Toxoplasma gondii in wild boars and domestic pigs in Sweden

Implications for food safety

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Abstract
Ingestion of undercooked meat is a major risk factor for human toxoplasmosis, a disease caused by the zoonotic protozoan parasite Toxoplasma gondii. Infected pork, mutton and game meat are considered important infection sources in Europe. This thesis aims to describe the epidemiology of T. gondii in two possible key species for human toxoplasmosis in Sweden, namely domestic pigs and wild boars.

Approximately half of the wild boars investigated were found to be T. gondii seropositive, and the seroprevalence was dependent on age, geographical region, and year of sampling. In conventional fattening pigs the seroprevalence was estimated to 1%, compared to 8% in pigs raised in organic management systems with pasture access (KRAV). A one-month increase in pasture access was associated with an 80% increase in the odds of being seropositive. This strengthens the hypothesis that pigs become infected via their natural rooting behaviour. The high level of T. gondii infection in wild boars, and the increasing demand for outdoor management systems for pigs, emphasise the need of consumer awareness of proper cooking procedures to prevent infection.

Other means to limit meat-borne transmission could include abattoir-based serological screening of pigs to reduce the amount of T. gondii infected meat distributed to consumers. Since it has been suggested that such screening systems could be based on meat-juice serology, a study was conducted to investigate aspects of the sampling and preparation of meat-juice samples. It was found that antibody levels in meat juice varied depending on the muscle used for extraction, and in meat juice obtained from the diaphragm, levels of anti-T. gondii IgG correlated poorly with levels in serum. However, the level of anti-T. gondii IgG in meat juice was associated with the total level of IgG, which could possibly be used to assess if enough antibodies have been distributed to the meat-juice sample for the test result to be reliable. However, implementation of meat-juice serology in risk-management programmes needs to be preceded by further evaluation and standardisation of sampling, extraction, and testing procedures.

Keywords: Toxoplasma gondii, wild boar, pig, meat juice, serology, organic

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Dedication

To my mother, for making this possible. And to Jonas and Bosing for supporting me all the way.

“If God didn’t want us to eat animals, why did he make them out of meat?”
Homer Simpson

“Never argue with an idiot. They will only bring you down to their level and beat you with experience.”
George Carling
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List of publications

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Abbreviations

2-ME  2-mercaptoethanol
AHD  Alkaline haematin detergent
CI   Confidence interval
CrI  Credibility interval
DAT  Direct agglutination test
DNA  Deoxyribonucleic acid
DT   Dye test
ECDC European Centre for Disease Prevention and Control
EFSA European Food Safety Authority
ELISA Enzyme-linked immunosorbent assay
FCI  Food chain information
Hb   Haemoglobin
IFAT Indirect fluorescent antibody test
IgG  Immunoglobulin G
IgM  Immunoglobulin M
KRAV Control Association for Alternative Cultivation (translated)
MAT  Modified agglutination test
NUTS Nomenclature of territorial units for statistics
OD   Optical density
P30  Tachyzoite surface antigen (SAG1)
PCR  Polymerase chain reaction
PI   Post infection
SAG1 Tachyzoite surface antigen 1 (P30)
Se   Sensitivity
Sp   Specificity
WHC  Water holding capacity
1 Introduction

The zoonotic protozoan parasite *Toxoplasma gondii* is the causative agent of toxoplasmosis. This infection can lead to abortion in primarily infected pregnant women and to neurological abnormalities in congenitally infected children (Olariu *et al.*, 2011). Life-threatening, acute or reactivated, *T. gondii* infection is a concern in immunosuppressed patients, e.g., those undergoing immunosuppressive cancer therapy, and transplant recipients (Derouin & Pelloux, 2008). Further, over the last few decades, *T. gondii* has been associated with behavioural changes and psychiatric illness such as schizophrenia, implying that latent toxoplasmosis may be a greater problem than previously recognised (Sutterland *et al.*, 2015). However, whether a causal relationship exists between *T. gondii* infection and psychiatric illness remains to be established.

The cost and suffering of human *T. gondii* infection is increasingly acknowledged (Batz *et al.*, 2012; Havelaar *et al.*, 2012; EFSA, 2007), indicating the benefits of limiting the spread of infection from possible sources such as food-producing animals. To succeed in this, control measures must be targeted to where they are most efficient, and this requires knowledge of the epidemiology of *T. gondii*.

As consumption of undercooked pork is suggested as one of the most important sources of human *T. gondii* infection (Tenter, 2009; Tenter *et al.*, 2000), the work presented in this thesis was performed to fill some of the knowledge gaps regarding the epidemiology of *T. gondii* in populations of wild and domestic pigs in Sweden. Attention is also drawn to the question of how *T. gondii* may efficiently be monitored during meat inspection of slaughtered pigs, with focus on the use of meat juice as a matrix for serological analysis.
2 Background

2.1 Toxoplasma gondii – history and life cycle

*T. gondii* is an intracellular protozoan parasite with an exceptionally wide host range that includes humans and most domestic animals. Its name is derived from the host species in which the organism was first found. In 1908, Charles Nicolle, a scientist working at the Pasteur Institute in Tunis, discovered the parasite in a North African rodent (*Ctenodactylus gundi*) in his search for *Leishmania* (Nicolle and Mancaux (2009), translated manuscript). The parasite, with its characteristic crescent shape, was further named after the Greek words for bow (*toxo*) and life (*plasma*). Independently, and almost simultaneously, the organism was also discovered in a rabbit in Sao Paulo, Brazil (Splendor (2009), translated manuscript).

The discovery of *T. gondii*, just over a hundred years ago, led to extensive research and important findings related to its life cycle, epidemiology, and pathogenesis (extensively reviewed by J.P. Dubey in 2008). However, it was not until about sixty years later that the complete life cycle, with felines as key players, was revealed (Dubey *et al.*, 1970; Frenkel *et al.*, 1970; Hutchison *et al.*, 1970).

While parasite replication occurs both sexually and asexually, the former is only possible in the intestines of the feline definitive host. During sexual reproduction, male microgametes merge with female macrogametes and oocysts are produced within the enterocytes. This occurs after infection with any of the three infectious stages of the parasite: 1) the sporozoite within oocysts, 2) the tachyzoite within many different host cells (or free), or 3) the bradyzoite within intracellular tissue cysts. Ingestion of tissue cysts is much more likely to cause production of oocysts than is ingestion of oocysts or tachyzoites, and the prepatent period is shorter (Dubey, 2006) – about three to ten days (Dubey, 2001; Dubey & Frenkel, 1972). After infection, millions of
oocysts are passed in the faeces, and after a 1 to 3-day sporulation time (depending on the environmental conditions), these oocysts become infectious (Dubey et al., 1970). The sporulated oocysts contain two sporocysts, which in turn contain four sporozoites each (Dubey et al., 1998).

Since the discovery of *T. gondii*, the presence of infectious tissue cysts has been demonstrated in a great variety of intermediate hosts (including humans), which is why it is believed that *T. gondii* is able to infect all warm-blooded animals (reviewed by Hill *et al.* in 2005).

The life cycle of *T. gondii* is complex in the way that infection can be maintained in populations of intermediate hosts without the presence of a definitive host. Intermediate hosts become infected when oocysts, tachyzoites, or tissue cysts in another intermediate host are ingested (Dubey, 1983). Upon this event, sporozoites or bradyzoites are released in the gut, transformed into rapidly multiplying tachyzoites and the acute stage of the infection is initiated. Tachyzoites are subsequently dispersed throughout the body via blood and lymph, likely exploiting the host’s immune cells as a Trojan horse (Lambert *et al.*, 2006). After a few intracellular multiplication cycles, the tachyzoites develop further into slowly multiplying bradyzoites, which are sheltered from the surrounding host immune system by an impenetrable tissue-cyst wall (Dubey *et al.*, 1998). This stage of infection is referred to as the latent stage and *T. gondii* infection is presumed to be lifelong in most species (Tenter *et al.*, 2000). Even though *T. gondii* has the ability to infect and replicate in almost any nucleated cell type, neural and muscular tissues are the predilection sites (Juránková *et al.*, 2014; Dubey, 1997a; Dubey, 1988; Dubey *et al.*, 1984).

During acute infection, tachyzoites may also pass the placental barrier (vertical transmission) to cause infection in the unborn foetus (Jungersen *et al.*, 2001; Dubey & Urban, 1990). The consensus view is that congenital transmission only occurs following primary infection during pregnancy. A previous infection is generally thought to confer lifelong immunity, which is presumed to prevent tachyzoites from crossing the placenta in subsequent gestations (Benard *et al.*, 2008; Montoya & Remington, 2008).

Contradicting this established belief, evidence of vertical transmission to offspring of chronically infected animals has been presented. For example, Rodger *et al.* (2006) studied vertical transmission in 31 chronically infected sheep and found serological evidence of transmission in two twin lambs. Further, Morley *et al.* (2008) found that 9 out of 29 ewes gave birth to congenitally infected lambs during several successive pregnancies and Parameswaran *et al.* (2009) showed evidence of *T. gondii* infection in offspring of chronically infected marsupials. Evidence has also been derived from experimental studies on chronically infected house (*Mus musculus*) and field
(Apodemus sylvaticus) mice (Owen & Trees, 1998). However, the occurrence of vertical transmission in successive pregnancies may be species-dependent. For instance, the phenomenon is less likely in rats than in mice (Dubey et al., 1997b). In humans, transmission to the foetus from chronically infected mothers has been documented in rare cases, when infection was reactivated due to immunosuppression (Montoya & Remington, 2008). The occurrence of congenital infection from chronically infected sows has rarely, if ever, been studied.

Transmission routes for T. gondii between different hosts and the environment are schematically shown in figure 1.
Figure 1. Transmission routes for *T. gondii*. Sources shown in black are considered more important than sources shown in grey (Jones & Dubey, 2012; Petersen *et al.*, 2010). Transmission by the three parasitic stages is illustrated with a symbol for each stage, for example, sporulated oocysts contaminating soil, tissue cysts in the muscles of pigs and tachyzoites transferred from mother to the foetus.
2.2 Humoral immune response

Protective immunity against *T. gondii* is mainly cell mediated, but circulating *T. gondii* antibodies play a significant role in clearance of extracellular stages of the parasite (Buzoni-Gatel & Kasper, 2007; Roberts *et al.*, 2007).

Studies of experimental *T. gondii* infections in pigs have shown that IgM appear quickly (after 3-7 days) and reach a peak at around ten days post infection (PI). Thereafter, the levels start to decline and return to baseline levels within a few weeks (Lind *et al.*, 1997). Similar results were also found by Verhelst *et al.* (2011), who showed that IgM levels in experimentally infected pigs decreased below cut-off levels (a level above which samples are regarded as positive) within six weeks PI.

*T. gondii* IgG have been shown to first appear after 1-2 weeks and to stabilise at high levels at around 3-6 weeks PI (Garcia *et al.*, 2006b; Lind *et al.*, 1997). Dubey *et al.* (1997a) found that the times at which detectable *T. gondii* IgG appeared (starting at one week PI) in experimentally infected pigs, varied depending on: 1) the choice of serological test, 2) the *T. gondii* strain used for inoculation, and 3) the individual pig. Detectable levels of *T. gondii* IgG in pigs are assumed to sustain for several years (Dubey *et al.*, 1997a).

In pigs, *T. gondii* antibodies are transferred from the sow to the newborn, across the gut epithelium, following colostrum intake after birth. There is no transfer of antibodies across the placenta during gestation (Dubey & Urban, 1990) and intake of colostrum is therefore necessary for protection against pathogens in the environment. Colostrum-derived antibodies, formed after experimental infection of sows, have been detected in their piglets up to three months of age (Dubey & Urban, 1990). In contrast, García-Bocanegra *et al.* (2010) showed that colostrum-derived antibodies in piglets born to naturally infected sows, disappeared within weeks. During foetal development, the immune system gradually matures. Production of *T. gondii* IgM and IgG is thus possible in unborn piglets, in the uterus, as a result of congenital infection (Jungersen *et al.*, 2001; Dubey & Urban, 1990).

2.3 Human *T. gondii* infection

2.3.1 Prevalence and clinical aspects

*T. gondii* is regarded as the most widely distributed parasite in the world (Tenter *et al.*, 2000). Globally, it infects a third of the total human population. However, local variations in prevalence is a characteristic feature of *T. gondii* infection (Halsby *et al.*, 2014; Hofhuis *et al.*, 2011; Petersson *et al.*, 2000;
Jenum *et al.*, 1998a; Ljungström *et al.*, 1995), and the seroprevalence varies between 0% and 100% depending on the population investigated (Pappas *et al.*, 2009; Tenter *et al.*, 2000). The risk of encountering the infection increases with time, and because it is a lifelong infection, the *T. gondii* prevalence in a population is highly dependent on the age composition.

One of the first studies of *T. gondii* prevalence in Sweden was performed in 1951 by Zeipel and Linder, who found a seroprevalence of 37% in 300 healthy blood donors. In 1953, Hedqvist studied the frequency of *T. gondii* infection in different age groups in Eskilstuna, a city located in the south-central part of Sweden. A difference in seroprevalence was found between children 0-4 years old (0%) and adults 20-50 years old (40%). Thirty years later (1982/1983), a similar overall seroprevalence of 40% was found in pregnant women in Malmö (Ahlfors *et al.*, 1989). Also in this study, an age-dependent seroprevalence was documented (36% in women <20 years and 50% in women >36 years). At about the same time (1987/1988), the seroprevalence in Stockholm (near Eskilstuna) was reported at 18% (Forsgren *et al.*, 1991). A similar prevalence level was found a few years later (1990-1994) by Birgisdóttir *et al.* (2006), who reported a seroprevalence of 23% in test subjects aged 20-44 years in the area of Uppsala. An apparently decreased seroprevalence was reported in both Malmö (26%) and Stockholm (14%) by Petersson *et al.* (2000), when the serological status of pregnant women was determined post-delivery. In summary, it seems as the *T. gondii* seroprevalence in humans has decreased in Sweden over recent decades and today, the parasite appears to infect approximately a fifth of the adult Swedish population. However, the methodology and populations in these studies differ, making the results difficult to compare. Still, a decreased seroprevalence in humans is also reported in other countries (Hofhuis *et al.*, 2011; Walker *et al.*, 1992).

One of the first reports of human toxoplasmosis in Sweden was published in 1948 (Magnusson & Wahlgren, 1948), in which 12 clinical cases were presented. After that, several cases have been described (Malm *et al.*, 1999; Norrby & Eilard, 1976; Sjövall & Köhler, 1963; Hedenström *et al.*, 1961; von Sydow, 1952). *T. gondii* infection was included as a notifiable disease in the first Communicable Disease Act in Sweden in 1968¹, but was removed when an updated act on communicable diseases was implemented in 2004². Currently, human toxoplasmosis is not monitored in Sweden. Therefore, a national overview regarding clinical toxoplasmosis is difficult to obtain.

Human toxoplasmosis includes a broad spectrum of symptoms, usually grouped into: 1) mild and transient disease in immunocompetent individuals, 2)

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¹. Smittskyddskungörelse 1968:234.
reactivation of latent infection in immunocompromised patients, 3) congenital infection in newborns with symptoms apparent either at birth or detected later in life, and 4) severe toxoplasmosis with ocular, neurological, and/or generalised symptoms as a result of postnatally acquired infection in immunocompetent persons (Montoya & Liesenfeld, 2004).

In immunosuppressed patients, such as those infected with human immunodeficiency virus (HIV), the cause of disease is usually not a primary infection, but rather reactivation of a latent infection (Kodym et al., 2015). Toxoplasmic encephalitis is a commonly reported manifestation, whereas extra-cerebral toxoplasmosis appears to be reported less frequently (Rabaud et al., 1994; Porter & Sande, 1992). More recently, the prevalence of HIV-associated toxoplasmosis has decreased because of HAART (highly active antiretroviral therapy). However, other groups of immunocompromised patients, such as organ or stem-cell transplant recipients and patients undergoing cancer therapy, are at risk for reactivation of latent T. gondii infection (Hakko et al., 2013).

Human congenital infection presents a broad clinical spectrum in those affected. The spectrum ranges from severe conditions, such as stillbirth, abortion and retinal and/or neurological lesions, to initially vague conditions like mental retardation or audiologic deficits (Wilson et al., 1980). Approximately 90% of congenitally infected children are born asymptomatic, of which many will develop clinical signs later in life (Guerina et al., 1994; Wilson et al., 1980). In the northern parts of Europe, the incidence of primary infection during pregnancy ranges between 5 and 25 of 10,000 pregnancies (Prusa et al., 2015; Jenum et al., 1998b; Lappalainen et al., 1995; Stray-Pedersen, 1980). In Sweden, the corresponding figure has been estimated to 4 to 6 of 1,000 pregnancies in 1989 (Ahlfors et al., 1989) and 5 of 10,000 in 2001 (Evengård et al., 2001). In recent years, the incidence of congenital infection in Europe has been estimated to between 1 and 18 cases in 10,000 pregnancies (Prusa et al., 2015; Röser et al., 2012; Kortbeek et al., 2009; Evengård et al., 2001).

T. gondii infection in immunocompetent individuals is usually asymptomatic, and only 10-20% experience clinical signs of infection (Montoya & Liesenfeld, 2004). Clinical symptoms are usually transient without any severe signs of disease – mimicking a mild influenza, a seasonal cold or glandular fever (Halsby et al., 2014; Bowie et al., 1997). However, recent reports of severe clinical disease in immunocompetent individuals (de Souza Giassi et al., 2014; Cuomo et al., 2013; Carme et al., 2002) challenge previous beliefs about toxoplasmosis.
Ocular toxoplasmosis, which in the worst case leads to blindness, may: 1) result from primary infection in immunocompetent individuals (Balasundaram et al., 2010; Burnett et al., 1998), 2) be an early result of, or a late sequel to, congenital infection (Guerina et al., 1994), or 3) be the result of reactivation of latent infection in immunocompromised patients (Holland, 1989). Congenital infection was long regarded to be the main source of ocular disease caused by toxoplasmosis. However, recent evidence has challenged this belief and ocular toxoplasmosis from infection acquired later in life is now considered more common (Gilbert & Stanford, 2000).

*T. gondii* was recently estimated to be one of the most important foodborne pathogens, being responsible for considerable disease burden and economical losses in The Netherlands and the United States (Batz et al., 2012; Havelaar et al., 2012; Hoffmann et al., 2012). Even so, the cost and disease burden of *T. gondii* infection are likely underestimated because: 1) it is difficult to estimate the disease burden of latent infections, where no, or only mild symptoms are present in the initial active phase 2) *T. gondii* infection usually occurs as sporadic cases (in contrast to outbreaks), which are less likely to engage the attention of any health care service, and 3) some disease outcomes may be vague and difficult to attribute to *T. gondii* infection (e.g., learning disabilities in congenitally infected children) (Jones et al., 2003; Wilson et al., 1980).

### 2.3.2 Risk factors

Numerous studies of risk factors for *T. gondii* infection have been conducted in Europe, and the result is a better understanding of sources involved in *T. gondii* transmission to humans. However, a cautious approach is needed when the results of these studies are compared and interpreted, because only the risk factors that are included in the studies are evaluated. This is evident from studies considering the attributable risk (Jones et al., 2009; Kapperud et al., 1996), where a considerable part of the risk cannot be explained by the factors included in the study (Petersen et al., 2010; Cook et al., 2000). It is also important to keep in mind that, for lifelong infections, exposure may have occurred early in life, and it may be difficult to recall specific sources of exposure at that time. Furthermore, a recognition of cultural differences between human populations, enables researchers to include relevant factors for analysis (Birgisdóttir et al., 2006; Cook et al., 2000).

**Oocysts in soil and water**

The oocyst stage of *T. gondii* has been found in the environment, both in soil (Gotteland et al., 2014; Afonso et al., 2008) and in water (Khan et al., 2013; de
Moura et al., 2006; Villena et al., 2004), where they can survive for a long time, depending on temperature and humidity (Lélu et al., 2012; Dubey, 1998). Contact with soil during gardening (Bobić et al., 2007; Cook et al., 2000) and consumption of raw or poorly rinsed vegetables (Baril et al., 1999; Kapperud et al., 1996) have been identified as significant risk factors for T. gondii infection in Europe.

Although less frequently reported, oocyst-contaminated water is also a possible source of infection, and it has been hypothesised that oocysts may enter water reservoirs through meltwater (Simon et al., 2013) and rain washings (Mullens, 1996). However, drinking unfiltered water (such as water from private wells and untreated surface water) has only been suggested as a risk factor in a limited number of studies – from outside Europe (Ertug et al., 2005; Jones et al., 2005; Bahia-Oliveira et al., 2003). In two European studies, drinking untreated water was a significant risk factor in univariable analysis, but not in subsequent multivariable analysis (Cook et al., 2000; Kapperud et al., 1996). Still, water-borne outbreaks have been reported from Panama (Benenson et al., 1982), Brazil (de Moura et al., 2006) and Canada (Bowie et al., 1997), which highlights the possible impact of water-borne toxoplasmosis.

**Cat ownership**

Several multivariable risk factor studies in Europe have identified cat ownership or direct contact with cat faeces as risk factors for human T. gondii seropositivity (Hofhuis et al., 2011; Kolbekova et al., 2007; Kortbeek et al., 2004; Baril et al., 1999; Kapperud et al., 1996). In contrast, just as many investigators have not been able to find this association (Ferguson et al., 2011; Bobić et al., 2007; Birgisdóttir et al., 2006; Cook et al., 2000; Bobić et al., 1998; Buffolano et al., 1996; Decavalas et al., 1990). Possible reasons for the latter are: 1) cats need to ingest infected intermediate hosts (or oocysts in the surroundings) for shedding to occur, 2) cats excrete oocysts only for a short time after primary infection (Dubey, 1995), 3) cats are usually resistant to re-infection and protected against re-shedding of oocysts for several years after primary infection (Lappin et al., 1996; Dubey, 1995), 4) excreted oocysts need to sporulate to become infectious and because this usually takes a minimum of 24 h, faeces deposited indoors is usually discarded before sporulation takes place, and 5) oocysts are rarely found in the fur of cats (Dubey, 1995).

In Sweden, the seroprevalence in pet cats was investigated by Uggla et al. (1990), who found that 42% were seropositive. This indicates that a large proportion of Swedish pet cats become infected at some point in their lives. Ryser-Degiorgis et al. (2006) showed that oocyst-shedding was very rare in the
wild feline host – the lynx (*Lynx lynx*) – in Sweden. The occurrence of oocyst-shedding domestic cats has not been studied in Sweden.

*Undercooked meat*

Undercooked meat has been identified as a major risk factor in several European countries, including Serbia (Bobić *et al.*, 2007), Norway (Cook *et al.*, 2000; Kapperud *et al.*, 1996), Italy (Cook *et al.*, 2000; Buffolano *et al.*, 1996), France (Baril *et al.*, 1999), The Netherlands (Hofhuis *et al.*, 2011), Czech Republic (Kolbekova *et al.*, 2007), Yugoslavia (Bobić *et al.*, 1998) and Belgium, Denmark and Switzerland (Cook, 2000). For example, Cook *et al.* (2000) found that consumption of undercooked beef, mutton, and game meat was responsible for between 30% and 63% of *T. gondii* infections in the six large European cities included in the study. Consumption of game meat (e.g., venison) was responsible for between 1% and 16% of the infections. Kapperud *et al.* (1996) found that inadequately cooked pork was associated with infection in pregnant women in Norway. Consumption of cured pork was a risk factor for recent *T. gondii* infection in women in Italy (Buffolano *et al.*, 1996). A recent study from The Netherlands showed that consumption of inadequately cooked pork was associated with *T. gondii* infection in the general population (Hofhuis *et al.*, 2011).

Although beef is generally considered a less important source of *T. gondii* infection – because of relatively rare findings of viable parasites in their tissues (Dubey, 2010; Tenter *et al.*, 2000) – beef has been suggested as a risk factor in a few studies in Europe (Opsteegh *et al.*, 2011a; Bobić *et al.*, 2007; Baril *et al.*, 1999).

Even though the consumption of mutton is relatively low in Sweden (1 kilo per person and year) (Lööv *et al.*, 2013), it may contribute in part to the *T. gondii* infection level, because it is usually eaten undercooked. Differences in attributable fraction between sources of infection in different studies may be due to consumption habits of the populations studied. In Sweden, pork is the main meat consumed (37 kilos per person and year), followed by beef (26 kilos) and poultry (18 kilos).

Undercooked meat is also implicated as the infection source in many descriptive case studies from different parts of the world (Demar *et al.*, 2012; Pomares *et al.*, 2011; Carme *et al.*, 2002; Choi *et al.*, 1997; McDonald *et al.*, 1990). For instance, Choi *et al.* (1997) described a case of acute toxoplasmosis resulting from consumption of raw liver and spleen from a wild pig.

It is unlikely that pork is a major source of human toxoplasmosis in countries where the majority of fattening pigs are reared in intensive management systems. However, pork from pigs reared in animal-friendly
management systems is thought to be a significant risk (Tenter et al., 2000). For an extensive review of the risk of contracting *T. gondii* infection from meat, please refer to Kijlstra and Jongert (2008).

Properly cooked meat does not involve a risk for infection, because *T. gondii* tissue cysts are killed at 65°C (Dubey et al., 1990). Also, proper freezing (-12°C) is effective in killing bradyzoites (Kotula et al., 1991; Dubey, 1988; Dubey, 1974), indicating that freezing of meat can be an effective method for reducing the number of meat-associated *T. gondii* infections.

Additional meat processing methods have been evaluated for their ability to kill bradyzoites (reviewed by Tenter (2009)). For instance, the effect of salting was described by Dubey (1997b), curing and smoking by Lundén and Ugglä (1992), and irradiation by Dubey et al. (1986a). More recently, Pott et al. (2013) studied the effect of curing and different levels of pH and sodium chloride concentration. The conclusion from these studies is that freezing and heating are the most reliable methods for killing *T. gondii* tissue cysts.

### 2.4 Fattening pigs and wild boars in Sweden

#### 2.4.1 Domestic pigs

Between 2013 and 2014, the number of conventional fattening pig farms in Sweden decreased by 4% (Swedish Board of Agriculture, 2014). Looking at Swedish pig production in further retrospect, the number of farms has decreased by 88% since 1990. At the same time, the mean farm size has increased from 119 pigs in 1990 to 795 pigs in 2014 (Swedish Board of Agriculture, 2014).

An increasing number of consumers demand pork from pigs reared under animal-friendly management systems. The framework for organic animal production within the EU is carefully regulated. However, in several member states there are additional voluntary rules of conduct – defined by different certification bodies – promoting environmental sustainability and animal welfare. Such conduct codes are developed by a variety of interest groups and vary between countries. In Sweden, the major certification body is KRAV (in translation, Control Association for Alternative Cultivation), whose standards (KRAV, 2015) extend beyond the EC regulations.

The number of fattening pigs in KRAV-certified production systems has increased from 17,500 in 2008 to 22,500 in 2013 and is expected to increase

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further (KRAV, 2014). Still, the number of KRAV-raised pigs constitutes only 1% of the total pig production in the country.

KRAV pigs are usually reared on smaller farms than conventional pigs. As an example, conventional pigs are distributed over 1,065 farms, and 84% of conventional pigs are raised on farms with more than 750 pigs (40% on farms with more than 2,000 pigs) (Swedish Board of Agriculture, 2014). In contrast, KRAV pigs are distributed over some 30 farms, and 39% are raised on farms larger than 750 pigs.

The KRAV regulations fulfil all requirements of organic production according to EU legislation, and set additional standards to ensure increased animal welfare. KRAV requires at least four months of pasturing during the summer, usually from the beginning of June to the end of September\(^5\). During the rest of the year, outdoor access is still required but may be provided in outside pens on a concrete floor (in accordance with EU legislation). The pigs’ natural rooting behaviour is satisfied all year around by providing deep straw bedding indoors. In contrast, conventional pigs are raised in closed confinement on a concrete floor, provided with straw for activation. Feeding of pigs with animal by-products is not allowed within the EU\(^6\).

2.4.2 Wild boars

![Figure 2. Number of hunted wild boars in Sweden during 2000-2014. The figure is based on information provided by the Swedish Association for Hunting and Wildlife Management (2016).](image)

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5. Mats Schörling, Jord på trynet, personal communication 2014-03-28
In the late 17th century, wild boars disappeared from the Swedish forests after deliberate eradication (Swedish Association for Hunting and Wildlife Management, 2016). However, wild boars were later reintroduced in enclosures from which escapes occurred in the 1970s and 1980s. Today, wild boars are again a part of the Swedish fauna, and the Swedish Association for Hunting and Wildlife Management (2016) reports that the Swedish wild boar population is increasing rapidly and spreading north. Figure 2 shows the dramatic increase in number of hunted wild boars in Sweden between 2000 and 2014.

The destructive behaviour of wild boars in agricultural areas (rooting, trampling and damage to different agricultural crops) has warranted enhanced hunting (Månsson et al., 2010). Increased hunting consequently leads to increased availability of wild-boar meat on the market. However, the distribution of wild-boar meat to restaurants and the wholesale market is partly hampered by high costs for meat inspection at game handling plants, which may lead to destruction of the meat or selling of wild-boar meat on the black market (Wretling Clarin & Karlsson, 2013). The Swedish Board of Agriculture has produced a report describing strategies to increase the proportion of meat that reaches the consumers (Wretling Clarin & Karlsson, 2013). In 2014, the estimated proportion was only 15%. Currently, key players are striving to increase the use of this valuable resource.

2.5  *T. gondii* infection in fattening pigs and wild boars

2.5.1 Prevalence of infection

*Domestic pigs*

The reported seroprevalence in conventional fattening pigs in Europe is usually low, especially in the northern countries. In Finland, Hirvelä-Koski (1992) estimated the seroprevalence to be 3% in 1984. In Scotland, a seroprevalence of 4% was reported in 1987 (Jackson et al., 1987). A similar seroprevalence of 5% was estimated in Ireland in 2007 (Halová et al., 2013) and Deksne and Kirjušina (2013) reported a seroprevalence of 4% in fattening pigs in Latvia in 2010/2011.

Countries in southern parts of Europe tend to have higher seroprevalences. For instance, in 2008, Berger-Schoch et al. (2011) found a seroprevalence of 14% in Switzerland and Veronesi et al. (2011) found 16% seropositive fattening pigs in Italy. Also Serbia and Portugal are reported to have similar seroprevalence levels (Lopes et al., 2013; Klun et al., 2006). However, a few studies deviate from the described tendency. For instance, Paštiu et al. (2013)
found no seropositive pigs when 660 fattening pigs were tested with IFAT in Romania in 2008-2010.

The seroprevalence in Swedish fattening pigs has been estimated twice previously. In 1982 and 1983, a total of 599 serum samples from fattening pigs were investigated from the north, the middle and the south of Sweden (Uggla & Hjort, 1984). The mean proportion of positives was 16%, with a range from 9% in the north to 37% in the south. The reported seroprevalence was higher than expected compared to previous estimates from nearby countries (Uggla & Hjort, 1984; Work, 1967). In 1999, meat-juice samples from 695 fattening pigs and 110 sows were collected at 10 abattoirs in Sweden (Lundén et al., 2002). The estimated seroprevalence was 3.3% and 17.3% in fattening pigs and sows, respectively.

![Figure 3. T. gondii seroprevalence in wild boars in Europe. The seroprevalence varies between 5% (Greece) and 44% (Spain). The map includes the most recent study available from each country. Because there are several discrepancies between the studies regarding the serological tests used and type of populations studied, firm conclusions are not advisable.](image-url)
Wild boars

Figure 3 shows a map of the *T. gondii* seroprevalence in wild boars in different European countries (Račka *et al.*, 2015; Touloudi *et al.*, 2015; Witkowski *et al.*, 2015; Coelho *et al.*, 2014; Deksnė & Kirjušina, 2013; Paštiu *et al.*, 2013; Ranucci *et al.*, 2013; Beral *et al.*, 2012; Jokelainen *et al.*, 2012; Berger-Schoch *et al.*, 2011; Closa-Sebastià *et al.*, 2011; Opsteegh *et al.*, 2011b; Richomme *et al.*, 2010; Antolová *et al.*, 2007; Lutz, 1997). The studies indicate seroprevalence levels that vary greatly between and within country (Beral *et al.*, 2012). The *T. gondii* seroprevalence has not previously been investigated in wild boars in Sweden.

2.5.2 Clinical aspects

Clinical toxoplasmosis in food-producing animals is mainly associated with abortions and stillbirths in sheep, where infection may cause great economical losses (Dubey & Kirkbride, 1989). Congenital infection in pigs occurs less frequently, but when it does, the signs may be dramatic (Kim *et al.*, 2009). It is associated with abortions, production of stillborn and/or mummified piglets, and piglets born weak (Dubey, 2009; Dubey & Urban, 1990).

In Sweden, only one case of clinical porcine toxoplasmosis has been reported (Hansen *et al.*, 1977). In 1964, a whole litter of piglets fell severely ill two weeks post partum. The route of infection was considered congenital, even though all piglets were healthy at birth. The farrowing gilt was not tested for *T. gondii* infection (or the results were not presented). An alternative route of infection could thus have been postnatal ingestion of oocysts (or ingestion of an infected intermediate host) from the environment.

2.5.3 Risk factors

Ingestion of oocysts has been proposed as the major infection route for pigs (Du *et al.*, 2012; Lehmann *et al.*, 2003; Lubroth *et al.*, 1983). Oocysts shed by infected cats may contaminate feed, water and pig-residing areas when cats are allowed access.

Outdoor access for pigs, which is often provided in animal-friendly management systems, has also been associated with a higher *T. gondii* prevalence (Deksnė & Kirjušina, 2013; van der Giessen *et al.*, 2007; Kijlstra *et al.*, 2004), possibly due to ingestion of oocysts during rooting and mud bathing. Ingestion of oocyst-contaminated feed and water on pasture is also possible. The occurrence of oocysts in soil is thought to be dependent on the density of cats in the surroundings (Du *et al.*, 2012) and several studies show porcine *T. gondii* infection to be more prevalent in farms where cats (particularly juveniles) are present (Meerburg *et al.*, 2006; Weigel *et al.*, 1995).
Beral et al. (2012) found a similar association between *T. gondii* infection in wild boars and the presence of European feral cats (*Felis silvestris*).

Ingestion of infected rodents is another possible infection route. In fact, rodents have been shown to play a key part in the transmission of *T. gondii* to pigs (Kijlstra et al., 2008).

Congenital infection as well as ingestion of tissue cysts through tail biting and cannibalism may be additional infection sources (Dubey et al., 1986b). Feeding of goat whey (Meerburg et al., 2006) and leftover food (Alvarado-Esquível et al., 2014; Feitosa et al., 2014) has also been associated with *T. gondii* infection in pigs. Still, the relative contribution of different infection routes for pigs is largely unknown and it is likely that transmission routes differ between geographical areas or even between farms in the same area.

As in humans, increased time of exposure is associated with increased probability of *T. gondii* infection. For European wild boars, an increased seroprevalence in older pigs has been documented in Spain (Ruiz-Fons et al., 2006), Slovakia (Antolová et al., 2007), The Netherlands (Opsteegh et al., 2011b), Finland (Jokelainen et al., 2012), Italy (Ranucci et al., 2013), Portugal (Coelho et al., 2014) and Corsica (France) (Richomme et al., 2010). A difference in seroprevalence between fattening pigs and sows is also evident in many studies (Lundén et al., 2002; Dubey et al., 1995c; van Knapen et al., 1995; Edelhofer, 1994). No studies of risk factors for porcine *T. gondii* infection have previously been performed in Sweden.

### 2.6 Methods for monitoring *T. gondii* infection in pigs

#### 2.6.1 Serology

Demonstration of anti-*T. gondii* antibodies is a useful indirect indication of *T. gondii* infection, because the infection is assumed to be lifelong in most host species (Tenter et al., 2000). However, the relationship between parasite abundance in muscles and specific antibodies in blood seems to differ between species. For pigs, the two seem to correlate well, and it has been shown that a higher antibody titre is associated with a higher probability of finding viable parasites in tissues using bioassay (section 2.6.3) (Dubey et al., 1995a).

Many different serological tests have been used for detection of *T. gondii* specific IgG and/or IgM. Some of these are: the Sabin-Feldman dye test (DT), the indirect fluorescent antibody test (IFAT), the direct agglutination test (DAT; also referred to as the modified agglutination test, MAT), the enzyme-linked immunosorbent assay (ELISA), the latex agglutination test (LAT), complement fixation (CF), and the indirect haemagglutination test (IHAT). DAT and ELISA are widely used and have been evaluated for pig
serum in many studies (Hill et al., 2006; Gamble et al., 2005; Dubey, 1997c; Dubey et al., 1997a; Dubey et al., 1996; Dubey et al., 1995b).

**DAT**

In DAT, the antigen (whole killed *T. gondii* tachyzoites of the RH strain) coalesce into a network in which the organisms are coupled by specific IgG, if present in the test serum. The outcome of the test (the titre) is the highest dilution of the sample at which agglutination occurs.

The DAT for *T. gondii* was developed in 1959 by Fulton and Turk. Initially, a low sensitivity (Se) and specificity (Sp) limited its use, until the test was further developed in 1980 (Desmonts & Remington, 1980). The Sp was improved by adding 2-mercaptoethanol (2-ME) to denature interfering natural IgM antibodies and the Se was improved by modifying the antigen production. Note that specific IgM activity is also inhibited by 2-ME, and therefore, DAT only detects *T. gondii* specific IgG. DAT may thus result in false negative results in recently infected individuals (Dubey et al., 1995b).

DAT is a simple and straightforward test; it does not require any specific equipment, and results are read after 5-18 hours. Serum samples should be tested in at least two dilutions (one dilution representing the decided cut-off and one higher dilution) to avoid false negative results caused by a phenomenon known as the prozone effect (Seefeldt et al., 1989; Desmonts & Remington, 1980). This effect is a result of excessive *T. gondii* specific IgG in the sample, which saturates binding sites on the antigen and thus prevents cross-linking of the antigen (agglutination). A disadvantage of DAT is that the results are read subjectively.

**ELISA**

In indirect ELISA tests, a solubilised antigen is absorbed to a plastic surface of high-binding capacity. Test samples are added to allow specific antibody-antigen binding. If present in the sample, specific antibodies are subsequently detected using an enzyme-linked secondary antibody. As a final step, a substrate solution for the enzyme is added and the interaction between the enzyme and the substrate generates a visible colour. The colour intensity is quantified objectively using a spectrophotometer and the result is presented as an optical density value (OD). Using a predefined cut-off value, samples are assigned as positive or negative. ELISAs for *T. gondii* come in a variety of designs including different types of antigens, conjugates and buffers etc., all of which affect the performance of the test.
2.6.2 Serology using meat juice

Meat juice is a serological matrix extracted from muscle samples by freezing and thawing (Nielsen et al., 1998). Although not very well characterised, meat juice consists of a mixture of blood, lymph and extracellular and intracellular fluids. Therefore, meat juice contains antibodies, originating in different amounts from the above-mentioned sources. However, the relative contribution of these sources is not well defined.

Because meat juice is readily available from animals at slaughter and from hunted game, it has been used in several serological assays to demonstrate antibodies directed at different pathogens (e.g., *Trichinella*, *Salmonella* and *T. gondii* in pigs) (Felin et al., 2014; Alban et al., 2010; Lundén et al., 2002). However, there is evidence of inferior Se, presumably due to relatively low levels of antibodies in meat juice compared to serum (Vico & Mainar-Jaime, 2011; Hill et al., 2006; Gamble et al., 2005).

2.6.3 Detection of parasites

Other means than serology are necessary to directly determine if parasites are present in the tissues of an animal. Investigations based on detection of parasite DNA (by the polymerase chain reaction (PCR)) are common, but may underestimate disease prevalence (Garcia et al., 2006a; Hill et al., 2006). *T. gondii* tissue cysts are distributed unevenly throughout the muscles of the host, and very small amounts of tissue (a few milligrams) are usually used in the PCR analysis. Thus, there is a risk that the sample does not contain tissue cysts. However, a method to analyse larger samples (100 grams) – by digestion followed by magnetic-capture PCR – has recently been developed (Opsteegh et al., 2010a).

With traditional PCR, it is impossible to determine if detected parasites are infectious (live) or not. For this, a bioassay can be used (Dubey et al., 1995a). In bioassay, tissues of potentially infected animals are inoculated (e.g., orally or subcutaneously) into cats and/or mice, which are then examined for evidence of infection. Bioassays using cats are more sensitive because larger amounts of tissue can be used (more than 500 grams in cats compared to approximately 0.25 grams in mice) (Dubey, 2010). The relative Se of bioassays in mice compared to bioassays in cats is about 50% (Dubey et al., 1995b). Bioassays are expensive, labour intensive, and in many cases, ethically questionable.
2.7 Test evaluation

The usefulness of a diagnostic test is determined by its ability to correctly classify individuals as positive (the probability of a diseased individual to test positive, i.e., Se) or negative (the probability of a disease-free individual to test negative, i.e., Sp). As shown in figure 4, the Se and Sp of a serological test are closely related and when the test cut-off is altered, the Se and Sp change in opposition to each other.

A great variety of diagnostic tests are continuously developed, modified and used to detect *T. gondii* infection in food-producing animals. Development of new tests is motivated by: 1) economical superiority, 2) suitability for prevailing laboratory conditions, and 3) inadequate properties (Se and/or Sp) of existing tests. All tests will produce some false results and without knowledge of a test's performance it is difficult to accurately interpret the test results. However, with an adequate evaluation, expected misclassifications can be estimated and accounted for.

2.7.1 Evaluation using reference tests

The characteristics of a new test (the index test) can be evaluated by comparing its results to those of a reference test. The reference test is considered a gold standard or a perfect reference test if it provides perfect classification of the infection status of the animal. If the reference test is assumed to be perfect – even though it is not – the estimated Se and Sp of the index test can never exceed those of the reference test. In other words, this may result in biased estimates of the test performance.

For the diagnosis of (subclinical) *T. gondii* infection in pigs, there is as yet no perfect reference test available. Many studies have evaluated serological tests against the cat or mouse bioassay (Hill *et al.*, 2006; Dubey *et al.*, 1995b). This may be problematic because negative bioassay results, due to low density and uneven distribution of tissue cysts in the animal, may give an erroneously low Sp of the serological test. Also, specific IgG is detected no sooner than approximately 1-2 weeks PI, while tachyzoites can be detected in blood from approximately day 4 PI (Dubey *et al.*, 1997c; Costa *et al.*, 1977). Thus, pigs sampled during the first weeks after primary infection can be bioassay positive while still being serologically negative.

2.7.2 Evaluation using experimentally infected animals

Because there are no perfect reference tests for *T. gondii* infection, diagnostic tests are sometimes evaluated using experimentally infected animals, i.e., animals with known infection status. However, there are some associated difficulties.
Figure 4. Illustration describing how the sensitivity (number of individuals that test positive of the diseased population) and specificity (number of individuals that test negative of the non-diseased population) are affected by changing the cut-off of a serological test.

Firstly, experimentally infected animals are often inoculated with very high parasite doses, causing a high infection burden and usually high antibody levels in positive individuals compared to negative controls. In contrast, the levels of detectable antibodies in naturally infected animals often extend across a broader spectrum, in which truly positive and truly negative individuals are not completely separated.

Secondly, animals kept in experimental settings usually lack concurrent infections, which in a natural environment might induce cross-reacting antibodies. Using specific-pathogen-free animals therefore risks overestimating the Sp. Thus, experimentally infected animals are not representative of naturally infected populations and results from these studies are difficult to infer to natural situations. To mitigate inferential problems, diagnostic tests should be evaluated on the population for which they are intended (Greiner & Gardner, 2000; Ransohoff & Feinstein, 1978).

2.7.3 Evaluation in the absence of a gold standard

Latent class analysis (Johnson et al., 2001; Enøe et al., 2000; Hui & Walter, 1980), using a Bayesian statistical approach, is increasingly applied to evaluate
tests used in veterinary parasitology (Mainar-Jaime & Barberán, 2007; Frössling et al., 2003). In contrast to evaluation methods relying on a gold standard, a latent class analysis enables evaluation without considering any of the tests to be more accurate than the other. Also, estimates for Se and Sp are obtained for all tests included in the analysis.

Another advantage with a Bayesian approach is the possibility to include prior knowledge of the variables into the model, thus making use of knowledge gained from previous research (Enøe et al., 2000). However, this may not only be an opportunity but a prerequisite, for models where the number of degrees of freedom provided by the data are outnumbered by the number of variables to be estimated. Selection of prior information needs to be carried out with outmost care and its effect on the posterior estimates needs to be evaluated (Toft et al., 2005; Georgiadis et al., 2003; Winkler, 1967).

The prior knowledge is included as distributions (e.g., beta) for each parameter in the model. When prior knowledge is unavailable (which is usually the case for the characteristics of the index test), it is possible to define the prior distributions as uniform, also known as non-informative. The prior distributions are combined with the information contained in the data, resulting in posterior distributions for the variables in the model, from which the mean and the 95% credibility interval can be used as posterior point estimates.

2.8 Monitoring of T. gondii in food-producing animals

2.8.1 Monitoring in EU member states

In 2007, the European Food Safety Authority (EFSA) published a scientific opinion stating the need of representative data on T. gondii in food-producing animals and in food in Europe (EFSA, 2007). According to an EU directive – on the monitoring of zoonoses and zoonotic agents7 – T. gondii should be monitored in animals, food, feed, and humans if warranted by the epidemiological situation in the member states. Toxoplasmosis is a notifiable disease in all animals in Latvia and Poland, and in Finland, in all animals except hares, rabbits and rodents. In Germany, toxoplasmosis is notifiable in pigs, dogs and cats (EFSA & ECDC, 2015). In the 2014 European Union summary report on trends and sources of zoonoses, zoonotic agents and foodborne outbreaks, 14 member states provided information of toxoplasmosis in animals (EFSA & ECDC, 2015).

Data on T. gondii infection in domestic food-producing animals can be acquired either by on-farm sampling or abattoir-based sampling. On-farm

sampling requires dealing with live animals, which is time consuming, involves high costs, and is strenuous compared to abattoir-based sampling. It is also associated with unnecessary stress for the animals. Abattoir-based sampling for *T. gondii* may be possible to integrate with already implemented sampling protocols for other foodborne pathogens (such as *Trichinella* and *Salmonella*), and provides a more accurate estimate of the burden of *T. gondii* distributed in the food-chain.

2.8.2 Monitoring in Sweden

EFSA has recommended the implementation of pre-harvest monitoring of *T. gondii* in sheep, goats, pigs, and game to collect representative data on *T. gondii* infection in animals (EFSA, 2007). No monitoring of *T. gondii* is currently ongoing in Sweden, and knowledge of the *T. gondii* epidemiology in food-producing animals is gained by cross-sectional studies. Serological studies have been performed, both pre-harvest and post-harvest, of different wild and domestic food-producing species during the last 30 years. The results from these studies are described below (except for those of pigs described in section 2.5.1).

None of the sera from 176 apparently healthy brown hares (*Lepus europaeus*) collected in 1984/1985 was *T. gondii* positive (Gustafsson & Uggla, 1994). In 1984, 600 cattle of various ages were sampled in three abattoirs in the north, middle and south of Sweden (Uggla & Hjort, 1984). The overall seroprevalence was 17% (10% in the north and 35% in the south). In the same study, 597 serum samples from sheep (collected from the same geographical areas) revealed a seroprevalence of 60% in the north and 69% in the south (Uggla & Hjort, 1984). In 1986/1987, 704 sheep were tested for *T. gondii* specific IgG, of which 19% were classified as seropositive (Lundén et al., 1992). A seroprevalence of 1% were found in 219 horses examined in 1990 (Uggla et al., 1990). A similarly low seroprevalence (0.5%) was detected in 1992/1993 when 414 horses were tested (Jakubek et al., 2006). A total of 417 moose (*Alces alces*) and 199 roe deer (*Capreolus capreolus*) were sampled in 2000-2005 and 1990-2007, respectively (Malmsten et al., 2011). Serum samples were tested using DAT and *T. gondii* antibodies were detected in 20% and 34%, respectively. The presence of tissue cysts was not investigated in any of these studies.
3 Aims

The overall aim of this thesis was to gain knowledge about the epidemiology of *T. gondii* in domestic pigs and wild boars in Sweden, and to explore the prospects for standardisation of meat-juice serology.

More specifically, the objectives were:

- To estimate the overall *T. gondii* seroprevalence in Swedish fattening pigs, to investigate whether there is any difference in seroprevalence between conventionally raised pigs and pigs raised with specific organic certification (KRAV).

- To estimate the overall *T. gondii* seroprevalence in Swedish wild boars, as well as age-related, geographical and temporal differences in seroprevalence.

- To evaluate an in-house ELISA and a commercial DAT for serological screening of wild-boar serum samples.

- To investigate how the antibody concentration in meat juice correlates to the antibody concentration in serum and to investigate possible differences in antibody concentration in meat juice from different muscles.

- To find out whether the concentration of *T. gondii* antibodies in meat juice is dependent on the blood content and/or content of total IgG in the sample.
4 Considerations on materials and methods

4.1 Extraction of meat juice (I, III)

In paper I, meat-juice samples were collected from fattening pigs and tested for antibodies against *T. gondii* for prevalence estimation. In paper III, pigs experimentally infected with *T. gondii* were used to study the association between antibodies in serum and meat juice from different muscles.

The experimentally inoculated pigs (III) were culled and exsanguinated while in a supine position, while in routine slaughter, bleeding is usually performed on hanging carcasses. The amount of blood lost at bleeding is associated with the position of the animal (bleeding while in a supine position results in a higher blood loss); however, the residual blood volume in the muscles seems to be unaffected (Warriss & Leach, 1978). Therefore, in terms of bleeding position, the experimental procedure is assumed to be representative of normal slaughter.

Muscle samples were excised from the diaphragm (I, III), heart (III), tongue (III), foreleg (III), and hindleg (III) and placed in meat-juice collecting tubes (Nielsen *et al.*, 1998) (figure 5). For paper I, the choice of muscle was influenced by the wide use of diaphragm muscle as a source of meat-juice in serological surveys. In addition, diaphragm sampling for *Trichinella* was already implemented in the abattoirs, so no additional sampling customisation was necessary.

4.2 Quantification of haeme proteins (III)

Meat juice contains haeme-containing proteins, of which haemoglobin (Hb) and myoglobin likely constitute the greater part (Ledward, 1992). One of the aims of paper III was to evaluate if the level of specific antibodies in a
meat-juice sample was dependant on the blood content. The alkaline haematin detergent (AHD)-575 method (Wolf et al., 1984; Zander et al., 1984) was used to measure the total content of haeme proteins, which was used as an approximation for the blood content. This method – which is simple and inexpensive – is based on conversion of all haeme groups to a stable end product (alkaline haematin), by addition of an alkaline solution (sodium hydroxide) of the non-ionic detergent Triton-X (AHD reagent). The absorbance of the end product is measured spectrophotometrically and compared to the absorbance of a standard solution with known concentration.

Alkaline haematin has an absorbance maximum ($\lambda_{\text{max}}$) at 575 nm regardless of the haeme species present in the sample (Hb or myoglobin) (Karlsson & Lundström, 1991). In the initial phase of paper III, we aimed to confirm the $\lambda_{\text{max}}$ for haematin originating from haeme species in meat-juice samples. The absorbance at 450-700 nm was measured in solutions made from: 1) pure chlorohaemin (0.1 mmol/l), 2) porcine haemoglobin (0.1 mmol/l), and 3) meat-juice from porcine heart. Figure 6 shows a broad absorbance peak at around 575 nm, which was obtained irrespective of the origin of the haeme species (haemoglobin, chlorohaemin or those present in meat-juice). Based on these results, it was concluded that measurements of meat-juice samples could be performed at 570 nm instead of 575 nm. The absorbance at 700 nm was measured for each sample and used as a blank.
Figure 6. Absorption spectrum (450-750 nm) of alkaline haematin produced from pure chlorohaemin dissolved in AHD reagent (0.1 mmol/l). The arrow indicates the absorbance maximum ($\lambda_{\text{max}}$) at around 575 nm.

4.3 Serological tests (I, II, III)

Three serological tests were used in this thesis. In paper II, an in-house ELISA was adapted from a previously published assay (Opsteegh et al., 2010b), with the aim to facilitate comparison with test results from other European studies using a similar ELISA (Opsteegh et al., 2011b). The ELISA was evaluated for use on wild-boar serum samples using Bayesian latent class analysis.

A commercial DAT (Toxo-Screen DA, bioMérieux SA, Marcy-l'Étoile, France) was used to test a subset (242 of 1327) of the wild-boar samples, for inclusion of the results as prior information in the latent class analysis. Posterior estimates of Se and Sp were obtained for the DAT, as well as the ELISA, from this evaluation.

Additionally, a commercial ELISA (ID Screen Toxoplasmosis Indirect Multi-species; IDvet Innovative Diagnostics, Montpellier, France) was used for preliminary seroprevalence estimations for inclusion in the latent class analysis. This commercial ELISA was later used to analyse serum and meat juice from 23 experimentally infected pigs (III) and meat juice from the 972 conventionally and KRAV-raised pigs (I).
4.3.1 Antigens (I, II, III)
The in-house ELISA uses an antigen produced from *T. gondii* tachyzoites of the RH strain, solubilised in saponin and octylglucoside (Hughes *et al.*, 1982). During this preparation, both internal and surface membrane antigens are released. In contrast, the DAT uses formalin-fixed tachyzoites, only presenting surface antigens, while the commercial ELISA uses a purified native surface antigen (P30/SAG1) of the *T. gondii* tachyzoite. Thus, discrepancies between DAT and ELISA results can be due to the specificity of the antigen preparations.

4.3.2 DAT (II)
The DAT was performed according to the manufacturer's instructions except that serum samples were diluted 1:20 instead of 1:40. This dilution has previously been suggested as the most accurate for pig serum (Dubey *et al.*, 1995b). Samples were additionally tested in a higher dilution (1:4,000) to avoid false negative results caused by the prozone effect (Desmonts & Remington, 1980). A prozone phenomenon, shown as a positive reaction in the 1:4,000 dilution and a negative or inconclusive reaction in the 1:20 dilution, was registered for 24 samples. This corresponds to approximately 27% of the positive samples. It is apparent that performing this test only at the lower dilution may considerably underestimate the seroprevalence.

According to the manufacturer, the samples should be classified as follows: 1) negative reaction – a round button (or a ring) at the bottom of the well, 2) positive reaction – agglutination as a mat covering approximately 50% or more of the bottom of the well, and 3) doubtful reaction – agglutination as a mat covering less than 50% of the bottom of the well. In paper II, doubtful reactions were further divided in 4) doubtful positive – agglutination as a mat covering between 25% and 50% of the bottom of the well (n = 22), and 5) doubtful negative – agglutination as a mat covering less than 25% of the bottom of the well (n = 13). Doubtful reactions were finally dichotomised into positive or negative, assigning the doubtful negative samples to the negative category and doubtful positive samples to the positive category.

The results of the DAT were read visually and were therefore subjective. The test results were interpreted by experienced laboratory staff.

*Evaluation of DAT using latent class analysis*
Based on latent class analysis the Se and Sp of the DAT were estimated to 82% and 87%, respectively. These estimates are in agreement with a previous evaluation of the DAT on 1,000 sow sera using the same cut-off, and bioassay in cats and mice as reference (Dubey *et al.*, 1995b). These results were further
supported by Gardner et al. (2010), who applied a Bayesian latent class analysis to the data provided in Dubey et al. (1995b). Gamble et al. (2005) retrieved a slightly higher estimate for Sp (95%), when the DAT was evaluated on 204 naturally exposed fattening pigs, negative in cat bioassay. In contrast, a lower Se (74%) was found by Hill et al. (2006), where 15 of 21 bioassay positive pigs were detected with the DAT.

4.3.3 Commercial ELISA (I, II, III)

This ELISA uses a multi-species conjugate and several publications report on serosurveys in various domestic and wild species using this test (Balea et al., 2012; Bártová & Sedláček, 2011; Roqueplo et al., 2011). A few studies have reported estimates of Se and Sp for analysis of serum from naturally exposed pigs (Steinparzer et al., 2015; Veronesi et al., 2012). These estimates ranged between 57-90% and 97-99%, for Se and Sp respectively. Veronesi et al. (2012) also evaluated the ELISA test performance using meat juice from naturally infected pigs and reported a Se of 65% and a Sp of 97%. They used the test results of paired serum samples as reference. The manufacturer of the commercial ELISA reports both the Se and the Sp of the test to be 100% (for serum samples from naturally exposed pigs). Their Sp estimation is based on pigs from disease-free and confined herds and the Se is estimated using IFAT as reference test. No estimates of Se or Sp are provided – when the test is performed on meat juice – by the manufacturer, even though meat juice is declared a suitable test matrix.

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*Figure 7. Cross-classified test results of meat juice and serum samples from 166 KRAV pigs tested in the ID Screen Toxoplasmosis Indirect Multi-species ELISA (A), and S/P values (see paper III) in meat juice and serum from the five positive pigs (B).*

Both serum and meat juice were collected from a subset (n = 166) of the KRAV pigs in paper I. These samples were used in a pilot study to obtain estimates for the test Se and Sp on meat-juice samples collected from the target population. A contingency table of the resulting classification is presented in
4.3.4 In-house ELISA (II)

Assumptions in latent class analysis

To estimate the characteristics (Se and Sp) of the in-house ELISA, a Bayesian latent class analysis, based on two dependent tests (DAT and ELISA) and two populations (wild boars >12 and <12 months of age) was performed. The DAT and ELISA were assumed to be dependent because they measure the same biological phenomenon (Georgiadis et al., 2003). Two important assumptions are made in this model: 1) the two populations have different disease prevalence, and 2) the test properties are equal in the two populations.

Prior information included in latent class analysis

The prevalence priors for the two groups were determined based on the results from the commercial ELISA, see section 4.3.3. This is not optimal because the prior distributions should be estimated independently of the data (Branscum et al., 2005). However, the prevalence in Swedish wild boars had never been estimated before and a third test provided the most suitable estimates.

The two populations in paper II were retrospectively constructed from the complete dataset by dividing it into pigs older than 12 months of age and pigs younger than 12 months of age. As mentioned in section 2.5.3, the prevalence in the adult population was expected to be higher than in the young one. Several strategies for constructing two populations have previously been reported (Györke et al., 2011; Opsteegh et al., 2010b; Mainar-Jaime & Barberán, 2007; Georgiadis et al., 2003) and subdivision by age has been recommended in the literature (Hui & Walter, 1980). Nevertheless, subdivision by age may negatively influence inference of the test properties. More specifically, inaccuracies in inference may stem from an invalid assumption of equal test properties in the two populations.

As older pigs presumably have encountered a greater number of pathogens (for which specific antibodies are produced that could cross-react during *T. gondii* serology), there is a theoretical possibility for a lower Sp in the adult population. Very little information is available regarding the age distribution in the Swedish wild boar population. However, it is likely that even the adults sampled are quite young. In a report from the Swedish University of
Agricultural Sciences and the Wildlife Damage Centre (Månsson et al., 2010), the age composition of a wild boar population is described as 75% piglets, 15-20% yearlings, and 5% older pigs.

In the latent class model, the difference in test Sp between the age categories was assumed to be insignificant. However, if this assumption is erroneous, and the true Sp in the adult population is lower, then posterior estimates of Sp may be biased towards the test Sp in the young population (the population with the lowest disease prevalence and thus providing more data for Sp estimation), i.e., an estimated Sp relatively higher than the true Sp (Johnson et al., 2009; Toft et al., 2005). Such bias will in turn give an estimate of Se that is relatively lower than the true Se (a false high Sp will lower the Se because false positive samples are not detected).

Prior information for the DAT properties was retrieved from the literature. After a search for valid estimates of Se and Sp, studies were chosen that used cat bioassay as reference test and serum samples from naturally exposed pigs. Only a few such studies were found, which in addition to these criteria, also applied a similar DAT cut-off (1:20) as in paper II (Hill et al., 2006; Gamble et al., 2005; Dubey, 1997c; Dubey et al., 1995b). The selected studies resulted in an interval for Se and Sp of 0.71-0.86 and 0.90-1.0, respectively. The final mode of the prior distributions for DAT Se and Sp were based on Dubey et al. (1995b) because this study had the largest sample size and its estimates were based on samples from sows and not marked-aged pigs (sows are assumed to resemble wild boars in age distribution).

Characteristics of the in-house ELISA and selecting the best cut-off

The measurement unit in an ELISA is an optical density (OD) value (which is measured on a continuous scale). Therefore, a positive cut-off can be chosen retrospectively, depending on the practical application of the test, prevalence of infection, costs of false test results, and availability of other tests.

The latent class analysis resulted in estimates for Se and Sp in the pre-specified cut-off range (0.30-0.60). The highest values for the combined Se and Sp (i.e., the Youden’s index = Se + Sp – 1) (Youden, 1950) were obtained in the cut-off range of 0.37-0.41 (maximum value at cut-off = 0.41). However, a higher Se was prioritised above the Sp when selecting the cut-off that best suited the purpose of the study, meaning that we intended to find as many of the positive animals as possible. At the selected cut-off (0.39), the Se and Sp of the in-house ELISA were estimated to 79% (95% credibility interval (CrI) 60-95) and 85% (95% CrI 74-94), respectively. A Se of 79% was considered adequate for a screening study. However, if the ELISA had been used to
classify individuals as positive or negative, the test properties would have been considered insufficient.

Latent class sensitivity analysis
To assess the sensitivity of posterior inferences (for the ELISA Se and Sp values) to prior assumptions, the model was also run using non-informative priors across the range from 0-1 (meaning that all values in this range had approximately the same probability). The DAT Se and the prevalence estimate for population 2 (older wild boars) were the only parameters affected by the prior information included in the model. The posterior estimates of ELISA Se and Sp were stable.

4.4 Sample quality (I, II, III)
It is possible that the outcome of a serological test is affected by the quality (e.g., haemolysis, bacterial contamination, decomposition etc.) of the samples used. Between 10% and 30% of the wild-boar samples (II) were excluded in the selection process due to poor quality (decomposed and discoloured in ways other than haemolysis), but a large proportion of included samples were mildly to moderately discoloured by haemolysis. For inherent reasons, meat-juice samples in papers I and III all contained large amounts of lysed erythrocytes. How the results of serological assays are affected by haemolysis is only sparsely documented and the results are inconclusive.

For example, Jokelainen (2013) showed that the outcome of the DAT was not affected by haemolysis of the samples analysed. In contrast, Boadella and Gortázar (2011) studied the effect of haemolysis and repeated freeze-thawing on the outcome of a commercial ELISA used on wild-boar sera. Three of seventeen individuals displayed different results (positive-negative, negative-positive, and negative-doubtful) in the clear and haemolysed samples, respectively. Due to limits in sample sizes it is difficult to draw firm conclusions, and the results may vary depending on which test is used.

In all three papers (I-III), antibodies were stored at room temperature at some point. In paper II, wild-boar blood samples were voluntarily collected by hunters and sent to the National Veterinary Institute. The time between sample collection and freezing was therefore unknown. However, because signs of gross deterioration were evident in many samples, a long time at ambient temperatures may be suspected. In papers I and III, meat samples were left to thaw (for extraction of meat juice) for 24 h at room temperature.

Jakubek et al. (2012) found a low stability of T. gondii IgG in carcass fluids stored at room temperature. The degradation of antibodies was measured as a
decreasing number of seropositive foxes per week of sample storage, and after four weeks only approximately 40% of the originally seropositive foxes were still positive. In contrast, Jakubek and Uggla (2005) found no reduction of antibody levels in bovine blood when samples were stored for two weeks at room temperature.

4.5 Study populations

4.5.1 Fattening pigs (I)

The aim of the sampling protocol in study I was to mimic the composition of fattening pigs that reaches consumers as pork. The nine largest abattoirs in Sweden were asked to participate in the study; these nine accounted for more than 80% of the total slaughter volume (conventional and KRAV pigs) in Sweden in 2010. The composition of fattening pigs sent to slaughter likely represents the composition of fattening pigs on farms, because fattening pigs are bred almost exclusively for delivery to abattoirs (usually within 6-7 months).

The number of samples to be collected at each abattoir was calculated based on their annual slaughter volumes. Sampling was carried out during eight months in 2011. The sampling period was divided in two (January-April and September-November) to include pigs that had been out on pasture as well as those kept from pasture during the entire fattening period. During the sampling period, slaughter logistics changed and a large batch of KRAV pigs originally destined for one abattoir was slaughtered at two different ones. Subsequently, the sample size for each abattoir was recalculated.

Samples from conventionally and KRAV-raised pigs were collected at the abattoir, either by abattoir personnel or by the study team. A detailed study description was provided for the sampling personnel, so that they would be familiar with and understand the importance of the project. A systematic sampling procedure was applied, in which sampling was to be carried out on a defined number of days each month and where pigs were sampled at specified intervals (to increase the probability that sampling was dispersed across farms). Ideally, sampling could have been directed at different farms in proportion to their supply, which would have provided us with an even better appreciation of the *T. gondii* prevalence in pork reaching consumers. However, this was not practically feasible.

4.5.2 Wild boars (II)

The wild boar serum samples in paper II were a subset of samples from a national surveillance programme for disease-freedom concerning specific
diseases in Swedish wild boars (National Veterinary Institute, 2011). This is a programme – ongoing since 2000 – to which samples are voluntarily submitted by hunters. This sample is considered a convenience sample. Consequently, the sample cannot be assumed to be representative of the entire population.

We have no reason to believe that the sampled wild boars differ from wild boars not sampled. However, wild boars may be managed differently in different geographical areas, and some areas may be over- or underrepresented in the sample due to behaviours of the local hunters. For example, the use of supplementary feeding may be associated with the behaviour of hunters, who may be more or less likely to send in samples from these regions. Furthermore, the use of supplementary feeding may affect the probability of *T. gondii* infection in wild boars. On the one hand, feeding at such sites may render wild boars less prone to rooting in the ground for feed (presumably resulting in less contact with oocysts). On the other hand, wild boars that dwell around feeding sites may be more likely to ingest rodents or small birds (increasing the risk of tissue-cyst ingestion).

Adding to the complexity, the wild boar population in Sweden increased to more than 100,000 individuals by the hunting season of 2010/2011 compared to about 40,000 in 2005/2006 (Swedish Association for Hunting and Wildlife Management, 2016). Also, the geographical distribution of wild boars has expanded considerably during the study period, from the southernmost area to the middle-eastern parts of the country. This kind of inconstant population structure needs to be considered when analysing the data, and far-reaching conclusions based on the whole data may be inappropriate.

The ages of the wild boars were registered at the time of sampling (only for those collected in 2010 and onwards). The age assessment was based on phenotype (colour and size), and individuals were grouped in 4 different categories: 1) 3-5 months, 2) 5-12 months, 3) 3-12 months, and 4) adults. In the data analysis, the pigs were regrouped into pigs >12 months and pigs <12 months.

### 4.6 Statistical methods (I, II, III)

All methods used for hypothesis testing are described by Thrusfield (2005). Pearson’s Chi2 test is applied to sets of categorical data (presented in a contingency table) and tests the null hypothesis that the variables are independent (the observed frequency is compared to the frequency that would be expected if the two samples were derived from the same distribution). The Fisher’s exact test is used for the same hypothesis testing, but for categorical data for which the expected cell count is less than 5 in any of the cells (a small
sample size or low counts in some cells). The Pearson’s Chi2 test was used for comparison between KRAV pigs and conventional pigs in paper I and for all analyses in paper II. Due to small samples size and expected cell counts of less than 5, Fisher’s exact test was used for comparisons between 1) KRAV pigs without access to pasture and conventional pigs, 2) KRAV pigs with and without pasture access, and 3) different NUTS2 regions in paper I.

The Kruskal-Wallis rank sum test is used as a non-parametric test (with the one-way ANOVA as its parametric counterpart) on data with one categorical variable and one measurement variable, which do not follow a normal distribution. It is performed on ranked data, and because some information in the data is lost in this process, the test is associated with less power and consequently a higher risk of making a type II error.

The Kruskal-Wallis rank sum test was used to study differences in variable medians between infection groups of pigs (n = 15-25) (III). Based on the results that no differences were detected between the infection groups, they were grouped together for further analyses. Because the impact of making a type II error was considered more serious than a type I in this case, a one-way ANOVA was performed (null hypothesis still not rejected, data not shown) to ensure that the result was not due to lack of power. The Kruskal-Wallis rank sum test was also used to investigate differences in variable medians in meat juice from different muscle types (III). Because a significant difference was detected for all variables (except myoglobin) between different muscle types, a post-hoc analysis was performed using the non-parametric Wilcoxon rank sum test (also called a Mann Whitney U test) for unpaired data.

Possible associations between variables were investigated using the Spearman rank correlation test, which – in contrast to Pearson’s correlation test – does not require the data to be linearly related.

4.6.1 Logistic regression model (I)

A logistic regression model describes the relationship between one or more predictors (explanatory variables) and an outcome (response variable), in the case when the outcome variable is dichotomous (Thrusfield, 2005). More than one explanatory variable can be included in the model, to account for potential covariates. The estimate of association for the variable of interest is then adjusted for the other variables in the model.

The overall aim of paper I was to investigate whether pigs raised in KRAV-certified production systems (providing outdoor access) had a higher risk of T. gondii seropositivity than pigs raised in confinement, an observation previously found for other types of organic production systems (van der Giessen et al., 2007). The variable “production type” was included in the
regression model to investigate its association with presence of *T. gondii* antibodies. However, the higher risk of *T. gondii* infection is probably only indirectly associated with the production type, and other factors within the production system (such as access to oocyst-contaminated pastures) may contribute to the increased *T. gondii* prevalence in organic pigs. Therefore, the variable “pasture length” was also included, allowing us to investigate, not only if pasture access contributes to the prevalence of infection, but also how the odds of infection change with time spent on pasture.

The number of months of pasture access for individual pigs was based on two assumptions: 1) a slaughter age of 6 months, and 2) a presumed pasture period of 4 months between the beginning of June and the end of September (figure 8). The variable “production type” was kept in the final model to capture other management factors characteristic for KRAV production. “Farm” was included in the model to account for the fact that pigs were clustered within farms.

4.6.2 Spatial analysis (I, II)

The geographical classification system used in paper I and II is called NUTS (Nomenclature of Territorial Units for Statistics), which is a single hierarchical classification system for statistical production across the European Union. The NUTS variable was used as an indirect variable in the absence of information on other biologically relevant factors (such as cat density, temperature, and precipitation).

The NUTS variable was used to investigate the possible association between seroprevalence in KRAV pigs and region, as evaluated by univariable logistic regression analysis (I). It was also investigated as a possible random variable in a mixed model (I).

In paper II, differences in seroprevalence between NUTS regions were investigated using the Pearson’s Chi2 test.

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Figure 8. Illustration of how the variable “pasture length” was calculated for individual pigs. The circle represents a year with the 4 months of assumed pasture in dark green. The numbers inside the circle show the estimated number of months on pasture for a 6 months old pig slaughtered at the end of corresponding month.
5 Results and discussion

5.1 *T. gondii* in domestic fattening pigs in Sweden (I)

The seroprevalence of *T. gondii* in fattening pigs in Sweden has been estimated at 37% in 1984 and 3.3% in 2002 (Lundén *et al.*, 2002; Uggla & Hjort, 1984). Although these studies are not strictly comparable, the results imply that the *T. gondii* prevalence in fattening pigs decreased considerably in the interim 15 years. The results of paper I show that the prevalence in conventional fattening pigs remains low (1%).

A decreased seroprevalence has also been recorded in fattening pigs in other European countries during the last decades (van Knapen *et al.*, 1995; Edelhofer, 1994). This decrease is usually attributed to an intensified livestock production with effective biosecurity protocols including, for example, rodent control and enclosed feed storage sites. The decrease in seroprevalence in pigs in Sweden could be the result of a similar process. A relatively high biosecurity level is reported in Swedish pig farms compared to other livestock species (Nöremark & Sternberg-Lewerin, 2014; Nöremark *et al.*, 2010). However, studies show that there may be room for improvement. Backhans *et al.* (2015) pointed to shortcomings in biosecurity in Swedish farrow-to-finish farms, where the lowest biosecurity level against introduction of pathogens to the farm (external biosecurity) was observed in the categories “removal of manure and dead animals” and “feed, water and equipment supply”.

The results presented in paper I show that pigs raised under KRAV management were more likely to be seropositive (8% compared to 1% for conventional pigs), which was expected. However, the risk of being seropositive seemed to be attributed specifically to pasture access. The hypothesis in paper I was that there would be a higher prevalence in KRAV pigs – with or without pasture access – compared to conventional pigs. However, the results did not support this hypothesis: KRAV pigs without
pasture access were not more likely to be *T. gondii* seropositive. This was surprising, because cats and rodents were assumed to have increased access also to indoor areas and outside pen areas in KRAV production. The low seroprevalence level in KRAV pigs raised during periods without pasture access may indicate that biosecurity measurements – to limit the spread of pathogens to the pigs – are also in place in many KRAV production systems. However, this cannot be concluded based on only one study.

Results from this project showed that a one-month increase in pasture access was associated with an increase in the odds of being seropositive by 80% (OR = 1.8). This is compatible with what is known about the life cycle of *T. gondii*. Possible sources of pasture-borne infection are oocysts in soil, infected rodents and small birds on pasture and near feeding stations, and oocyst-contaminated feed and water on pasture. Thus, pre-harvest efforts to reduce *T. gondii* infection may include means directed at sources of *T. gondii* infection on pasture. The feasibility and effectiveness of pre-harvest preventive measures for *T. gondii* infection in outdoor reared animals have been discussed by others (Opsteegh *et al.*, 2015; Kijlstra *et al.*, 2009).

### 5.1.1 Pre-harvest prevention strategies

Pre-harvest on-farm prevention programmes would have to include a strict control of rodents (Kijlstra *et al.*, 2008). Backhans *et al.* (2015) showed that Swedish pig farmers are aware of the importance of rodent control, but since rodents and birds may thrive around feeding stations on pasture, this could be difficult to achieve outdoors, if at all possible. To what degree rodents contribute to the spread of *T. gondii* infection to Swedish pigs is unknown. However, no serological evidence of *T. gondii* was found in 148 rodents caught around pig and chicken farms (Backhans *et al.*, 2013).

Eliminating cats from near-farm areas has been proposed to limit the infection in pigs (Du *et al.*, 2012; Kijlstra & Jongert, 2009). However, even though it is possible to restrict the number of cats residing on the farm, it is probably more difficult to eliminate visits from cats residing in off-farm areas. Also, oocysts may be dispersed long distances due to rain washing (Gotteland *et al.*, 2014) and meltwater (Simon *et al.*, 2013).

Vaccination of on-farm cats, with an experimental live vaccine that prevents oocyst-shedding upon infection, has been shown to reduce the *T. gondii* seroprevalence in fattening pigs (Mateus-Pinilla *et al.*, 1999). Hypothetically, on-farm cats – if immune against oocyst shedding – could also serve as an efficient means for rodent control. This hypothesis deserves to be evaluated further. No vaccine is yet on the market (Opsteegh *et al.*, 2015), and even if an effective and safe vaccine, for prevention of oocyst-shedding in cats, were to
become available (Innes et al., 2009; Jongert et al., 2009), it would be difficult to administer it to stray cats.

Vaccination of pigs to prevent tissue-cyst formation (Burrells et al., 2015) could also have a positive effect on limiting the disease burden in humans. Development of such a vaccine for administration to specific risk groups of pigs is warranted (Opsteegh et al., 2015).

In conclusion, pre-harvest management procedures may not be enough to ensure a low T. gondii prevalence in pigs kept on pasture. Therefore, post-harvest management strategies are recommended to ultimately reduce human T. gondii infection from infected pork. These strategies are freezing or heat treatment of pork, and educating consumers of the risk of eating pork undercooked. Post-harvest risk management is further discussed in section 5.3.

5.2 T. gondii in wild boars in Sweden (II)

5.2.1 Seroprevalence
The T. gondii seroprevalence in wild boars in Sweden seems to be relatively high, estimated at 34% in young pigs and 55% in adults. This is similar to the recently estimated seroprevalence in neighbouring Finland. Jokelainen et al. (2012) investigated 197 farmed wild boars and found a seroprevalence of 29% in young pigs and 48% in adult pigs. A similar seroprevalence in the two countries is not unexpected, because Finland (in many geographical areas) may resemble Sweden in preconditions such as climate, composition of wild and domestic cat populations, and occurrence of rodent species.

In paper II, we found a surprisingly high seroprevalence in the youngest age category (3-5 months) (figure 9). Maternally derived antibodies are expected in the serum from piglets up to a few months of age, but are usually not present after three months (Dubey & Urban, 1990). It is therefore unlikely that the piglets in paper II still had maternally derived antibodies in their serum. Thus, it is concluded that a considerable environmental T. gondii pressure exists, which probably leads to infection early in life.

5.2.2 Wild boars as sentinels
Animals that are free to roam, and which also have close contact with soil, have been proposed as suitable sentinel animals for monitoring the infection pressure from the environment (Beral et al., 2012; Opsteegh et al., 2011b; Richomme et al., 2010). Therefore, we sought to explore how the environmental infection pressure varied over time, as measured by the seroprevalence in wild boars sampled during five different years (II).
Figure 9. *T. gondii* seroprevalence (including 95% confidence intervals) in wild boars aged 3-5 months (n = 44), 5-12 months (n = 231), and >12 months (n = 205) in Sweden.

A significantly higher seroprevalence was seen in wild boars sampled in 2005 (72%) compared to the following years from 2008 to 2011 (50%-38%). Any changes in environmental infection pressure are probably associated with changes in specific risk factors for *T. gondii* infection in wild pigs. Unfortunately, no information about such environmental factors was available for consideration in the study.

Few studies have investigated long-term temporal changes in wild-boar seroprevalence, but an overview of studies from the Czech Republic shows that the seroprevalence increased from 15% in wild boars sampled 1981-1990 (Hejliček et al., 1997), 26% in wild boars sampled 1999-2005 (Bártová et al., 2006), to 40% in wild boars sampled in 2008-2010 (Račka et al., 2015). Similarly, information retrieved from two studies conducted in Spain shows that the seroprevalence was higher in wild boars sampled in 2004-2007 (44%) (Closa-Sebastià et al., 2011) than in those sampled in 1993-2004 (21%) (Gauss et al., 2005). However, firm conclusions are inappropriate due to differences in the methodologies and populations in the studies. In contrast, a stable and homogeneous environmental infection pressure was concluded from results from wild-boar samples collected in 2002/2003 and 2007 from the same areas in The Netherlands (Opsteegh et al., 2011b).
Based on the literature and the results of paper II, a fluctuating environmental infection pressure is likely in Sweden, rather than a continuous trend in any direction. For instance, the seroprevalence in a dynamic Swedish sheep flock varied greatly when sampled twice annually for six subsequent years (Lundén et al., 1994). Sheep were sampled in spring before turnout to pasture and then again in autumn after housing. The seroprevalence varied between 10% and 45% (figure 10). The majority of seroconversions occurred on pasture, and the monthly incidence varied between years. This supports the hypothesis that the environmental contamination with infectious oocysts is fluctuating between years.

![Figure 10. Seroprevalence in a dynamic flock of Swedish sheep, sampled twice annually during 6 subsequent years. Sheep were tested before turnout to pasture in the spring (first bar of each year) and again after housing in autumn (second bar of each year). Based on data provided in Lundén et al. (1994).](image)

It is also likely that the degree of fluctuation differs between regions, depending on the dynamics of the risk factors. For instance, variations in factors (e.g., temperature, humidity, precipitation and snow coverage) influencing oocyst survival, oocyst dispersion, or thriving of rodent populations could have a considerable impact on the *T. gondii* prevalence in affected populations. This hypothesis is supported by Richomme et al. (2010), who found that the seroprevalence in wild boars in Corsica differed significantly between two consecutive sampling years, and that the level of change in seroprevalence differed between areas.
In Sweden, a higher *T. gondii* seroprevalence in the south, compared to other parts of the country, has been recorded in several host species, e.g., humans (Evengård et al., 2001), cattle (Uggla & Hjort, 1984), fattening pigs (Uggla & Hjort, 1984), lynx (Ryser-Degiorgis et al., 2006), and moose (Malmsten et al., 2011). A geographical difference in seroprevalence was further supported by the results in paper II, where a higher seroprevalence was seen in the counties of Skåne and Blekinge, in the very south of Sweden, compared to other regions. Ljungström et al. (1995) found a decline in seroprevalence from south to north in humans, and discussed possible associations between temperature and oocyst survival. Comprehensive studies clarifying the association between environmental factors and *T. gondii* seroprevalence in wild boars may provide a better understanding of under what circumstances *T. gondii* is transferred from the environment to intermediate hosts, including humans.

5.3 Post-harvest risk management of *T. gondii* infection in pigs

5.3.1 Fattening pigs (I)

Pigs reared with pasture access have a higher risk of being *T. gondii* infected. Options to limit the rate of *T. gondii* transmission to humans from pork include: 1) prevention of infection in pigs, 2) decontamination of infected pork at the abattoir, and 3) decontamination of infected pork before consumption.

In pigs reared with outdoor access, pre-harvest risk management may not be practically feasible and thus, information of the risk of consuming undercooked pork from such pigs needs to be available for consumers. A fundamental piece of information is that porcine meat of unknown *T. gondii* status should not be eaten raw or undercooked, i.e., heated to less than 65°C. Interestingly, the dilemma between the perceived health benefits of consuming organic meat and the associated risks has been discussed by Kijlstra et al. (2009). The authors pointed to the potential risk of not properly communicating to consumers the food-safety issues associated with outdoor management.

In 2011, EFSA delivered a scientific opinion, which concluded that the current use of Food Chain Information (FCI) does not include all indicators needed to classify pigs in relation to relevant public health risks post-harvest (such as *T. gondii*) (EFSA, 2011). As *T. gondii* tissue cysts are macroscopically invisible and undetectable by palpation, the infection cannot be detected during standard meat inspection of the carcass. To overcome this, it was suggested by EFSA that “incoming batches of pigs can be categorised into those from *T. gondii*-free herds and infected herds”, with subsequent risk management.

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(e.g., freezing or heat treatment) directed at pig carcasses from high-risk batches. Pigs from *T. gondii*-free farms would be exempted from this treatment (EFSA, 2011). Potential benefits and problems associated with implementing such management programmes have been discussed by Opsteegh *et al.* (2015)

Considering that over 2,500,000 conventional fattening pigs are slaughtered annually in Sweden (Swedish Board of Agriculture, 2015), the seroprevalence of 1% (I), albeit low, cannot be ignored. Further, it is likely that some conventional farms (or groups of pigs within farms) have a higher *T. gondii* prevalence than others, and the prevalence of infection may be due to on-farm management factors. These farms could be targeted specifically for risk management based on historical data on seroprevalence and information on *T. gondii*-associated management factors, as suggested by EFSA (EFSA, 2011). However, whether it is possible to achieve a “*T. gondii*-free” status must be evaluated further.

Based on the increased risk for *T. gondii* infection in KRAV pigs raised with pasture access (I), it seems appropriate to categorise this group as high risk. Even though the results of paper I indicated that KRAV pigs reared without pasture access were not at higher risk of *T. gondii* infection than conventional pigs, it may be unadvisable to categorise such pig batches on the basis of historical prevalence data. For pigs in “free-range” and “outdoor” management systems (even when pasture access is not provided), the *T. gondii* infection status could change rapidly with the sudden introduction of infection sources. However, the information of a relatively low seroprevalence in non-pasture KRAV pigs deserves further attention, and valuable epidemiological information could be gained by understanding the transmission routes on such farms.

5.3.2 Wild boars (II)

As shown, the seroprevalence of *T. gondii* in wild boars in Sweden appears to be high (II). Currently, there is no way of identifying *T. gondii* infected individuals during meat inspection at the game-handling plant. This clearly shows that measures are needed for prevention of *T. gondii* infection through consumption of wild-boar meat.

Information about the risk and risk management should be made readily available for groups who traditionally have consumed wild-boar meat frequently, e.g., hunters and their families. However, information should also be directed at the general public due to the increased availability of wild-boar meat.

Cooking practices have changed in Sweden in recent years, with a generally increased culinary interest and a curiosity for new foods. Game meat is
included in several recipes without distinction between species (Swedish Association for Hunting and Wildlife Management, 2015). Therefore, there may be a risk that wild-boar meat is prepared in similar ways as suggested for meat traditionally cooked rare (e.g. venison and beef). Also, because wild-boar meat, intended for distribution outside the hunter’s family, must be tested for *Trichinella*\(^{10,11}\), there could be a risk that *Trichinella*-free wild-boar meat is perceived as “safe” to eat undercooked.

5.3.3 Standardisation of meat-juice serology (III)

EFSA proposes that “categorisation can be based on historical testing results e.g. by serological testing of meat juice”, as a substitute for serum samples (EFSA, 2011). Categorisation of pig herds based on meat-juice serology is also proposed by others (Felin *et al.*, 2014; Meemken *et al.*, 2014).

Benefits of abattoir-based sampling of meat juice as compared to serum include: 1) blood sampling in the often narrow exsanguination area is avoided, 2) the carcass number tattooed in each pig is clearly visible only after entry of the carcass into the clean area of the abattoir, and 3) no additional sampling system (personnel) is needed in countries with already existing sampling protocols for *Salmonella* or *Trichinella* (using meat juice).

How well the level of antibodies in meat juice correlates with the level in serum is dependent on several factors. Firstly, antibodies are distributed to the meat-juice sample from blood and lymph within the muscle and thus, differences in the vascularisation between muscles may be critical. Secondly, the retention of blood in the muscles after bleeding at slaughter may be more or less extensive (reviewed by Warriss (1984)). For instance, the residual blood volume in muscles of rats depends on the level of stress around the time of killing (Warriss, 1978). Thirdly, the muscle ability to retain water (water holding capacity, WHC) possibly determines how much the meat-juice sample is diluted (Nielsen *et al.*, 1998). This ability is affected by post-mortem changes, such as decreased pH (Huff-Lonergan & Lonergan, 2005), and varies greatly between animals (Gil *et al.*, 2008), muscles, and parts of muscles. The WHC is also influenced by genetic factors, animal handling before slaughter, and meat processing.

In paper III, it was apparent that the level of specific antibodies in meat juice is dependent on the muscle chosen for extraction. Similar differences in antibody levels in meat juice from different muscles have been demonstrated previously (Forbes *et al.*, 2012; Ranucci *et al.*, 2012; Wallgren & Persson, 2000; Nielsen *et al.*, 1998). Consequently, if meat-juice serology is to be

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implemented for classification of pig batches in the food chain, then standardisation is necessary of the sampling protocol, including the type of muscle sampled.

Meat juice from heart muscle contained the largest amounts of specific IgG compared to the other muscles. Meat juice from heart also contained the largest amounts of Hb, partly reflecting the rich blood supply to this muscle. This is supported by others. For instance, O’Brien et al. (1992) found that porcine heart muscle contained about three times as much Hb than M. longissimus dorsi. Finally, our results showed that the correlation between antibody levels in meat juice and serum was highest for heart samples. Thus, it appears as heart would be the muscle best suited for meat-juice extraction. However, in regions were the heart is sold for consumption, it may not be considered suitable or available for sampling.

The diaphragm is the most widely used muscle for meat-juice extraction in serological surveys (Račka et al., 2015; Deksne & Kirjušina, 2013; Berger-Schoch et al., 2011; Lundén et al., 2002). Although the correlation of specific antibodies between meat juice and serum was shown to be less for diaphragm than heart samples, serological testing of T. gondii using meat juice from diaphragm could still be useful for estimation of population seroprevalence or risk-classification of pig farms (or groups of pigs). However, the overall conclusion of study III is that meat juice from diaphragm cannot be used to classify individual pigs as free from T. gondii infection.

In 1998, Nielsen et al. described the relationship between serum and meat-juice ELISA values for Salmonella. They concluded that the antibody OD values in meat juice and serum were associated with each other by a ten-fold dilution difference. This is one of the most cited studies regarding the dilution of meat-juice samples for serology, and assays designed for analysis of serum are often adapted to meat juice on the basis of this ten-fold dilution difference (Meemken et al., 2014; Richomme et al., 2010). However, in paper III, approximately 70% of the diaphragm samples had T. gondii antibody levels less than one-tenth of the serum level. A similar proportion was seen for total IgG (figure 11). Consequently, the number of positive samples is likely to be underestimated if a ten-fold dilution is consistently used in meat-juice serology without prior evaluation of the suitable dilution factor for each test.

Adjusting the dilution of samples to compensate for a low level of blood has been suggested as a way to increase the sensitivity of meat-juice serology (Mecca et al., 2011). Interestingly, our results showed a perfectly linear relationship between each of the parameters (Hb, haematin and total IgG) and the T. gondii antibody level in samples originating from the same pig (figure 12).
Figure 11. Total IgG ratio (serum/meat juice) for meat-juice samples from diaphragm, heart, semitendinosus, triceps and tongue.

Figure 12. Relationship between the *T. gondii* titre (see paper III for details) and levels of Hb, haematin, and total IgG in three representative pigs.
This initially suggested a predictive ability of each of these parameters for specific antibody distribution to meat-juice samples. However, the linear relationship was reduced when results for each type of muscle from all pigs were grouped together. This is most likely due to the relatively wide range of values for these parameters, normally occurring in pigs. For haemoglobin, values have been reported in the range of 100-170 g/l (Chittavong et al., 2013; Friendship et al., 1984) and for total IgG between 12-39 g/l (Wattrang, 1996). In our study (III), the range of IgG in serum was 8-26 g/l. The level of Hb in blood was not measured.

Thus, the results (III) do not support dilution of samples to adjust for differences in blood level, as suggested by Mecca et al. (2011). On the contrary, our results indicated that a low Hb level is not suitable as an indicator of a low antibody level in meat juice, when the method is inferred to a large population. Not surprisingly, the haemin measurement was less predictive than the haemoglobin measurement because haemin is composed of all haeme-containing proteins (e.g. Hb, myoglobin and cytochromes).

A more promising indicator was the level of total IgG, which could be expected, as it considers the antibody distribution from several possible sources (blood, lymph and extracellular spaces).

Future studies need to determine the practical implication of total IgG measurements in meat-juice serology. However, to implement dilution of meat-juice samples according to their total levels of IgG may not be suitable in post-harvest management strategies. Instead, the level of total IgG could be used as an indication of a low antibody distribution to the sample, which could then be assigned as unreliable.
6 Conclusions and future perspectives

- Based on the estimated *T. gondii* seroprevalence in wild boars in Sweden, it appears as the infection is relatively common. Infection can be expected in up to every other pig. Thus, information regarding the risks of consuming undercooked wild-boar meat and how to prevent infection should be made available for consumers. A fundamental piece of information to consumers is that porcine meat of unknown *T. gondii* status should not be eaten raw or undercooked, i.e., heated to less than 65°C.

- The seroprevalence in wild boars differed between years and regions, indicating a variability in the infection pressure from the environment. This is possibly influenced by factors affecting, for instance, the distribution and survival of oocysts, occurrence of rodents, and/or the feeding behaviour of pigs. To learn more about the epidemiology of *T. gondii* and routes of infection to intermediate and definitive hosts, such factors (temperature, humidity, altitude, precipitation, snow coverage etc.) need to be further evaluated regarding their impact on *T. gondii*.

- It has been suggested that abattoir-based screening could be used to classify batches of pigs as high or low risk regarding *T. gondii* infection. The *T. gondii* seroprevalence was significantly higher in pigs that had access to pasture compared to conventionally raised pigs. This indicates that pigs raised with pasture access could be classified as high risk. Freezing at the abattoir could be a strategy for post-harvest risk management for such high-risk batches of pork. However, before implementing management strategies, the risk of infection from eating
undercooked pork from such pigs needs to be assessed, and the cost-benefit of management programmes should be evaluated.

- Meat-juice has been proposed as a serological matrix for abattoir-based screening. However, factors affecting the distribution of antibodies to the meat-juice sample should be further evaluated to enable harmonisation of sampling, extraction, and testing. For example, it was found that the level of antibodies in meat juice varied depending on which muscle the sample was extracted from, which highlights the need of defining this parameter. Furthermore, it was indicated that the levels of total IgG could be used as an indication of a low antibody distribution to the meat-juice sample, which could then be assigned as “unreliable”.

- A cost-benefit analysis needs to be performed for human health in relation to implementation of any preventive strategies (e.g., abattoir-based risk management systems). A comprehensive approach is thus necessary for mapping the *T. gondii* epidemiology in the Swedish human population. In this context, consumer awareness of food safety – especially how to safely prepare meat – deserves to be investigated.

- Pre-harvest factors – likely to be associated with *T. gondii* infection in pigs – need to be characterised under Swedish conditions to allow a comprehensive assessment of the risk of *T. gondii* infection in different farms or groups of pigs. Further, future research should be directed at prevention of oocyst-shedding by cats and prevention of tissue-cyst formation in edible tissues of food-producing species.
7 Populärvetenskaplig sammanfattning


Parasiten sprids via katter (huvudvärd) som utsöndrar parasiten med avföringen. När dessa kommer ut i miljön kan de infektera människor och andra varmblodiga djur (mellanvärdar) via förorenat foder, mat, vatten eller via jord. Parasiten kan även spridas mellan olika mellanvärdar genom intag av infekterat kött som innehåller mikroskopiska så kallade vävnadscystor. Den främsta orsaken till toxoplasmainfektion hos människor i Europas anses vara konsumtion av otillräckligt upphettat infekterat kött, framförallt från gris, får och jagat vilt.

Avhandlingen baseras på tre vetenskapliga studier som beskriver förekomsten av *T. gondii* hos vildsvin respektive slaktsvin i Sverige, samt utvärderar köttsaft för användning vid undersökningar av antikroppar (serologi) mot *T. gondii* hos dessa djur. Förekomst av antikroppar anses vara en bra indikation på att grisar bär på parasiten, och att påvisa antikroppar i köttsaft istället för i blodprov är en metod som har praktiska fördelar, exempelvis vid provtagning för livsmedelsburna smittämnen i samband med slakt.
Förekomsten av *T. gondii* hos svenska slaktsvin undersöktes senast 1999. Då hade drygt 3 % av grisarna antikroppar mot parasiten. Under senare år har det blivit vanligare att grisar har tillgång till utevistelse, vilket kan öka risken för infektion. Därför jämfördes nu förekomsten mellan konventionellt uppfödda grisar och grisar uppfödda under ekologiska förhållanden (enligt KRAVs regler). Förekomsten av antikroppar hos konventionella slaktsvin var fortsatt låg (1 %), medan den var högre (8 %) hos KRAV-grisar. Resultaten visade också att den ökade förekomsten hos KRAV-grisar var förknippad med betesgång snarare än till övriga faktorer inom driftsformen och att varje månads förlängning av betesperioden ökade oddsen för grisen att bli infekterad.

Förekomsten av *T. gondii* hos vildsvin har inte tidigare undersökt i Sverige. Kött från vildsvin konsumeras i allt större utsträckning i Sverige eftersom dessa djur ökar kraftigt i antal. En stor andel av de undersökta vildsvinen i den här avhandlingen hade antikroppar mot *T. gondii*: 34 % av de unga (<1 år) och 55 % av de äldre djuren. Vildsvin som skjutits i södra Sverige hade oftare antikroppar än vildsvin från övriga landet och förekomsten varierade mellan provtagningsår.

Vid jämförelse av antikroppsnivåerna i blod och i köttsaft från infekterade grisar påvisades stora skillnader mellan köttsaft från olika muskler. Nivåerna av *T. gondii*-antikroppar var beroende av den totala mängden antikroppar (IgG) i köttsaften. Resultaten visade att undersökning av köttsaft kan vara ett verktyg för att uppskatta infektionsnivån i djurbesättningar, eller grupper av djur, men att det inte är lämpligt för att avgöra om enskilda individer är infekterade eller inte.
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