens. In this review, we will focus on the mechanisms of parasite persistence in its host, the transmission of *T. gondii* from animals to humans, the importance of different *T. gondii* genotypes, and two important clinical presentations of human toxoplasmosis, i.e. ocular and congenital toxoplasmosis, which still pose major medical problems. In this review, we will not focus on immune responses to toxoplasmosis and disease in HIV-infected patients, as these have been excellently reviewed before (Dupont et al., 2012; Montoya and Liesenfeld, 2004).

Life cycle of Toxoplasma gondii

A key factor to understand the biology and clinical relevance of *T. gondii* is the parasitic life cycle. *T. gondii* exists in different stages: *Oocysts* are the product of the parasite's sexual cycle in the intestine of its definitive host, the felidae (cat family). Oocysts are excreted in cat feces and following sporulation in the environment, sporozoites become infective (Fig. 1). Upon oral uptake of sporulated oocysts by new hosts, sporozoites transform to the invasive *tachyzoite* stage. Tachyzoites actively penetrate all nucleated cells and replicate rapidly in an intracytoplasmic vacuole. Following repeated intravacuolar replication, host cells are disrupted and tachyzoites invade neighbouring cells. The tachyzoite form causes tissue destruction and is therefore responsible for clinical manifestations of the disease. The ensuing immune response is accompanied by the transformation of tachyzoites into slowly replicating intracellular *bradyzoites* that form persisting *cysts* (Lyons et al., 2002; Weiss and Kim, 2000). Tissue cysts, which are found in the retina, brain, skeletal and heart muscles, are the infective stages for intermediate and definitive hosts via

* Corresponding author at: Institute of Medical Microbiology, Infection Control and Prevention, Otto-von-Guericke-University Magdeburg, Germany.
Tel.: +49 (0)391 6713393; fax: +49 (0)391 6713384.
E-mail address: dirk.schlueter@med.ovgu.de (D. Schlüter).

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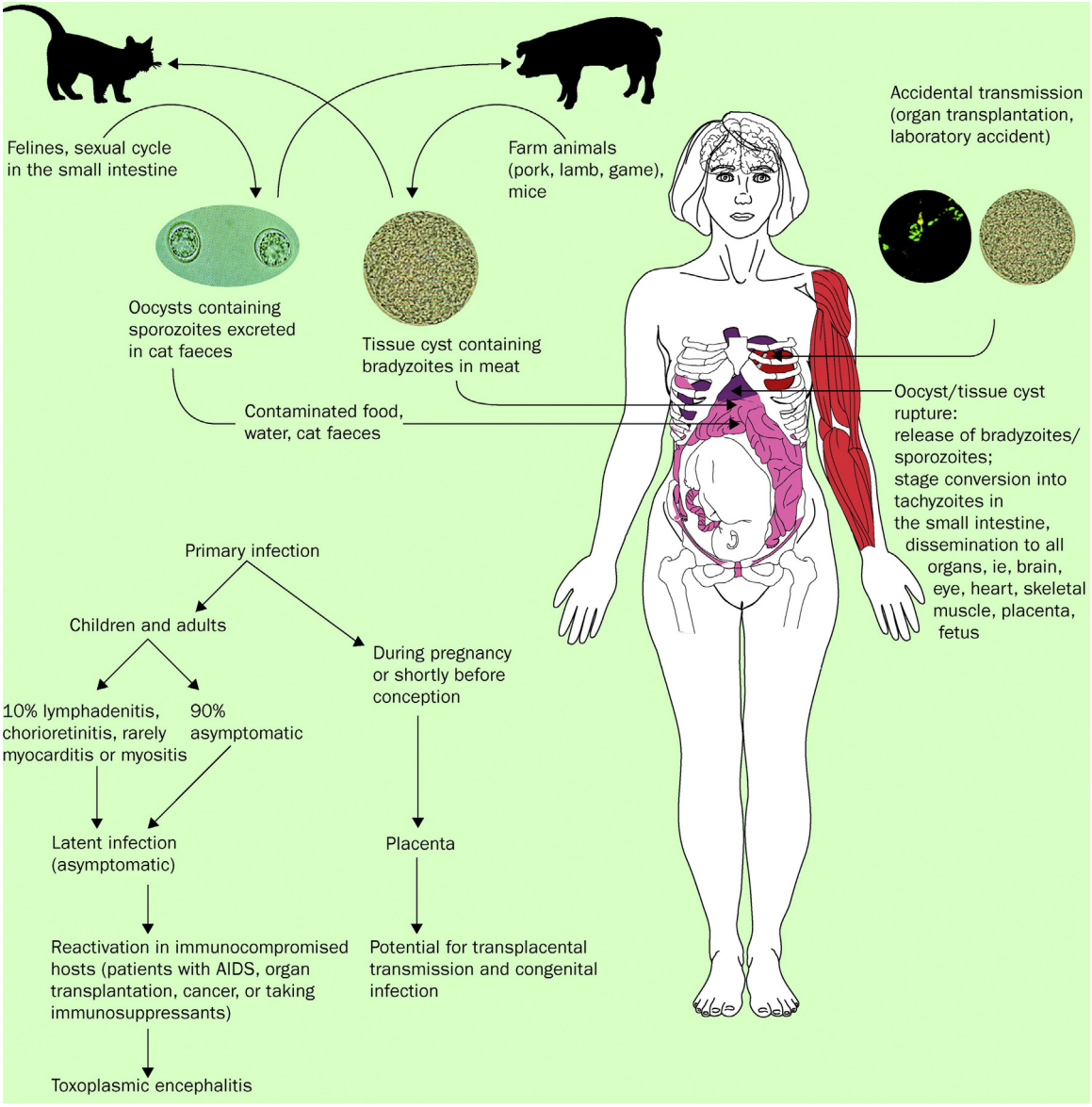

Fig. 1. Life cycle of *T. gondii*; routes of parasite transmisson and pathology after infection [reproduced with permission from Montoya JG & Liesenfeld O; Lancet 2004].

consumption of muscle or brain tissue (Fig. 1). Similar to oocysts, infective tachyzoites develop from bradyzoites, which are released from lysed cysts in the intestine. Humans can get infected by consumption of undercooked cyst-contaminated meat products or by sporulated oocysts (Fig. 1), which can be found in water, soil or vegetables. Since humans are a dead end in the life cycle of the parasite, the natural cycle comprising domestic animals and wildlife is crucial for survival of *T. gondii* and human infection. Thus, human toxoplasmosis would not exist without animals.

Mechanisms of parasite persistence

A European multi-centre study suggested that 30–63% of primary infections in pregnant women result from the consumption of raw, cured or undercooked meat products (Cook et al., 2000). Eating meat contaminated with persisting bradyzoites has also been identified as a significant risk factor for acquiring a *Toxoplasma* infection in the USA (Jones et al., 2009; Jones and Dubey, 2012). In addition, persisting parasites in brain, retinal and muscle tissue and their reactivation underlies the pathogenesis of cerebral and ocular toxoplasmosis (OT) and *Toxoplasma* polymyositis in immunocompromised patients (Montoya and Liesenfeld, 2004). Thus, parasite persistence in wildlife and livestock is a key factor for parasite transmission from animals to humans and for pathogenesis of human disease, since human is the “dead end” in *Toxoplasma* life cycle. Parasite persistence includes at least three distinct processes: (i) differentiation of the rapidly multiplying tachyzoite stage to the metabolically inactive bradyzoite stage (a process called stage conversion), (ii) formation of intracellular tissue cysts, and (iii) long-term survival in the immunocompetent host.

Although tissue cysts can be found in multiple organs *in vivo*, they are more prevalent in neural and muscular tissues, i.e. brain, eyes, skeletal and cardiac muscle (Dubey et al., 1998; Tenter et al., 2000). This can be either due to a preferred bradyzoite formation in these tissues or alternatively to an equal rate of stage conversion in all tissues, but a preferred long-term survival of tissue cysts in neural and skeletal tissues while parasites are largely eliminated in other organs. Stage conversion appears to be developmentally regulated since tachyzoites which emerge from infections with bradyzoites or sporozoites start to differentiate to bradyzoites following ~20 cell divisions (Jerome et al., 1998; Radke et al., 2003). Bradyzoite formation is preceded by a slower growth rate

and a prolongation of the parasites' cell cycle with appearance of a population of near-diploid parasites (DNA content 1.8-2N), i.e. in the interphase (G2) of the cell cycle (Radke et al., 2003). Since bradyzoites in mature tissue cysts are uniformly haploid (1N), it is assumed that parasites in G2 are intermediate stages which undergo mitosis and cytokinesis before entering a cell cycle-arrested G1/G0 phase (Radke et al., 2003).

Stage conversion is associated with up- and down-regulation of numerous stage-specifically expressed genes (Behnke et al., 2008; Naguleswaran et al., 2010). They encode various surface proteins, heat shock proteins, enzymes including some glucose metabolism enzymes, excretory-secretory proteins, cyst wall proteins, transcription factors and others (Manger et al., 1998; Naguleswaran et al., 2010; Radke et al., 2005). Recently, transcription factors with AP2 DNA binding domains have been identified, which are up-regulated during bradyzoite development (Radke et al., 2013; Walker et al., 2013). Among them, TgAP2IX-9 and TgAP2XI-4 are able to regulate expression of bradyzoite-specific genes in a negative or positive manner, respectively. This indicates that both transcriptional repressors and activators are required for regulating stage conversion. The transcriptional reprogramming during the tachyzoite-to-bradyzoite transition is also governed by extensive chromatin remodelling (Bougdour et al., 2009; Hakimi and Deitsch, 2007), and factors which regulate the chromatin state in *T. gondii* have been identified (Naguleswaran et al., 2010; Olguin-Lamas et al., 2011; Saksouk et al., 2005).

In vitro, bradyzoite formation can be induced or accelerated in the presence of external stress. This has led to the assumption that stage conversion is a response of the parasite to a hostile environment. Bradyzoite formation *in vitro* is associated with phosphorylation of eIF2α (eukaryotic initiation factor-2), which favours expression of stress-response and bradyzoite-specific proteins (Narasimhan et al., 2008; Sullivan et al., 2004). Among them, heat shock proteins including BAG1 (also designated HSP30), HSP70 and HSP90 are upregulated or differentially localized during stage differentiation (Bohne et al., 1995; Echeverria et al., 2005; Parmley et al., 1995; Weiss et al., 1998). Whether external stress is indeed required and does suffice to trigger bradyzoite formation is however unknown. Proinflammatory cytokines including IFN-γ induce bradyzoite formation in mouse macrophages *in vitro* (Bohne et al., 1994; Ferreira-da-Silva Mda et al., 2008) and might represent physiological stressors for triggering stage conversion *in vivo*. However, IFN-γ is ineffective in triggering stage conversion in other cell types including human fibroblasts, rodent neurons, astrocytes, microglia or skeletal muscle cells (Jones et al., 1986; Lüder et al., 1999; Takacs et al., 2012). Therefore we rather favour the hypothesis that the extensive morphological, transcriptional and biochemical remodelling that accompanies differentiation of *T. gondii* imposes significant pressure on the parasite which requires an adequate stress response.

We and others have recently shown that *T. gondii* efficiently transforms to bradyzoites in skeletal muscle cells (SkMC) (Ferreira-da-Silva Mda et al., 2009; Guimaraes et al., 2008; Swierzy et al., 2014; Takacs et al., 2012). Importantly, such stage conversion is observed without applying external stress. This suggests that *T. gondii* can differentiate spontaneously to bradyzoites depending on the cell type it resides in and supports the hypothesis that stage conversion is indeed differentially triggered in various host tissues. Remarkably, the differentiation state of SkMC was critical in inducing stage conversion. Whereas polynucleated and cell cycle-arrested myosin heavy chain-positive myotubes readily supported stage conversion, undifferentiated, proliferating myoblasts did not (Swierzy et al., 2014). Comparative transcriptomics indicated that expression of bradyzoite-specific gene expression was also readily induced in primary embryonic neurons but not in astrocytes or fibroblasts (Swierzy, Händel, Schlüter and Lüder, unpublished data). This opens the intriguing possibility that *T. gondii* senses a distinct environmental factor, which is particularly present in terminally differentiated, long-lived SkMC and neurons and which then initiates stage conversion. Artificial overexpression of cell division autoantigen (CDA)-1 suffices to efficiently induce stage conversion in human fibroblasts, and knockdown of CDA-1 abrogates stage conversion of *T. gondii* that has been triggered in these cells by a trisubstituted pyrrole (Radke et al., 2006). CDA-1 has pro-fibrotic and anti-proliferative activities (Pham et al., 2010) and inhibits cell growth through up-regulation of the cell cycle inhibitor $p21^{Waf1/Cip1}$ (Tu et al., 2007). Interestingly, the mouse ortholog of CDA-1, namely Tspyl2, is up-regulated during differentiation of SkMCs and knockdown of Tspyl2 in SkMC inhibits myotube formation and bradyzoite formation (Swierzy and Lüder, unpublished data). This indicates that Tspyl2 itself or a Tspyl2-regulated factor triggers spontaneous stage conversion of *T. gondii* in SkMC. High levels of Tspyl2 in SkMC and neurons might thus explain the preferred occurrence of *T. gondii* tissue cysts in neural and muscular tissues.

Persisting *T. gondii* bradyzoites are located in a robust intracellular structure, the so-called tissue cyst. Tissue cysts gradually increase in size and mature; young tissue cysts may contain only 2 bradyzoites and measure 5 μm in diameter whereas after several weeks to months, they may increase to sizes of 40–100 μm and may contain up to ~1000 bradyzoites (reviewed by Dubey et al., 1998). The cyst wall is believed to confer physical strength avoiding cyst rupture and may protect bradyzoites from hostile environmental conditions including host immune defences. It consists out of the parasitophorous vacuole membrane and membrane-bound vesicles embedded in a layer of granular material beneath the membrane. Recently, the cyst wall protein CST1 has been identified as being critical for the cyst wall integrity, resistance against mechanical stress, and bradyzoite persistence (Tomita et al., 2013).

Epidemiology and parasitology of *T. gondii* in animals

Risk of human infection by cats (oocysts)

An important factor for the transmission of *T. gondii* to humans is the number of infected felids and resulting oocyst prevalence in the environment. Remarkably, a single cat can pass more than 100 million non-sporulated oocysts, which become infective within 1 to 5 days after. Seropositivity rates in wild felids are in general very high and may be close to 100%, whereas the global seroprevalence for *T. gondii* in domestic cats is 30–40% (Elmore et al., 2010). Survival of sporulated oocysts in the environment is favoured by humidity and under optimal conditions sporulated oocysts may remain infective for more than 1 year. This might be one reason why seroprevalence in humans is higher in humid tropical as compared to dry climate.

Noteworthy, the quality of water plays another important role in human infection with oocysts. Use (particularly drinking) of unfiltered surface water bears a high risk of infection, especially in countries with a humid climate and high numbers of cats, which may contaminate water with oocysts. Interestingly, contaminated water has been reported to be a source of small epidemics of OT (Bowie et al., 1997; de Moura et al., 2006). Whether this correlation between oocyst contaminated water and OT really indicates that this mode of transmission favours the development of OT in comparison to other routes of oocyst ingestion is at present unclear and requires further epidemiological studies.

Several studies analysed risk factors for human infection with oocysts. These studies identified several specific risk factors for oocyst infection including: playing in sand boxes and school playground (dos Santos et al., 2010), contact to soil and gardening without gloves (Cook et al., 2000), contact with contaminated water

(Bahia-Oliveira et al., 2003), insufficient washing of vegetables and fruits (Kapperud et al., 1996a). Only cooking (>55 °C) but not freezing or disinfection reliably destroys sporulated oocysts, which have contaminated vegetables and other food. In water, oocysts remain infective for long time and are not reliably destroyed by freezing and moderate temperatures, chemical and physical treatments including chlorination and ozone treatment (Dumetre et al., 2008).

The prevalence of *T. gondii* cysts in wildlife and livestock and the consumption of raw meat are major factors influencing the rate of human infections with *T. gondii*. In the following chapter, the prevalence of *T. gondii* in livestock and wildlife animals is discussed in detail.

Risk of human infection by wildlife and livestock (bradyzoites and tachyzoites)

Toxoplasma gondii infects in addition to humans a wide range of warm-blooded animals world-wide (Dubey, 2010). Both, domestic as well as wildlife animals are important as reservoirs for *T. gondii* but also as sources for human infection via the ingestion of viable tissue cysts (Dubey, 2010; Kijlstra and Jongert, 2008). Risk factor studies for humans suggest that in addition to pork, also meat from ruminants, including mutton or lamb and also beef might significantly contribute to human infection (Baril et al., 1999; Cook et al., 2000; Kapperud et al., 1996b; Kijlstra and Jongert, 2008). One epidemiological study observed that also the consumption of meat from other sources including venison, horse, rabbit, whale, and game birds was associated with an increased risk for humans infection (Cook et al., 2000). Overall findings suggest that not all animal species are of the same relative importance as sources for human infection. This relative importance is likely to be influenced (i) by the prevalence of infection in a given animals species, (ii) the potential of *T. gondii* to establish as tissue cysts in this species, (iii) the density of such tissue cysts in meat, and finally (iv) the regional preferences of humans regarding the sources and the preparation of meat. Especially the preference to consume raw or undercooked meat is regarded as the most important risk factor for humans to become infected by *T. gondii* (Baril et al., 1999; Cook et al., 2000; Kapperud et al., 1996b).

In Germany, about 60% of the meat consumed is pork, 20% poultry and about 15% beef but only about 1% is coming from sheep and goats. (http://www.lfl.bayern.de/mam/cms07/iem/dateien/teilauszug_vieh_und_fleischr.pdf). Nevertheless, sheep and goats are highly susceptible to *T. gondii* infection and recent seroprevalence studies conducted in Europe (Bartova et al., 2009; Dumetre et al., 2006; Fusco et al., 2007; Halos et al., 2010; Jokelainen et al., 2010; Klun et al., 2006; Mainar et al., 1996; Opsteegh et al., 2010; Vesco et al., 2007) showed high prevalences of *T. gondii* antibodies in sheep and goats.

Relatively high *T. gondii* seroprevalences are also reported for cattle world-wide (Dubey, 2010) and the consumption of beef was identified as a potential risk factor for human infection (Baril et al., 1999; Cook et al., 2000). Although *T. gondii* DNA is rarely detectable in European cattle (Opsteegh et al., 2011b; Wyss et al., 2000), occasionally high numbers of DNA-positive calves were observed (Berger-Schoch et al., 2011). Although, viable parasites could not be detected in beef of naturally infected cattle in Germany and in North America (Dubey et al., 2005a,b; Hellmann and Tauscher, 1967) it is necessary to further analyze the relative importance of beef for human *T. gondii* infection.

Pigs are highly susceptible for *T. gondii* infections (Dubey, 2010) but modern industrialized pig production no longer favors *T. gondii* to establish its lifecycle on farm. However, a study in The Netherlands showed that on farms that are producing pork under animal friendly condition, i.e. by providing access of animals to enclosures outside of a barn, unusual high seroprevalences of 2.6% on organic and 5.6% on free-range farms could be observed (Giessen et al., 2007). In addition, relative high seroprevalences were recently observed on German conventional farms in fattening pigs 2.5% (Görlich, 2011, Dissertation; http://d-nb.info/1012834549). The relative importance of pork for human *T. gondii* infection should be regarded as high also due to the fact that it is not an unusual habit to consume raw pork in Europe, especially Germany.

Poultry is also susceptible for *T. gondii* (Dubey, 2010) and studies conducted world-wide suggest that especially in elder chicken the prevalence of *T. gondii* infection is high (Dubey, 2009). Most of the poultry meat is consumed cooked or fried. However, there is an increasing proportion of poultry used for the production of raw meat products like sausages which might represent sources of infection for humans. Although most of the chicken and turkey production is highly industrialized, a recent German study indicated a surprisingly high *T. gondii*-seroprevalence of 20.2% in turkey (Koethe et al., 2011) and high *T. gondii* seroprevalences of 5.7% and 25.2% were also observed in German ducks and geese (Maksimov et al., 2011).

As stated above, the consumption of meat from other sources than ruminants, pigs and poultry including venison and game birds was associated with an increased risk for seropositivity (Cook et al., 2000). Seroprevalence studies conducted in wild animals showed high sero-prevalences in wild boars in Germany (Lutz, 1997) which were further confirmed by other studies in neighboring countries (Antolova et al., 2007; Bartova et al., 2006; Beral et al., 2012; Opsteegh et al., 2011a). Our own studies conducted in foxes, which might – due to their carnivorous life style – be exposed to *T. gondii* infecting a large variety of wild animals, also revealed a high seroprevalence and in many foxes *T. gondii*-DNA was detected (Herrmann et al., 2012b).

To reduce the risk of humans becoming infected via meat of *T. gondii* infected domestic animals, it is necessary to implement efficient measures on the farm level to exclude the transmission of infectious *T. gondii* via water, fodder or other routes into livestock animals. Information on putative on-farm risk and protective factors of infection were obtained by epidemiological studies in pigs (Görlich, 2011, Dissertation; http://d-nb.info/1012834549; Davies et al., 1998; Garcia-Bocanegra et al., 2010a, 2010b; Giessen et al., 2007; Klun et al., 2006; Meerburg et al., 2006; Villari et al., 2009; Weigel et al., 1995), sheep and goats (Klun et al., 2006; Lopes et al., 2010; Tzanidakis et al., 2012; Vesco et al., 2007) and ducks or geese (Maksimov et al., 2011). These studies revealed a large number of specific farm-related factors which may favor *T. gondii* infection in livestock animals. Risk factors often identified in these studies were the number or presence of cats on farms (Görlich, 2011, Dissertation; http://d-nb.info/1012834549; Garcia-Bocanegra et al., 2010a, 2010b; Lopes et al., 2010; Meerburg et al., 2006; Skjerve et al., 1998; Vesco et al., 2007; Weigel et al., 1995). On pig farms, poor rodent control (Garcia-Bocanegra et al., 2010b; Villari et al., 2009), or a high *T. gondii* seroprevalence in mice (Weigel et al., 1995) turned out to be an important risk factor, too. Rodents are potential intermediate hosts for the parasite and their presence could either increase the risk of farm cats shedding *T. gondii* oocysts or – on the other hand – these rodents could serve as a directly source of infection for the omnivorous pigs. Other factors not only observed on pig farms but also for sheep and goats were related to feeding and watering practices and measures to prevent contamination of fodder and equipment with *T. gondii* (Görlich, 2011, Dissertation; http://d-nb.info/1012834549); Lopes et al., 2010; Meerburg et al., 2006; Tzanidakis et al., 2012; Vesco et al., 2007; Villari et al., 2009).

Although a large number of information is already available for the implementation of efficient control measures on farms, important information on the safety of certain dietary products is still scarce. Thus, infected goats and sheep may shed *T. gondii* in milk (Fusco et al., 2007; Sacks et al., 1982; Skinner et al., 1990), and,

therefore, the safety of sheep and goat milk and milk products has to be further studied.

Different *T. gondii* sero- and genotypes in animals

Due to the large variety of farm and wild animals infected in Europe, it is open whether there are differences in the *T. gondii* genotypes observed in various animal species. However, Europe is dominated by the *T. gondii* clonal lineage, II (Table 1) independent whether farm or wild-life animals were analyzed. In addition to clonal type II also type III and mixed or atypical genotypes were observed in a small number of domestic as well as in wildlife animals (Table 1). Genotyping is biased by the restriction to those animals and tissues in which high concentrations of *T. gondii*-DNA are available or from which the parasite could be *in vitro* isolated. Based on the very limited genetic diversity between lineages in Europe, it was possible to identify peptide sequences unique for the individual *T. gondii* lineages (Kong et al., 2003; Maksimov et al., 2012b; Morisset et al., 2008; Peyron et al., 2006; Sousa et al., 2008) which may allow to extent typing studies also to non-diseased but infected individuals. Several working groups have developed tools to use peptides with type-specific sequences for the examination of type-specific serological reactions i.e. for serotyping *T. gondii* infections. By serotyping a dominance of type II, specific antibody response was observed in cats in Germany (Maksimov et al., 2013) which is in accord with result obtained during genotyping studies (Herrmann et al., 2010, 2012a).

Immunity to *T. gondii*

In general, primary *Toxoplasma* infection is asymptomatic in most humans. In contrast, severe disease may develop in immunocompromised individuals. Clinical cases of toxoplasmosis dramatically increased with the AIDS pandemic and mainly manifest as *Toxoplasma* encephalitis. In the vast majority of AIDS cases, toxoplasmosis results from the reactivation of intracerebrally persisting cysts. Toxoplasmosis may also become reactivated in recipients of bone-marrow and solid organ transplantation. In the latter, cysts may be transferred with the donor organ, especially heart, and become reactivated in the immunocompromised recipient.

The high incidence of reactivated toxoplasmosis in immunocompromised patients illustrates that immunity to *T. gondii* is mainly mediated by T cells which are primed and activated by interleukin-12-producing dendritic cells. Clinical and experimental studies have shown that parasite-specific $CD4^+$ and $CD8^+$ T cells produce interferon-γ, which induces anti-parasitic effector molecules in infected cells. In addition to T cells, B cells and *Toxoplasma*-specific IgM, IgG, IgA and IgE antibodies contribute to protection and prevent parasite transmission to fetuses in primary infection of pregnant women.

Most data on immunity to *T. gondii* are derived from experimentally infected mice. However, the effector mechanisms directed against *T. gondii* in mice and men are quite different. For example, the induction of the inducible nitric oxide synthase (iNOS) is more intense in murine than in human cells (Däubener et al., 2009). *In vivo*, iNOS activity in murine cells mediates effects against Type 1 and Type II strains (Meisel et al., 2011; Takacs et al., 2012). In addition, interferon inducible "immune-related GTPases" (IRG proteins, also known as p47 GTPases) are important in the control of type 2 strains but fail to control the proliferation of type I strains in mice. Importantly, immune-related GTPases are represented by 23 genes in the murine genome. In contrast, humans have only a single full-length p47 GTPase, which carries no interferon-inducible elements in the promoter and which is expressed constitutively in the mature testis (Bepken et al., 2005). Therefore, p47 GTPases are not responsible for the inhibition of *T. gondii* growth in man (Spekker et al., 2013; Niedelman et al., 2012; Hunn et al., 2011). In human cells, the induction of the IFN-γ induced tryptophan degrading enzyme indoleamine 2,3-dioxygenase is an important antimicrobial effector mechanism, but up to now no direct anti-parasitic effects of this enzyme were detected in experimental settings using murine cells (Pfefferkorn, 1984; Heseler et al., 2008). Thus, the most frequently used animal model is not indicative for the course of *T. gondii* infections in man and animal models that properly mimic human toxoplasmosis are not available as yet.

Human toxoplasmosis

Clinical importance of human toxoplasmosis

Approximately 25–30% of the human population is infected with *T. gondii*. However, seroprevalence varies greatly between different countries (from 10 to 80%) and even within countries. Low seroprevalence has been reported from South East Asia, North America (Dubey and Jones, 2008) and Northern Europe (10–30%). Prevalences between 30 and 50% have been reported for Central and Southern Europe, whereas high seroprevalences are observed in Latin America and in tropical African countries (Robert-Gangneux and Darde, 2012). Of note, seroprevalence increases with age due to the lifelong risk of infection.

In immunocompetent children and adults primary toxoplasmosis is asymptomatic in most patients. Only some patients develop a lymphadenopathy, especially of cervical and occipital lymph nodes. Severe disease including myocarditis, pneumonia, encephalitis, and hepatitis is very rare in immunocompetent patients. Infection of immunocompetent children and adults as well as congenital infection may result in OT. In addition to OT, infected fetuses may suffer from other severe pathologies, especially of the brain. The clinical aspects of OT and congenital infection will be discussed in detail, since several new findings suggest that diagnostics, patient management and therapy can be improved in these patient groups.

Congenital toxoplasmosis

Primary *Toxoplasma* infection acquired during pregnancy might result in congenital toxoplasmosis. Clinical symptoms of congenital toxoplasmosis vary from mild to severe including abortion. The classical triad of hydrocephalus, intracerebral calcification and chorioretinitis is rare and in many cases clinical symptoms including chorioretinitis and mental retardation become apparent months or years after birth. The incidence of congenital toxoplasmosis has been evaluated in different countries and is thought to be 3.3 per 10,000 births in France and 1 per 3000 births in Brazil (Neto et al., 2000; Villena et al., 2010). The time point of maternal toxoplasmosis plays an important role for the risk and severity of fetal infection. The risk of fetal infection increases but the severity of clinical symptoms declines over time.

If timely diagnosed and treated, diaplacental transmission of the parasite and clinical manifestations in the newborn can significantly be reduced (Hotop et al., 2012). Since indicative symptoms in adult woman are very rare, diagnosis is mainly enforced in cases where ultrasound analyses result in conspicuous findings or is just based on serological screening during pregnancy. However, such screening programs currently exist only in France and Austria. Diagnosis of acute toxoplasmosis during pregnancy is challenging because IgM antibodies either might persist at low concentrations for months if not years or might be unspecific especially if directed against low-molecular weight antigens of *T. gondii* (Dao et al., 2003). In this case, the use of recombinant antigens in serological assays,

Table 1
Genotyping results in animals from Europe.

Country	Number analysed	Genotype				Animal	Method (# loci)	Reference
		Type I	Type II	Type III	Atypical/mixed			
CH	1	0	1	0	0	Cat	RFLP (11)	[Spycher et al., 2011]
FIN	6	0	6	0	0	Cat	MS (7)	[Jokelainen et al., 2012]
CH	1	0	1	0	0	Oocysts, cat	RFLP (11)	[Berger-Schoch et al., 2011]
GER	22	0	22	0	0	Oocysts, cat	RFLP (4–9)	[Schares et al., 2008], [Herrmann et al., 2010]
ITA	10	1	0	7	2	Goat	RFLP (11)	[Mancianti et al., 2013a]
AUT	19	0	19	0	0	Chicken	RFLP (9)	[Dubey et al., 2005a]
ITA	3	0	3	0	0	Chicken	RFLP (9)	[Dubey et al., 2008b]
POL	2	0	0	0	2	Chicken	RFLP (9)	[Dubey et al., 2008b]
CH	5	0	4	0	1	Sheep	RFLP (5–11)	[Berger-Schoch et al., 2011]
FRA	8	0	8	0	0	Sheep	MS (5)	[Dumetre et al., 2006]
FRA	46	0	45	1	0	Sheep	RFLP (3) + MS (6)	[Halos et al., 2010]
NOR	55	0	46	7	2	Arctic fox	RFLP (4–11)	[Prestrud et al., 2008b]
NOR	1	0	1	0	0	Arctic fox	RFLP (10)	[Prestrud et al., 2008a]
GER	2	0	2	0	0	Beaver	RFLP (4–9)	[Herrmann et al., 2013]
UK	10	0	10	0	0	Ferret	RFLP (4)	[Burrells et al., 2013]
BEL	26	0	25	1	0	Fox	RFLP (3) + MS (6)	[De Craeye et al., 2011]
FRA	9	0	9	0	0	Fox	RFLP (3) + MS (6)	[Aubert et al., 2010]
GER	26	0	24	1	1	Fox	RFLP (4–9)	[Herrmann et al., 2012a]
ITA	7	0	0	0	7	Fox	RFLP (5–9)	[Verin et al., 2013]
FIN	18	0	18	0	0	Hare	MS (4–6)	[Jokelainen et al., 2012]
UK	5	0	5	0	0	Mink	RFLP (4)	[Burrells et al., 2013]
FRA	1	0	1	0	0	Moufflon	RFLP (3) + MS (6)	[Aubert et al., 2010]
POR	12	1	9	2	0	Pigeons	MS (5)	[Waap et al., 2008]
UK	7	0	6	1	0	Pole cat	RFLP (4)	[Burrells et al., 2013]
FRA	1	0	1	0	0	Red deer	RFLP (3) + MS (6)	[Aubert et al., 2010]
FRA	12	0	12	0	0	Roe deer	RFLP (3) + MS (6)	[Aubert et al., 2010]
FIN	3	0	3	0	0	Squirrel	MS (7)	[Jokelainen et al., 2012]
FRA	21	0	21	0	0	Wild boar	RFLP (3) + MS (6)	[Richomme et al., 2009]
GER	3	0	3	0	0	Wild cat	RFLP (4–9)	[Herrmann et al., 2013]
FRA	1	0	1	0	0	Wild ducks	RFLP (3) + MS (6)	[Aubert et al., 2010]
ITA	3	0	0	0	3	Wild ducks	RFLP (11)	[Mancianti et al., 2013b]
Total numbers	346 (100%)	2 (0.6%)	306 (88.4%)	20 (5.8%)	18 (5.2%)			

Results were selected on those specimens in which T. gondii had been typed in at least four independent loci. Abbreviations: AUT, Austria; BEL, Belgium; CH, Switzerland; FIN, Finland; FRA, France; GER, Germany; ITA, Italy; POL, Poland; POR, Portugal; UK, United Kingdom; RFLP, Restriction fragment-length polymorphism; MS, microsatellite.

determination of IgA antibodies or IgG avidity should be considered. Direct detection of the parasite from amniotic fluid using the polymerase chain reaction (PCR; Wallon et al., 2010) has been placed into the diagnostic algorithm in some countries; however, in Germany, such prenatal diagnosis is only performed in 12.1% of suspected cases (Hotop et al., 2012). Based on low sensitivities, determination of IgM or IgA antibodies from prenatal cordocentesis has not gained acceptance worldwide (Foulon et al., 1999).

Therefore and if not diagnosed before by conspicuous ultrasound findings (e.g., ventricular dilatations, hydrocephalus, or intracerebral calcifications) (Fig. 2), diagnosis of congenital *T. gondii* infection is often based on microbial examinations of the newborn. Detection of specific IgM and/or IgA antibodies during the first weeks or months of life are important mainstays for serological diagnosis (Lebech et al., 1996). However, based on the time of infection during pregnancy, these isotypes might not be detectable shortly after delivery. Therefore, an increase of specific IgG antibodies within the first 12 months of life or IgG persistence beyond this time is proof for congenital infection. When using the immunoblot technique, a direct comparison of IgG reactive protein bands present in the child with those of the mother (comparative IgG profile between mother and child; CGMC test) might eventually allow diagnosis already at the time of delivery (Gross et al., 2000). Definite proof of diagnosis is achieved when the parasite is detected using the PCR (in rare cases, animal inoculation or cell culture-based assays are used as well) in either body fluids (e.g. cerebrospinal fluid or blood) of the child or tissue (e.g. placenta, cord tissue).

An IFN-γ release assay based on crude *T. gondii* antigens is a recently published and promising approach for diagnosing congenital infection in infants. First results indicate a sensitivity and specificity rate well above 90% (Chapey et al., 2010). Like with serological assays, the future application of recombinant parasite antigens might improve the accuracy of this new test format.

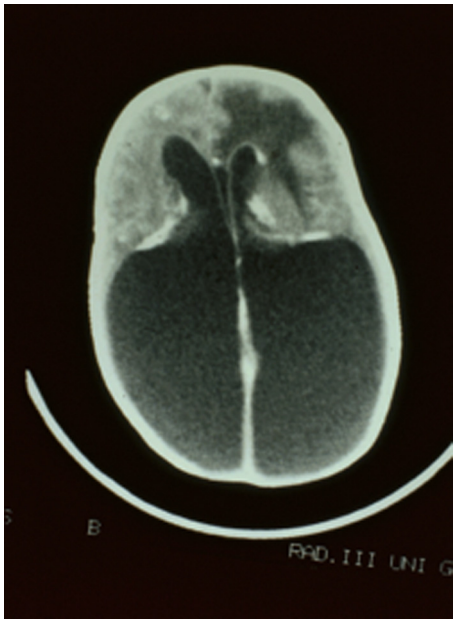

Fig. 2. Massive hydrocephalus with enlargement of the ventricular system in a child with congenital toxoplasmosis. Courtesy of Prof. W. Bommer.

Since decades, treatment of congenital toxoplasmosis in the infant is based on pyrimethamine, sulfadiazine and folinic acid. However, in the absence of large randomized trials, the precise treatment schemes differ not only from country to country but might even be modified from center to center. In many German centers, treatment of congenitally infected, asymptomatic newborns based on the combination of pyrimethamine (1 mg/kg body weight/day) and sulfadiazine (50 mg/kg body weight/day) and folinic acid (2× 3 mg/week) is given for the first 3 months of life. Newborns with discrete symptoms receive this combination with a higher dose of sulfadiazine (100 mg/kg body weight/day) for 6 months. This treatment is extended to 12 months in children with severe symptoms, such as seizures or retinochoroiditis. Irrespective of the duration of therapy, pyrimethamine and sulfadiazine blood concentrations should be regularly determined and possible adverse effects controlled (Hotop et al., 2012). Since this scheme might result in severe neutropenia, an alternative approach has been suggested by a referral center in Toulouse, France, which administers pyrimethamine with sulfadoxine every 2 weeks for 2 years, resulting in less toxicity. First data indicate that the efficacy as judged by the rate of new eye lesions, may even be better (Berrébi et al., 2010). However, since sulfadoxine is not available in Germany, adequate adaptations and modifications of the German treatment scheme will be an open field for future discussions (Pohl-Schickinger et al., 2012).

Ocular toxoplasmosis

Following primary infection of intestinal epithelial cells, *T. gondii* disseminates via the blood stream throughout the host and has the ability to cross vascular barriers, e.g. the blood ocular barrier (Feustel et al., 2012; Lachenmaier et al., 2011). Clinically, *T. gondii* remains a major cause of retina infection (posterior uveitis). Worldwide, the prevalence of OT varies between 4% and 18% of all uveitis patients (Glasner et al., 1992; Jakob et al., 2009). A common feature of the affected patients is their relatively young age (around 25 years).

The typical manifestation of infection with *T. gondii* is its presentation as necrotising retinochoroiditis. Often it has a characteristic clinical presentation to such a degree that further diagnostic workup is not needed (Fig. 3 A, B). Still primary lesions may vary in their appearance regarding size of the lesion, the inflammatory tissue response and secondary retinal vasculitis. This variation is considered to be dependent on parasite virulence, mode of infection e.g. congenital vs. postnatal acquired infection and the hosts immune response (summary Maenz et al., 2014). In the immune competent patient, these active lesions commonly "heal" within 2–4 months with a hyper-pigmented scar as a result of retinal pigment epithelium disruption.

Frequently, acute retinal lesions are associated with adjacent old scars indicating recurrent attacks. The overall recurrences rate in Europe is up to 80% of all OT patients followed for more than 5 years (Bosch-Driessen et al., 2002; Hovakimyan and Cunningham, 2002). In a Dutch study more than 70% of patients with OT at their first clinical visit already presented with a combination of an active lesion with a healed retinal scar. This indicates that previous (peripheral) retinitis often remains unnoticed (Bosch-Driessen et al., 2002). This also indicates that the functional impact of OT is mainly depending on the location of active retinitis. If it is centrally located, legal blindness may result, whereas peripheral lesions may not cause any symptoms.

The global annual incidence of congential toxoplasmosis is estimated to be 190,100 cases (Torgerson and Mastroiacovo, 2013). Therefore, congenital toxoplasmosis remains an important health burden that may lead to dislabeling damage. Interestingly, even in well-documented congenital infection, ocular involvement is often

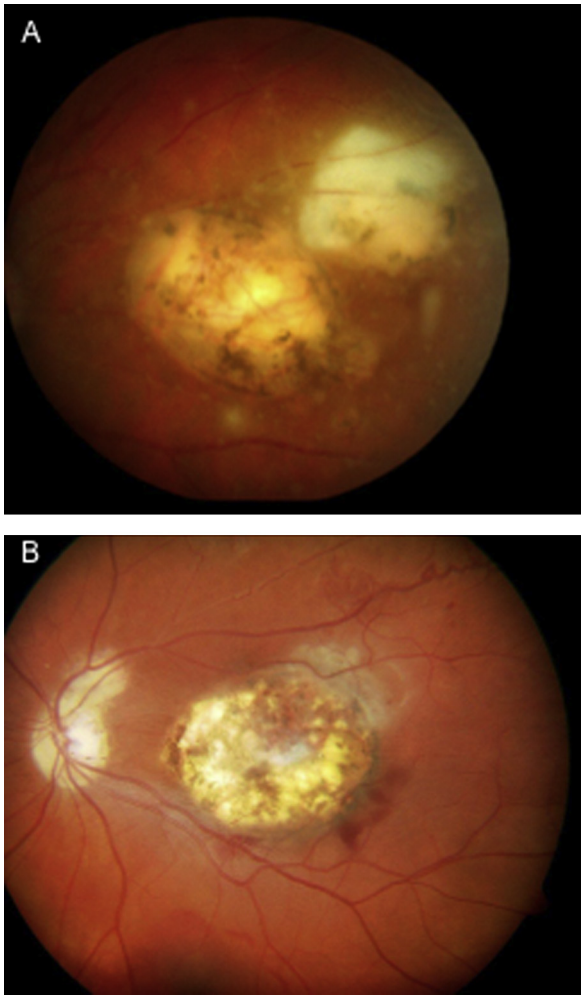

Fig. 3. (A) Fundus photography of a patient with peripheral located retina lesions suggestive for recurrent OT. Notice: Pigmented "old" retina lesion and neighboring active retinitis. Intraocular fluid analysis confirmed the presumed diagnosis by detection of locally produced *T. gondii*-specific antibodies (Goldmann-Witmer coefficient >5). (B) Fundus photography of a 26-year old patient affected by congenital OT. Bilateral central retinochoroidial lesions result in significant vision loss and severe visual field impairment.

delayed in clinical onset. Unfortunately, there exists no validated laboratory test to differentiate the route of infection. In addition, also on clinical grounds it is difficult to clearly differentiate congenital and postnatal acquired OT. Some authors suggested that the often critical central location of retinal infection is more common in congenital OT. This assumption is supported by a prospective longitudinal cohort series of 38 new borns with congenital *T. gondii* infection and ocular involvement. More than 52% developed central retina lesions (Phan et al., 2008). This rate is substantially higher than the expected number if lesions were distributed randomly. Also when retinal lesions are already present at birth, a higher risk ($p < 0.02$) of macular involvement was found during continuous follow up (Faucher et al., 2012). As an explanation for the different anatomic localisations, variations in the micro-vascular supply between the peripheral and central retina have been suggested.

Given the eminent role of the host immune system in *T. gondii* infection, an impact on the disease course can be expected in immunocompromised individuals. In fact, OT is far more severe in immunocompromised individuals. Often they suffer an atypical, fulminant clinical course of OT. Clinical presentations include simultaneous multifocal retinitis, active bilateral lesions and extensive areas of retinal necrosis. Similar problems and atypical

clinical presentations of OT may develop in patients receiving immunosuppressive therapy, e.g. following organ or bone marrow transplantation (Chung et al., 2008). The prevalence of OT in this population at risk is not known, but careful monitoring of infections in this increasing population is advised (Edvinsson et al., 2008).

Also the more severe course of OT in elderly patients has been attributed to alterations in host immunity. Aging is associated with complex changes in both adaptive and innate immune mechanisms that increase the prevalence and severity of many infections. These changes involve several components of the host defence against *T. gondii* including lymphocytes, natural killer cells and macrophages.

Laboratory assays using intraocular specimen are extremely helpful for the definite diagnosis of OT, especially when atypical ocular manifestations hinder the clinical diagnosis. The diagnosis is based either on the determination of intraocular production of *T. gondii*-specific antibodies or the detection of parasite DNA. Briefly, intraocular antibody synthesis is determined by the *Goldmann-Witmer* coefficient (GWC), which is based on the comparison of the *T. gondii*-specific antibodies in aqueous humour and in serum in relation to the immunoglobulin titres in the same fluids (Goldmann and Witmer, 1954). Definite laboratory confirmation of OT is achieved by the detection of *T. gondii* DNA in aqueous humour or vitreous fluid by the polymerase chain reaction (PCR). However, to date, neither the PCR protocols nor the parasite DNA fragment to be amplified have been standardised. When both methods are compared, real-time PCR seems to have an inferior sensitivity (Errera et al., 2011). An important consideration is timing of the diagnostic procedure. Whereas PCR is positive within the first days of clinical onset, the interval between intraocular infection and aqueous humour tap strongly influences the detection of intraocular antibody synthesis (De Groot-Mijnes et al., 2006; Errera et al., 2011; Talabani et al., 2009; Westeneng et al., 2007).

Although OT is a very common and sight-threatening cause of infectious posterior uveitis, treatment remains highly controversial. This relates to several limiting factors. First, in many patients *T. gondii* infection is a self-limited disease that has been considered to need no treatment. Second, the parasites are able to form cysts that are impenetrable to medications and host enzymes, therefore they cannot be eliminated from retinal tissue. However, despite the limited evidence of treatment effects an increasing number of experienced ophthalmologists treats patients with active OT (Holland and Lewis, 2002). Several surveys of uveitis specialists indicate that even experts differ in their therapeutic approaches. Whereas some will only care for sight threatening lesions, others will treat all lesions independent on its location (Basu et al., 2011; Holland and Lewis, 2002; Torun et al., 2008). Despite a lack of published evidence for effectiveness, most ophthalmologists elect to treat patients with OT that threatens visual acuity.

"Classic therapy" consists of oral pyrimethamine and sulfadiazine, plus systemic corticosteroid. Substantial toxicity of this drug combination has spurred interest in alternative antimicrobials, as well as local application of therapeutic agents. At this time, however, no therapeutic approach is curative of OT. In a Cochrane review, Gilbert et al. (2002) identified only three prospective, randomised, placebo-controlled clinical trials. Interestingly, there was a lack of evidence that antibiotics (short- or long-term) prevented vision loss in all three studies. A recent survey confirmed these findings (Kim et al., 2013). Only one study evaluating individuals infected with probably more aggressive South American strains of *T. gondii* demonstrated that long-term antibiotics (14 months) reduced the number of recurrences (Silveira et al., 2002).

Because of toxicity and lack of effectivity, alternative agents including clindamycin (Lakhanpal et al., 1983; Baharivand et al., 2012; Lasave et al., 2010; Soheilian et al., 2011), azithromycin (Rothova et al., 1998) and atovaquone (Pearson et al., 1999) were introduced as treatment, but have not gained widespread acceptance. In addition, trimethoprim/sulphamethoxazole combination has been successfully used as a long term prophylaxis over at least 12 months (Silveira et al., 2002; Felix et al., 2014). However, still significant uncertainty with regard to proper medication by experts in the field exists. This is reflected by several surveys of uveitis specialists in the US, Germany and India, indicating that at least nine separate drugs in even more combinations are currently used in daily practice (Basu et al., 2011; Holland and Lewis, 2002; Torun et al., 2008).

Impact of parasite strain on the course of human toxoplasmosis

The majority of *T. gondii* strains in Europe and North America belong to one of three distinct clonal lineages. The virulence of these different lineages in mice is quite different, high virulent strains were defined as type I strains, while low virulent or avirulent strains were clustered into lineages II or III. It is an attractive assumption that the clonal type of the parasite might also define the virulence of the parasite in human beings (Boothroyd and Grigg, 2002; Saeij et al., 2005). The possibility of genotyping and serotyping *T. gondii* strains in addition to ELISA tests detecting strain-specific antibodies allowed detailed studies on the role of different parasite strains involved in the infection of animals and of human beings (Herrmann et al., 2012a; Maksimov et al., 2012a, 2012b). Studies in Europe and the United States indicate that type II strains were most frequently found in animals and humans (Howe et al., 1997; Fuentes et al., 2001). However, highly virulent type I strains were more frequently detected in congenital infection cases whereas type III strains of *T. gondii* were only rarely found in human diseases (Howe et al., 1997; Fuentes et al., 2001). On the other hand, Ajzenberg et al. (2009) analysed 88 *T. gondii* isolates from immunocompromised patients and did not find significant type specific differences in the outcome of the infections. In addition, the same author did not find type I like strains in specimens from more than 500 patients with differential clinical signs of toxoplasmosis (Ajzenberg, 2010). In summary, no clear evidence has been published that specific forms of clinical disease are associated with specific genotypes of *T. gondii*. This might be explained by the fact that in addition to the genotype of the parasite individual immune reactions might influence the course of the disease.

Most *in vivo* data on the virulence of different *T. gondii* strains were obtained with experimentally infected mice. However, since a direct correlation between the canonical *T. gondii* strains and the severity of infections in human beings is not clearly detectable, it might be of great interest to identify the different *T. gondii* genotypes infecting man. This is important since it was uncovered that a successful reinfection of chronically infected mice by a different *T. gondii* genotype is possible (Dao et al., 2001). This might also have important implications for and lead to a reconsideration of the old thinking that women of child bearing age which were seropositive for *T. gondii* do not transmit the infection to their fetus.

In Europe and in the North America, three major clonal lineages of *T. gondii* dominate the majority of human infections (Howe and Sibley, 1995). Remaining strains belong to other groups and are sometimes summarized as atypical strains. These atypical strains are much more frequently found in other regions of the world, for example in Central and South America (Ajzenberg, 2010). Especially in Brazil, the *T. gondii* population was found to be highly diverse. However, a few successful clonal lineages with different mouse virulence expanded in wide geographic areas (Pena et al., 2008). These local strains in Brazil (BrI-V) are not related to type

II strains, which are predominant in Europe. Meanwhile, atypical strains have been described to cause congenital toxoplasmosis (Boughattas et al., 2011) and symptomatic reactivation in immunosuppressed individuals (Stajner et al., 2013). In addition, recent reports suggest that these atypical strains are able to induce severe toxoplasmosis in immunocompetent adults (Sobanski et al., 2013). Atypical strains might even be able to cause congenital toxoplasmosis occurring in seropositive mothers. In addition the new, local genotypes of *T. gondii* found in Brazil are also capable of causing congenital toxoplasmosis and the strain BrII was found in up to one third of congenital toxoplasmosis cases (Carneiro et al., 2013). It has also been assumed that that pregnant women with immunity against typical genotypes of *T. gondii* may fail to prevent transmission of newly acquired atypical strains to their offsprings (Lindsay and Dubey, 2011).

In France, the majority of congenital toxoplasmosis and OT cases is caused by type II strains (Fekkar et al., 2011). However, antibodies in the serum of German OT patients frequently failed to recognize serotyping peptides. These results were, at least in part, confirmed by multilocus PCR genotyping (Shobab et al., 2013). In addition, it was reported that recurrent OT was more common in patients with nontypeable strains (Shobab et al., 2013). Comparable to these data, more severe congenital toxoplasmosis was associated with non-Type II serotypes in the United States (McLeod et al., 2012). Therefore, evidence accumulates that atypical *Toxoplasma* strains or strains, which could not be serotyped, might more frequently cause severe diseases. However, different geno- and serotyping methods were used and better test systems to positively define the causative *Toxoplasma* strain need to be developed to discriminate between strains of low and high virulence infecting human beings.

The interpretation of clinical symptoms during toxoplasmosis as an indicator of parasite type dependent effects is further complicated by the fact that human beings might be infected by the oral uptake of tissue cysts especially with undercooked meat or by the oral uptake of environmental resistant, sporulated oocysts shed by infected cats. In a given individual it is nearly impossible to distinguish which stage of *T. gondii* was responsible for the infection. Therefore, it is difficult to define a potential different clinical course of toxoplasmosis after infection with oocysts or tissue cysts. However, waterborne transmission of *T. gondii* to humans is probably caused by ooystes derived from infected cats. A contamination of drinking water with sporozoites can cause local outbreaks of toxoplasmosis (Jones and Dubey, 2010; Baldursson and Karanis, 2011). Some waterborne outbreaks have been described in Canada (Bowie et al., 1997), India (Palanisamy et al., 2006; Balasundaram et al., 2010) and Brazil (Vaudaux et al., 2010). A high rate of ocular infections was described for all of these outbreaks. However, these findings were difficult to interpret, since the *T. gondii* strains causing these outbreaks were not defined with a few exceptions (Vaudaux et al., 2010). Thus, even infections with different *T. gondii* strains in a given single outbreak situation are possible. Furthermore, the infection dose taken up by individuals as well as the time point of infection are often unclear and both factors have the potential of modifying the observed clinical signs. Therefore, it remains unclear whether an infection with sporulated oocysts or with tissue cysts causes different courses of clinical toxoplasmosis. Animal models for the comparison of cyst and oocyst infections are available but none of these models is representative for human infections. Only a critical evaluation of future waterborne toxoplasmosis including geno/serotyping of causative *T. gondii* strains will possibly give a hint on how the mode of *T. gondii* infection impacts on the course of disease. Furthermore, novel test systems including the definition of cyst and oocyst specific antigens and the detection of antibodies directed against these specific structures may help to differentiate between oocyst and cyst induced infections in the future.

Conclusions

Due to its ubiquitous distribution and its high prevalence, *T. gondii* remains one of the most important zoonotic agents of high medical and veterinary importance. During recent years, considerable progress has been made in various aspects of the parasite biology including characterization of novel parasite genotypes, evaluating the relative contribution of risk factors for human infection, unravelling novel mechanisms of parasite interaction with its host (cell), and determining parasite and host factors that regulate tissue cyst formation and persistence. However, further efforts are clearly needed to ultimately translate our increased knowledge into an improved health management of toxoplasmosis patients. For example, although current data argue for a contribution of the parasites' genotype on the outcome of infection in humans, no conclusive evidence has been obtained yet. This may be in part due to the fact that genotyping is often not feasible and serotyping is still not a mature diagnostic method often leading to the identification of non-typeable strains only. In addition, the impact of different routes of infection, i.e. oocyst versus tissue cyst infection remains to be evaluated. Finally, the contribution of the hosts' (immune) responses particularly during OT and OT recurrence needs to be further delineated. Further progress in these questions may lead to the definition of prognostic markers for severe outcomes of infection and ultimately enable an individualized health care of toxoplasmosis patients.

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