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Review paper

Toxoplasma gondii infection in pigs in Serbia

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ABSTRACT

Toxoplasma gondii is a common zoonotic intracellular parasite in livestock raised for human consumption and is a public health concern. The mode of transmission is ingestion, and meat is considered to be a major vehicle for human and animal infection. As *T. gondii* is environmentally transmissible, other important vehicles in particular for animals include vegetation, soil and water. The seroprevalence of *T. gondii* infection in pigs is Serbia has been determined in several studies over the past two decades. It has been established that it varies considerably, primarily based on husbandry, with strictly to mostly indoor animals having a lower prevalence (below 20%) than animals raised outdoors, where prevalence exceeds 60%. Experimental data suggests that different genotypes of the parasite vary in virulence, but the significance of virulence in terms of pathology and disease manifestations is still being investigated. Genotypes of *T. gondii* isolated from pig tissues in Serbia to date are ToxoDB#1 (archetype II) and ToxoDB#2 (archetype III). Archetype II is predominant and, based on historical reports and recent findings, low to intermediately virulent. The virulence phenotype and mechanisms of archetype III, however, have not been extensively studied, but recent data suggests that its virulence may vary considerably. This review will also summarize the current knowledge regarding the virulence of archetypes II and III and evaluate it in the context of the pig host.

1. Introduction

Toxoplasma gondii is a food and waterborne protozoan parasite of notable significance to public health, as it can infect all warm-blooded species and cause chronic disease, ranging from mild in immunocompetent hosts, up to severe in hosts with inadequate (newborns), or suppressed immunity (*Djurković-Djaković*, 1998; *Montoya and Liesenfeld*, 2004; *Bouwknegt et al.*, 2018). Host immunity alone, however, is not sufficient to predict disease severity, as pathology depends significantly on the genotype of the parasite, which is defined most commonly by multilocus (11 loci) restriction fragment length polymorphism analysis (PCR-RFLP), multi-locus sequence typing (MLST) (10–11 loci) and microsatellite typing (15 loci) (*Ajzenberg et al.*, 2010; *Su et al.*, 2010). Aside from these defined loci, other undefined and unique single nucleotide polymorphisms (SNPs) scattered throughout the genome

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can endow individual strains with slightly different biological characteristics which modify their virulence for particular hosts. Domestic animals raised for meat are of paramount importance for the transmission of the parasite to humans and are, thus, often the focus of epidemiological studies to determine *T. gondii* infection prevalence and if possible, isolate strains and identify the genotype. The lion's share of the data on genotypes from domestic animals in Serbia are based on isolates from pigs (15/70), which are only two genotypes, ToxoDB#1 (archetype II) and ToxoDB#2 (archetype III) which may have varying degrees of virulence. Symptoms of the disease are generally non-specific and can occur only during the short acute phase of infection, while the chronic phase is clinically inapparent. Aside from known outcomes of infection in most animal species, such as abortion and/or stillbirth which can result from a primary infection in early gravidity, chronic disease in pigs is thought to be essentially without sequelae. This review will summarize our current knowledge regarding *T. gondii* infection with ToxoDB#1 and ToxoDB#2 in pigs.

1.1. Life cycle of T. gondii

The life cycle is complex and involves intermediate and definitive hosts (Felidae only) and progression through three infective life stages, the tachyzoite, the bradyzoite and the sporozoite (*Hutchison et al*., 1969, *Tenter et al*., 2001). Tachyzoites, which are essential for infecting host cells and propagation through tissues, can only be naturally transmitted vertically (from the mother to the foetus), while bradyzoites and sporozoites are transmitted by food and water. In all hosts, tachyzoites convert to bradyzoites after several rounds of asexual division and form cysts, most often located in neurons and myocytes. Tissue cysts increase in size slowly as the bradyzoites divide and persist essentially for the lifetime of the host. Oocysts form after sexual reproduction of the parasite in the gut of the definitive hosts and are shed in faeces, while maturation (sporulation) of oocysts, which facilitates the development of sporocysts and sporozoites, occurs naturally in the environment (*Frenkel et al.* 1970, F*erguson et al*., 1974). Definitive hosts are solely responsible for the contamination of the environment with oocysts, while the robustness of the oocyst wall ensures their maturation and survival after various physical and chemical stresses (*Yilmaz and Hopkins*, 1972, *Dubey*, 1998).

1.2. Transmission modes and vehicles

T. gondii is naturally transmitted by ingestion only or is transmitted vertically from the mother to the foetus (*Tenter et al.,* 2001). Other ways of direct host-to-host transmission include organ transplantation and transfusions. Meat (muscle tissue) containing tissue cysts has repeatedly been shown to be the major source of human infection in Europe, while vegetables and greens contaminated with oocysts are secondary and water is an exceedingly rare vehicle (*Bobić et al.*, 1998, *Deng et al.*, 2021). As bradyzoites can be killed by heat, freezing and salting (dehydration), human infection is due to improper preparation of meat and/or meat products or culinary habits, such as consumption of raw meat dishes, or the inadequate washing/cleaning of vegetables and greens, that fail to remove oocysts. In animals, meat is a primary vehicle for strict carnivores, while strict grazers acquire infection from vegetation and/or soil and omnivores and birds may be infected via either. Water is a common vehicle for all animals. Commercial diagnostic kits for the detection of specific anti-*T. gondii* antibodies in sera are not capable of distinguishing whether infection has occurred due to ingestion of bradyzoites or sporozoites, and thus, the vehicle and source of infection are mainly inferred through epidemiological questionnaires and evidence from scientific studies on the presence of the parasite in the environment. Determining transmission routes and attributing the source of infection have been shown to be important in explaining uncharacteristically severe disease and pathology in several human cases around the globe (*Carme et al*., 2009; *Schumacher et al*., 2021).

1.3. Population structure and genotype virulence

Populations of genotypes of *T. gondii* which circulate in the domestic and sylvatic environment are distinct, and sylvatic genotypes appear to be more virulent (*Dardé*, 2008; *Galal et al*., 2019). Although the genus *Toxoplasma* consists of a single species, the global population structure is complex, with over three hundred genotypes derived from multiple lineages, grouped into six clades (*Shwab et al*., 2014). The three globally distributed lineages (types) are I, II and III represented by reference genotypes ToxoDB#10, ToxoDB#1 and ToxoDB#2, respectively, also known as the archetypes. Archetype II (ToxoDB#1) is the most frequent genotype isolated from humans and animals in Europe,

followed by archetype III (ToxoDB#2), while archetype I (ToxoDB#10) is extremely rare. In Serbia, archetype II (ToxoDB#1) represents 33% of the genotype population in intermediate hosts, while archetype III (ToxoDB#2) represents 9% (*Uzelac et al.*, 2021). The three archetypes differ in virulence, and their pathology phenotype, lethal dose (LD) and mortality in laboratory mice has historically served to experimentally define the three virulence phenotypes: low (archetype III)-$LD10^5$, up to 30% mortality; intermediate (archetype II)-$LD10^3$, 30–99% mortality; and acute (archetype I)-$LD10^1$, 99–100% mortality (*Su et al.*, 2002). Although the phenotype definition is based on a single host species, the archetypes' virulence can be replicated in other species, but not the LD or mortality. However, recent findings suggest that perhaps only the acute phenotype is well defined, as the virulence phenotype and parameters of all of the global archetype I isolates are nearly identical in most laboratory mouse strains, while the virulence phenotype and parameters of the other two archetypes can differ from the definition.

1.4. Virulence of ToxoDB#1 and ToxoDB#2

Recently, the virulence phenotype of a ToxoDB#1 isolate from human fluids in Serbia was shown to be low, without mortality in Swiss-Webster mice, while an isolate of ToxoDB#2 from a free-ranging Iberian pig in Spain (TgPigSp5) was shown to have an intermediately virulent phenotype with nearly 90% mouse mortality, which is exceedingly high (*Fernández-Escobar et al.,* 2020; *Uzelac et al.,* 2020). Some of the differences can be attributed to adaptations induced by laboratory conditions like in vitro culture in rapidly growing cell lines and repeated freezing and thawing of parasite stocks over many years, all of which can change the replication rate, an important factor which contributes to virulence (*Saeij et al*., 2005). This could be the most plausible explanation for this Serbian isolate, given that it was tested against a laboratory-adapted strain, the reference strain ME49, which has a much higher proliferation rate than most other ToxoDB#1 isolates and thus may cause greater mortality. Interestingly, however, the virulence of archetype III may be naturally variable, as both the highly virulent TgPigSp5 and another isolate from a sheep in Spain (TgShSp24), which killed over 20% of mice, were low-passage isolates with different growth rates, thus excluding laboratory adaptation as a possible confounding factor (*Fernández-Escobar et al.*, 2020; *Fernández-Escobar et al.*, 2021).

1.5 Virulence in the pig host

Archetype III (ToxoDB#2) is less frequently isolated from human fluids and tissues but seems to be globally frequent in animals. In Serbia, archetype III (ToxoDB#2) has been detected in pigs, golden jackals and foxes, while archetype II has been found in all thus far examined host species, wild and domestic, including humans. Archetype II (ToxoDB#1) is predominant in pigs, which is expected given the population structure of *T. gondii* in Serbia (Table 1). Pigs are an important link in the transmission of *T. gondii* to humans, as they are

Table 1. *Toxoplasma gondii* seroprevalence and population structure in pigs in Serbia

Sample origin	N of pigs	Seroprevalence (%)	N of isolates	Genotype	References
Farms; Abattoirs	605	28.9	Not stated	-	*Klun et al.,* 2006
Abattoirs	488	9.2	Not stated	-	*Klun et al.,* 2011
Private slaughterhouse	18	66.7	3	ToxoDB#1 (n=3)	*Kuruca et al.,* 2016; 2019
Abattoirs	182	17	6	ToxoDB#1 (n=4) ToxoDB#2 (n=2)	*Kuruca et al.,* 2017; 2019
Abattoirs	825	16.5	6	ToxoDB#1 (n=6)	*Betić et al.,* 2022; *Uzelac et al.,* 2023
TOTAL	**2118**	**18.8**	**15**	**ToxoDB#1 (n=13)** **ToxoDB#2 (n=2)**	

omnivorous and, thus, can ingest both tissue cysts and oocysts, and depending on husbandry, may traverse between the domestic and sylvatic environments (*Klun et al.*, 2011; *Kuruca et al.*, 2017; *Uzelac et al.,* 2023).

2. Materials and methods

All published papers on *Toxoplasma gondii* infection in pigs in Serbia in the last 17 years have been included in this review and the data on seroprevalence and isolated parasite strains have been re-analysed in the context of interpreting *T. gondii* archetype II and III virulence in the pig host.

3. Results and discussion

The cumulative seroprevalence of *T. gondii* infection in Serbian pigs is 18.8%. The lowest seroprevalence of 9.2% was observed in market-weight pigs slaughtered in the Belgrade area abattoirs (*Klun et al.,* 2011), and the highest, of 66.7%, in an outdoor herd of indigenous Mangulitsa breed pigs butchered at a private slaughterhouse (*Kuruca et al.,* 2016). To date, 15 isolates have been obtained from pig tissues, and only two genotypes, archetype II (ToxoDB#1) and archetype III (ToxoDB#2), have been identified (*Kuruca et al.,* 2019; *Uzelac et al.,* 2023).

All sampled pigs were visibly healthy, i.e., in addition to being accompanied with a valid transport manifest and veterinary health certificate, they had been examined for the presence of clinical signs prior to slaughter. The fact that a certain number of animals harboured latent *T. gondii* infection is not surprising, since a number of research studies have shown that adult pigs in the majority of cases do not develop overt clinical signs of toxoplasmosis, thus appearing healthy at routine health check-up. Globally, pigs, especially adults, are considered fairly resistant to toxoplasmosis (*Stelzer et al*., 2019), with the exception of several recent reports from China, and one from Italy, where clinical disease was observed, with dyspnoea being the most common symptom (*Dubey et al*., 2020). Suckling piglets are more susceptible, and in pregnant sows infection can cause congenital disease and piglet mortality.

There are, however, sporadic reports on clinical toxoplasmosis in pigs from Europe. Severe cases of toxoplasmosis were reported nearly 30 years ago on fattening farms in Italy, where pigs exhibited fever, depression and cyanosis, followed by death 2–4 days later (*Gelmetti et al.,* 1999). Another more recent case was recorded in Germany, with dyspnoea and sudden death in a young pig, which was, however, concurrently infected with porcine circovirus-2 (*Klein et al.,* 2010). Unfortunately, as parasite isolation and genotyping was not performed, it is impossible to know whether these were caused by the two archetypes, II and III. All other reported clinical cases in pigs originate from China or South America, where atypical and/or highly virulent *T. gondii* strains are known to circulate (*Galal et al.,* 2019; *Dubey et al.,* 2020). Circulation of atypical, recombinant and variant genotypes, possibly virulent to certain hosts, in wildlife in Europe has been reported (*Fernández-Escobar et al.,* 2022). As *T. gondii* is a generalist parasite (i.e., of low host specificity), it is expected that infected intermediate hosts of any species may transmit the infection to another, provided the chance. Pigs arguably may be particularly susceptible to wildlife strains as outdoor rearing and semi-feral husbandry is practiced in many countries in Europe, thus allowing for strain transfer between ecosystems. A recent report on *T. gondii* infection in wild boars in Italy identified the presence of atypical genotypes, thus possibly explaining some of the deaths in earlier years (*Sgroi et al*., 2020).

Genotyping of parasite strains or their DNA in asymptomatic slaughter animals was performed in several studies. In Portugal, as in Serbia, type II and III were detected (*de Sousa et al.*, 2006). In France (*Djokic et al.*, 2016), Czech Republic (*Slany et al*, 2016) and Belgium (*Gisbert Algaba et al.*, 2020), only archetype II was detected; while in Poland, only partial genotyping was accomplished (*Sroka et al*., 2020). Interestingly, several studies in Italy, including both those with complete and incomplete typing, showed or indicated the presence of more abundant genetic diversity, with DNA from all global lineages (I, II, III) detected in pigs from organic as well from intensive type farms (*Bacci et al*., 2015; *Papini et al*., 2017; *Santoro et al*., 2017; *Pipia et al.,* 2018; *Vergara et al*., 2018).

Of note, in cases of abortion, stillbirth, or premature birth of piglets, the aetiological agent is sometimes unidentified. However, even if *T. gondii* infection had been detected by serology or at necropsy in these cases, parasite isolation is not routinely performed, and therefore, there is no possibility to identify potential genotypes. Genotyping is a technique still associated primarily with scientific research and is not a part of a diagnostic and/or pathological workup, even in human cases, while exper-

imental protocols for virulence determination have only been standardized a few years ago (*Saraf et al.*, 2017). In Serbia, there are no records of *T. gondii*-induced clinical or congenital disease in pigs to date, despite a rather high prevalence particularly in some herds. However, parasite isolation, genotyping and experimental determination of virulence would need to be performed in order to confirm disease aetiology and ascertain the virulence of certain genotypes for pigs. Thus, major conceptual and practical changes in the current standards in pig rearing and veterinary check-ups are necessary in order to raise farmer awareness of *T. gondii* infection and communicate the importance of diagnostics in cases of abortion and mortality among piglets and adults, in order to gain a better understanding of the true cost of toxoplasmosis in pigs. Moreover, once again, the importance of an interdisciplinary approach and cooperation between scientists, veterinarians, policy makers and pig farmers is emphasized.

All *T. gondii* genotypes cause infection that can be clinically severe in the immunologically incompetent, including the foetus (congenital toxoplasmosis). As viable tissue cysts have been isolated from practically all muscles and organs of experimentally infected pigs (*Dubey*, 1988), and up to day 875 post infection, which is much longer than the age at which market-weight pigs are brought to slaughter (at 6–8 months), these data point to a realistic and very present risk of infection for the consumers.

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