Experimental and immunological comparison of *Trichinella spiralis* and *Trichinella nativa*

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Academic dissertation

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1. ABSTRACT

Trichinella is a widely spread zoonotic nematode parasite. Human infection occurs after eating under-cooked meat, typically pork, wild boar or horse, containing infective *Trichinella*-larvae. Heavy infection may be fatal.

Eleven genotypes of *Trichinella* have been differentiated by PCR-based methods. In Finland, four species have been confirmed: *Trichinella spiralis, T. nativa, T. britovi and T. pseudospiralis*. *Trichinella spiralis* occurs in the domestic cycle, whereas *T. nativa* is the most common species infecting sylvatic animals in Finland. These two species have differences in their infectivity in different hosts and also in their resistance to freezing; *T. spiralis* does not survive at –20ºC, but in certain host species *T. nativa* does.

To learn more about the two most common *Trichinella* species in Finland, *T. spiralis* and *T. nativa*, these species were compared in this thesis both *in vivo* and *in vitro*. Raccoon dogs (*Nyctereutes procyonoides*), common *Trichinella* hosts in the Finnish fauna, and complement factor C6-deficient rats, in which the complement membrane attack complex was inactivated, were used as *in vivo* experimental models.

Raccoon dogs are favourable hosts for *Trichinellae* – they do not suffer from any clinical signs of trichinellosis even when the infection doses are fairly high. No significant differences in the course of infection were noted between *T. spiralis* and *T. nativa*. A peak of eosinophilic granulocytes was observed on the second week of infection, and weight loss and anaemia were more common in the infected group than in controls. Clear morphological differences between the species were observed in the tissue capsules. *Trichinella spiralis* capsules were lemon-shaped, whereas the capsules of *T. nativa* were more spherical and had more intense inflammation around them. Specific antibodies were recognized after two weeks of infection in both ELISA and Western blot analysis.

The role of the complement system in the host's defence against *Trichinella* was evaluated in an experimental infection of normal and complement factor C6-deficient rats with both *T. spiralis* and *T. nativa. Trichinella nativa* has a lower infectivity in rats. The survival of larvae in normal and C6-deficient serum was also observed *in vitro*. No

effect of C6 deficiency was noted in either species *in vivo* or *in vitro*. When exposed to human serum, no binding of complement factors C1q, C3, C8 or C9 to the outermost layer of the cuticle of adults, newborn larvae or muscle larvae was observed. This suggests that *Trichinellae* have mechanisms for evasion of complement, and that membrane attack complex does not explain the different infectivity of these two species in rats.

To investigate differences between *T. spiralis* and *T. nativa*, soluble proteins of their crude larval extracts were analysed by two-dimensional electrophoresis. Clearly different protein patterns were seen. Freezing was also shown to cause some changes in protein patterns. After MALDI-TOF analysis, we were not, however, able to identify the different proteins in database searches. Immunological differences were observed in two-dimensional Western blot analysis.

2. ABBREVIATIONS

2DE	two-dimensional electrophoresis
2D PAGE	two-dimensional polyacrylamide gel electrophoresis
Ab	antibody
ALAT	alanine aminotransferase
ASAT	aspartate aminotransferase
CK	creatine kinase
DAB	diaminobenzidine
DIGE	difference gel electrophoresis
ELISA	enzyme-linked immunosorbent assay
ES	excretory-secretory
FITC	fluorescein isothiocyanate
Hb	haemoglobin
HRP	horseradish peroxidase
IEF	isoelectric focusing
IgA	immunoglobulin A
IgE	immunoglobulin E
IgG	immunoglobulin G
mAb	monoclonal antibody
MALDI-TOF	matrix-assisted laser desorption / ionization – time of flight
MBL	mannose-binding lectin
MCH	mean corpuscular haemoglobin
MCHC	mean corpuscular haemoglobin concentration
MCV	mean corpuscular volume
MS/MS	tandem mass spectrometry
Mw	molecular weight
NBL	newborn larvae
OD	optical density
PCR	polymerase chain reaction
PCV	packed cell volume
pI	isoelectric point
RAPD	random amplified polymorphic DNA
RBC	red blood cell, erythrocyte
RCI	reproductive capacity index
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
T1	*Trichinella spiralis*
T2	*Trichinella nativa*
T3	*Trichinella britovi*
T4	*Trichinellapseudospiralis*
T5	*Trichinella murreli*
T6	*Trichinella* genotype 6
T7	*Trichinella nelsoni*
T8	*Trichinella* genotype 7
T9	*Trichinella* genotype 8
T10	*Trichinella papuae*
T11	*Trichinella zimbabwensis*
TIBC	total iron-binding capacity
VEGF	vascular endothelial growth factor
WBC	white blood cell, leucocyte

3. LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles, referred to in the text by their Roman numerals:

I Näreaho A, Sankari S, Mikkonen T, Oivanen L, Sukura A. Clinical features of experimental trichinellosis in the raccoon dog (*Nyctereutes procyonoides*). Veterinary Parasitology 2000, 91: 79-91.

II Sukura A, Näreaho A, Mikkonen T, Niemi M, Oivanen L. *Trichinella nativa* and *T. spiralis* induce distinguishable histopathologic and humoral responses in the raccoon dog (*Nyctereutes procyonoides*). Veterinary Pathology 2002, 39: 257-265.

III Näreaho A, Saari S, Meri S, Sukura A. Complement membrane attack complex formation and infectivity of *Trichinella spiralis* and *T. nativa* in rats. Veterinary Immunology and Immunopathology (submitted).

IV Näreaho A, Ravanko K, Hölttä E, Sukura A. Comparative analysis of *Trichinella spiralis* and *T. nativa* proteins by two-dimensional gel electrophoresis. Parasitology Research 2006, 98: 349-354.

4. REVIEW OF THE LITERATURE

4.1 Introduction

The *Trichinella* nematode, one of the biggest intracellular parasites (Despommier, 1990), is zoonotic and spread by ingestion of infected meat. Vertical transmission from a female host to offspring also occurs (Webster; Kapel, 2005). *Trichinella* has a wide range of hosts and an extensive geographical distribution. Over 150 mammalian species as well as birds and reptiles have been found to be infected worldwide (Bolas-Fernandez; Wakelin, 1989; Pozio, 2005). Even fish have been reported to serve as paratenic hosts to *Trichinella* (Moretti et al., 1996), although experimental infections have been unsuccessful (Pozio, 2005).

Taxonomically, the genus *Trichinella* belongs to the phylum *Nematoda*, class *Adenophorea*, order *Trichinellida* and superfamily *Trichinelloidea* (Noble et al., 1989). Two main clades are recognized within the genus: encapsulating and non-encapsulating (Pozio; Zarlenga, 2005). To date, 11 genotypes of *Trichinella* (Pozio et al., 2002), distributed by climate zones or by the host species, have been identified. For instance, *T. nativa* is found in the arctic zones and in sylvatic cycle, *T. nelsoni* in Africa and *T. spiralis* in the domestic cycle (Table 1). The genotypes are differentiated from each other by molecular biological methods. In Finland, four species have been confirmed: *T. spiralis, T. nativa, T. britovi* and *T. pseudospiralis* (Kapel et al., 2001; Oivanen et al., 2002). *Trichinella spiralis* occurs in the domestic cycle, whereas *T. nativa* is the most common species infecting sylvatic animals in Finland (Oivanen et al., 2002). These two species have a noticeable difference in their phenotype: *T. spiralis* does not survive at freezing temperatures, unlike *T. nativa*, which can stay alive in the muscles of certain species.

In the European Union, all slaughtered pigs and horses are at the moment examined for *Trichinella*. In human *Trichinella* epidemics, it would also be valuable to serologically identify the infecting species. This would help in searching for the infection source and, in some cases, in evaluating the severity of the infection; different species are known to cause different infection intensities. This kind of species diagnostics from muscle biopsies is complicated in human patients.

In Finland, *Trichinella* is annually found in pig meat inspections. We also have a high prevalence of *Trichinella* in wildlife, e.g. 38% in raccoon dogs (Oivanen et al., 2002). Human cases are, however, rare; the last unofficial report is from 1977, when three people became infected after eating bear meat (Salmi, 1978).

Table 1

	Species	Capsule	Geographical distribution	Confirmed in Finland	Freeze resistance[b]	Common host
T1	*T. spiralis*	yes	Cosmopolitan	yes	-	Pig
T2	*T. nativa*	yes	Arctic, subarctic	yes	+++	Bear, wolf, fox
T3	*T. britovi*	yes	Europe, Asia	yes	+	Fox
T4	*T. pseudospiralis*	no	Cosmopolitan	yes	-	Birds, marsupials
T5	*T. murrelli*	yes	North America	no	-	Bear, raccoon
T6		yes	North America	no	++	Bear, wolf
T7	*T. nelsoni*	yes	Africa	no	-	Hyena, lion
T8		yes	South Africa	no	-	Hyena, lion
T9		yes	Japan	no	-	Bear, raccoon dog
T10	*T. papuae*	no	Papua New Guinea	no	-	Reptiles
T11	*T. zimbabwensis*	no	Africa	no	-	Reptiles

Summary of *Trichinella* species and genotypes[a]

[a]Data according to Dupoy-Camet et al., 1994; Pozio, 2000; Kapel et al., 2001; Oivanen et al., 2002; Pozio et al., 2004.
[b]For details, see page 15.

4.2 History of *Trichinella spp.* and trichinellosis

Although *T. spiralis* was first discovered by medical student James Paget in anatomic dissection of human muscle tissue in London in 1835, trichinellosis and its connection to pork consumption were probably known much longer; the ancient dietary laws of early Israelites, for instance, prohibited the eating of pork (Gould, 1970). Predating Paget's observation, there had been some descriptions of oval-shaped structures in muscle specimens, which were later thought to be *Trichinella* capsules (Gould, 1970). Sir Richard Owen coined the term *Trichina spiralis* for this nematode in 1835 (Gould, 1970). Since the *Trichina* name had previously been given to a genus of flies, Railliet in 1895 renamed the worm *Trichinella* (Gould, 1970).

In 1846, Joseph Leidy found *Trichinellae* in a hog muscle. He recognized the capsules easily since he had seen the same organisms several times in human bodies in the dissection room (Gould, 1970).

Herbst was the first to establish that *Trichinellae* can invade the muscles through consumption of meat containing *Trichinella* capsules in it. He found these capsules in a cat in 1845, and conducted the first experimental infections with a badger and dogs. At the end of 1850, Leuckart and Virchow also performed experimental infections by feeding infected human muscles to dogs, thereby discovering the life cycle of *Trichinellae* (Gould, 1970).

In 1860, trichinellosis was determined to be fatal to humans. Zenker observed a large number of *Trichinellae* by microscope when examining the autopsy muscle samples of a 20-year-old woman. She had died 33 days after the onset of illness, which occurred just four days after eating pork. Sexually mature worms were also found, indicating that the entire developmental cycle had transpired in the same host (Gould, 1970). Several thousand people were simultaneously infected in European countries, hundreds of them subsequently dying (Blancou, 2001).

After *Trichinella*'s life cycle and the connection to eating meat had been established, the next step was to take action to protect consumers. Obligatory microscopic examination of pork was instigated in Prussia in 1879, but even before this, in1863, it was practised in some provinces. Inspection was performed by compression of the samples between glass plates (Gould, 1970). Finland too, has had a long history of *Trichinella* examination in meat inspection; inspection started in 1867, but official obligation to *Trichinella* inspection for pork came into force in 1923 (Rislakki, 1956).

4.3 Life cycle

4.3.1 Infection and adult stages

All of the developmental stages of *Trichinella* occur within a single host (Fig. 1). After ingestion of meat containing *Trichinella* larvae, the larvae are freed from their capsules during the digestive process. They penetrate the columnar epithelial cells of the small intestine. Development to the adult stage (from L1 to L5) in this intramulticellular niche

is rapid, taking approximately 30h (Despommier, 1983). An adult male is 1.4-1.6 mm long, and a female 3-4 mm, with some variation according to *Trichinella* species. After copulation, the male dies and the female starts to produce larvae (Soulsby, 1982). Eggs hatch inside the uterus, and L1 larvae 0.1 mm long are born.

4.3.2 Newborn larvae and migration

Larval production begins one week after the infection and continues for several weeks. However, females are the most fecund during the first week of larval production (Marti; Murrell, 1986). Their fecundity depends on the *Trichinella* species, as well as the site in the small intestine. Females recovered from the jejunum produce more larvae than ones recovered from the ileum (Sukhdeo, 1991). The newborn larvae (NBL) enter the lymph, reach the circulation and spread all over the body. *Trichinella* larvae settle into striated muscle and induce a nurse cell (and capsule) formation around them.

4.3.3 Muscular stage and nurse cells

When entering a muscle, *T. spiralis* larvae cause local damage first to the capillary and then to the muscle cell. The capillary is repaired without angiogenesis because the damage is minor. Muscle-specific proteins, such as creatine kinase (CK) and myoglobin, are released in detectable amounts in heavy human infections (Capo et al., 1998).

Larvae begin to coil inside the nurse cell as they grow (Soulsby, 1982). Their growth is rapid from day three to a couple of weeks after entry into the muscle (Despommier et al., 1991). In addition, the nuclei of infected cells increase in volume, reaching a maximum size in eight days. Each nurse cell contains an average of 40 enlarged nuclei derived from myocytes and the nurse cell complex accompanying satellite cells (Despommier et al., 1991). Recently, expression of apoptosis-related genes has been reported to be up-regulated during muscle cell transformation, suggesting their involvement in nurse cell formation (Wu et al., 2005). The inflammatory cellular infiltrates around the nurse cell are mainly mononuclear cells controlled by interleukin-10 (IL-10) (Beiting et al., 2004).

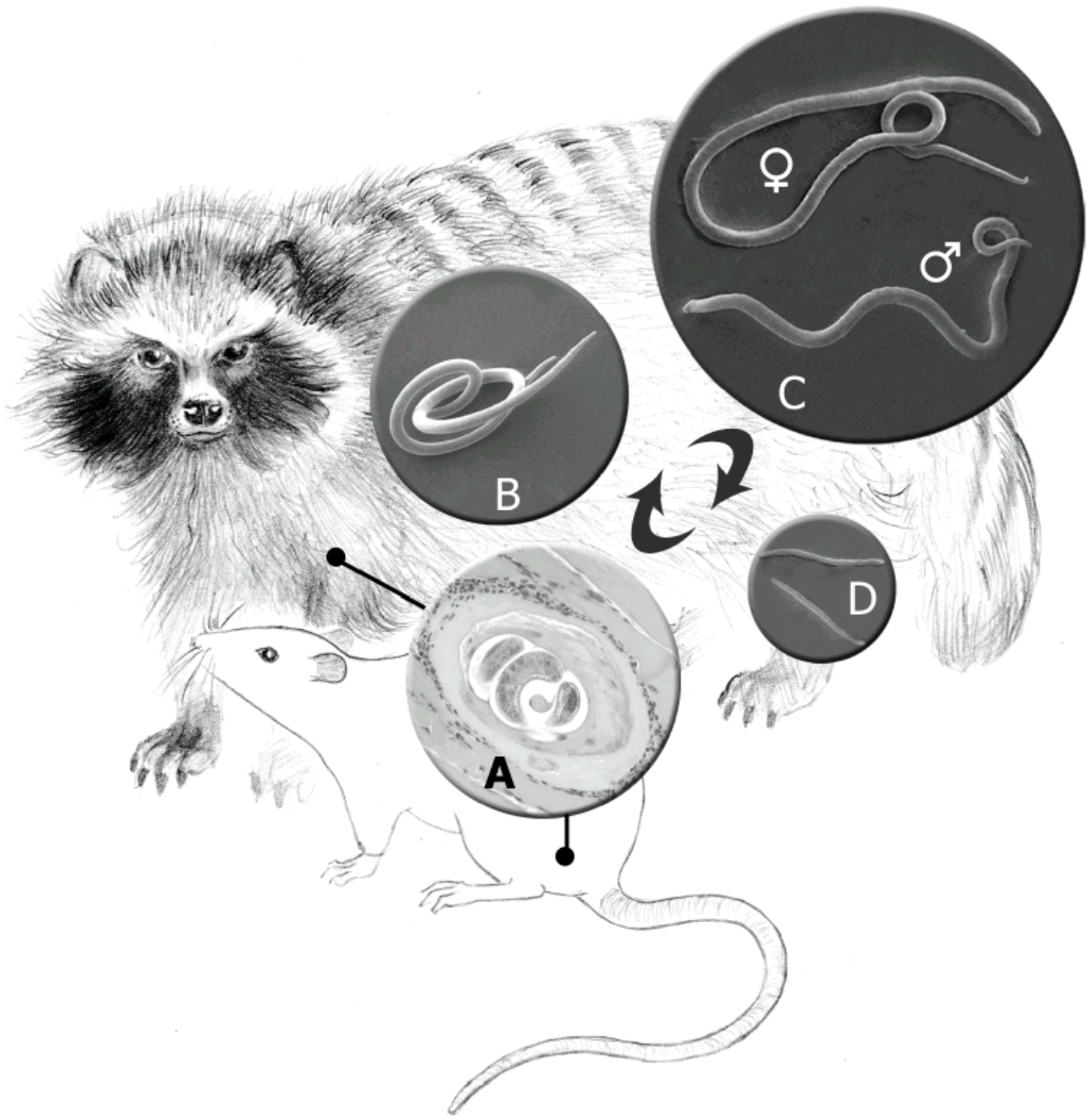

Figure 1. Life cycle of encapsulated *Trichinella spp.* A) Infective *Trichinella* larvae are encapsulated in the muscle (light microscopic photograph). B) After ingestion, larvae are freed from the capsule in the new host's ventricle (scanning electron microscopic (SEM) photograph,). C) Fast molting from first-stage larvae (L1) through L2, L3, L4 and L5 to reproductive adult females and males (SEM). D) Newborn larvae (SEM) begin to migrate and settle into the muscle A). Figure by S. Nikander & S. Saari.

Angiogenesis around the nurse cell starts at approximately day 12 post-infection. It is preceded by a hypoxic event, which triggers the up-regulation of vascular endothelial growth factor (VEGF). As the parasite and the nurse cell grow, hypoxia and the need for nutrients increase: neovascularization continues. In addition, the parasite stimulates the

nurse cell to synthesize collagen IV and VI to form a capsule around the nurse cell (Polvere et al., 1997; Capo et al., 1998).

The metabolism of infective L1 larva is anaerobic. Due to a larger-than-normal vessel diameter, oxygen exchange is minimal and anaerobic conditions are maintained, even though the nutrients and waste products are transported efficiently. Thus, the parasite must constantly secrete proteins to keep the nurse cell in a differentiated state (Capo et al., 1998). The anaerobic metabolism becomes valuable when the host dies, enabling the parasite to remain infective in the decaying tissue until eaten by another host (Despommier, 1998).

The development cycle of a nurse cell varies depending upon the *Trichinella* species. *Trichinella nativa* is the most rapid to encapsulate; nurse cell formation occurs between 20 and 30 days post-infection. *Trichinella murrelli* (genotype T5) is the slowest, with nurse cells formed around 60 days post-infection. The earliest nurse cells are, however, detected in *T. spiralis*, at 16 days post-infection, and the slowest species to start developing nurse cells is *T. nelsoni*, at 34 days post-infection (Pozio et al., 1992b). The differences in encapsulation times are probably the result of different maturation and reproduction rates since the capsule becomes apparent around day nine after the arrival of the NBL to muscle (Li; Ko, 2001). Once in the muscle, *Trichinellae* may survive for several years before being eaten and starting the cycle again in a new host.

4.4 Hosts

Trichinellae have a wide variety of hosts. Some of these hosts are permissive, such as the raccoon dog, mouse and human; several *Trichinella* species can infect and reproduce successfully in these hosts (Pozio et al., 1992a; Oivanen et al., 2002). Others are more selective, like the rat and pig, in which *T. nativa*, for example, is almost non-infective (Pozio et al., 1992a).

Due to this selectiveness, two distinct infection cycles occur: the domestic and sylvatic cycles. In the raccoon dog, four species existing in Finland have been confirmed, and mixed infections with two species have been found (Oivanen et al., 2002). *Trichinella* epidemiology in Finland has been described by a *Trichinella* study group at the Faculty

of Veterinary Medicine, University of Helsinki, in a recent doctoral thesis by Oivanen (2005) and in some related articles (Mikkonen et al. 1998; 2005; Oksanen et al., 1998).

The non-encapsulated species also have a difference in infectivity; *T. pseudospiralis* infects mammals and birds, whereas *T. papuae* and *T. zimbabwensis* infect mammals and reptiles (Kapel et al., 1998a; Pozio et al., 1999; 2002; 2004; Pozio, 2005) (Table 1). In addition, marine mammals may be infected (Forbes, 2000).

4.5 Resistance to freezing

Even before the different *Trichinella* species were recognized, muscle larvae originating from arctic animals were observed to survive freezing, whereas larvae from temperate areas died (Dick; Belosevic, 1978; Kjos-Hanssen, 1984). *Trichinella nativa* tolerates freezing considerable better than *T. spiralis*, especially in the natural host's muscle, but resistance is reduced in experimental mice (Pozio et al., 1992b). Thus, the host species has an effect on freeze resistance. Infective *T. nativa* larvae have been found in fox muscle after four years of storage at –18ºC (Kapel et al., 1999). In rat muscle, no infectivity remained after four weeks of storage at –18ºC, although a low reproductive capacity index (RCI) was observed after one week of freezing (Malakauskas et al., 2001).

T. spiralis can tolerate temperatures of –18ºC in pork for 2.8 days, but not for 5.6 days (Kotula et al., 1990), and in lamb for four weeks (Theodoropoulos et al., 2000). In rat muscle, *T. spiralis* was no longer infective after storage for one week at –18ºC (Malakauskas et al., 2001). The studies indicate that the host species is also important when freeze resistance is evaluated. These features must be taken into account when regulations are set; only pork can be considered *Trichinella*-safe after freezing in certain conditions (Kapel, 2005). The age of larvae also has an influence on resistance to freezing; 12-month-old muscle larvae survived better than two-month-old larvae (Pozio et al., 1993).

In liquid nitrogen, muscle larvae survive, but their infectivity is diminished (Jackson-Gegan; James, 1988). Cryopreservation of *Trichinellae* would reduce the need for

laboratory animals in *Trichinella* strain maintenance; however, a prerequisite for the method is that larvae remain reliably infective during the freezing.

Studies with free-living terrestrial nematodes in the Antarctic have clarified some of the mechanisms of cold tolerance, such as cryoprotective dehydration and recrystallisation inhibition, that work together depending on the conditions (Wharton, 2003). Although *T. nativa* is a parasitic, not terrestrial nematode, it might share some of these protective methods. In fact, one of the recrystallization-inhibiting proteins is a member of nematode-specific proteins (Wharton, 2003).

4.6 Trichinellosis

4.6.1 Trichinellosis in humans

Recent trichinellosis outbreaks have been caused by consumption of game and horsemeat rather than pork, although pork remains an important source of infection globally (Pozio et al., 1998; Schellenberg et al., 2003; Møller et al., 2005). Between 1991 and 2000, over 20 000 human trichinellosis cases have occurred in Europe (Bruschi; Murrell, 2002). The worldwide estimation of prevalence in people is as high as 11 million (Dupouy-Camet, 2000).

Symptoms of trichinellosis vary, which complicates the clinical diagnosis. Moreover the amount of ingested larvae, the infecting *Trichinella* species and the age and status of the patient can affect the symptoms (Bruschi; Murrell, 2002). Ingestion of 500 larvae or more causes clinical or even life-threatening disease (Murrell, 1985). Five larvae per gram of body muscle is reported to produce death (Faust and Russel, 1957, cited by Gould, 1970), but as many as 1000 larvae / g body weight have been found in people who died of other causes (Nolan and Bozicevich, 1938, cited by Gould, 1970). Diarrhoea, rash, fever, abdominal pain, vomiting, swellings, malaise, myalgia, elevated CK and eosinophilia are typical findings (Dupouy-Camet et al., 2002; Gomez-Garcia et al., 2003; Schellenberg et al., 2003). Death due to trichinellosis is rare but may result from myocarditis, encephalitis, pneumonitis, hypokalemia or adrenal insufficiency (Bruschi; Murrell, 2002).

4.6.1.1 Therapy

Mild cases of trichinellosis are asymptomatic and do not require medical therapy. Treating clinical trichinellosis in time can be problematic; the diagnosis is often set late due to the delayed onset of non-specific symptoms after an infected meal (Nunez et al., 2003). Antiparasitic drugs are most effective during the intestinal phase. The drugs used to treat trichinellosis are benzimidazole anthelmintics and corticosteroids (Dupouy-Camet et al., 2002; Schellenberg et al., 2003). Mebendazole is usually administered at a daily dose of 5 mg/kg, but higher doses (up to 20-25 mg/kg/day) are recommended in some countries. Albendazole is used at 800 mg/day (15 mg/kg/day) administered in two doses. These drugs should be taken for 10-15 days. The use of mebendazole or albendazole is contraindicated during pregnancy and not recommended in children aged < 2 years. The most commonly used steroid is prednisolone, which may alleviate the general symptoms of the disease. It is administered at a dose of 30-60 mg/day for 10-15 days (Dupouy-Camet et al., 2002). Otherwise, treatment is given according to symptoms. Antiparasitic drugs and prednisone appear to be effective in limiting the severity and duration of the illness (Schellenberg et al., 2003).

4.6.2 Trichinellosis in animals

Trichinella infection rarely causes clinical signs in the parasite's natural hosts, unless they are infected with a very large number of larvae (Bruschi; Murrell, 2002). For research purposes, *Trichinella* strains are maintained in laboratory animals because of the parasite's poor infectivity and survival after freezing preservation – infection is thus well defined in the mouse and rat (Stewart et al., 1978; Bell, 1998; Torrents; Vergara, 2000; Malakauskas et al., 2001). Rats are excellent hosts for *T. spiralis*. *Trichinella britovi* and *T. pseudospiralis* also yield high infection intensities, but *T. nativa* does not reproduce well in the rat (Malakauskas et al., 2001). Rats are asymptomatic, except for a decrease in food consumption and weight gain during the first two weeks of infection with a dose of 7500 *T. spiralis* larvae (Torrents; Vergara, 2000).

Experimental infections with other animal species have revealed differences between hosts (Kapel et al., 1998a; Pozio et al., 2004). These experiments usually clarify the infection cycle in local fauna and, through this, the possible risks to humans. Some experimental studies of *Trichinella* infection in pigs focus more on the distribution of larvae in muscles and serology instead of clinical signs (Kapel et al., 1998b; Kapel;

Gamble, 2000). Even if an infection dose is quite high, clinical signs do not necessarily appear. *Trichinella spiralis* infects pigs very efficiently, *T. britovi, T. nelsoni* and *T. pseudospiralis* moderately, and *T. nativa, T. murreli* and T6 only barely. After an initial rise, the antibody response corresponded to the infection intensities (Kapel; Gamble, 2000). Similar observations have also been made with wild boar (Kapel, 2001).

Studies in foxes have clarified *Trichinella*'s predilection sites in carnivorous animals in natural (Kapel et al., 1995; 1999) and experimental (Kapel et al., 1994) infections. Reports have indicated a lack of clinical signs in experimental infections with 500 and 5000 *T. spiralis* larvae/fox (Kapel et al., 1994) and with 10 000 *T. papuae* larvae/fox (Webster et al., 2002). Thus, foxes can carry heavy *Trichinella* burdens without manifesting any clinical signs, making them important reservoirs.

In an experimental infection, dogs (*n*=6) suffered from mild gastrointestinal disturbances during the first week of infection with a dose of 7500 infective larvae/dog. Eosinophil values in peripheral blood were within the reference range, but the blood sampling was performed as late as 26 days post-infection. In the same experiment, two medical treatments for trichinellosis were tested: albendazole (50 mg/kg, BID for 7 days) significantly reduced the number of larvae even if it was administered at the muscle phase, but milbemycin oxime (1.25 mg/kg, BID for 10 days) administered during larval shedding by females did not have an effect on the number of recovered larvae. The results were similar with cats (Bowman et al., 1993). Neuromuscular signs have been described in naturally infected dogs (Lindberg et al., 1991).

4.6.3 Eosinophilia in trichinellosis

Trichinella infection, like many other endoparasitic helminth diseases, elevates the number of eosinophils in blood. The reaction is dose-dependent; the higher the number of infectious larvae, the stronger and earlier the peak. T-cells (Basten et al., 1970) and genetic factors (Wakelin; Donachie, 1983) also have an influence on eosinophilic response. In rodents and rabbits, the peak typically occurs at the second or third week of infection, depending on the host species (Bell, 1998). In man, eosinophilia may occur as early as at the seventh day of infection or it may be delayed even up to the fifth or sixth week (Gould, 1970).

Although eosinophils have been shown to have marked killing activity for NBL *in vitro* in antibody-dependent cellular cytotoxicity (ADCC) experiments, they have no protective role *in vivo* (Bruschi F., 1999; Bruschi, 2002).

4.6.4 Antigens and immunity

Trichinella is able to infect many different kinds of hosts. This suggests that the genus is only little immunogenic or is not affected by the host's immunity (Wakelin, 1996). Bell (1998) has simplified *Trichinella's* immunity to three basic host responses whose consequences are rejection of infectious larvae, rejection of adult worms, and immunity against NBL. Evidence indicates that in swine encapsulated muscle larvae can be killed (Bell, 1998), while in mice the immunity is mostly expressed in the intestine (Goyal et al., 2002) and is primarily T-cell-mediated (Wakelin; Goyal, 1996), T-helper 2 (Th2) type (Khan; Collins, 2004). Host-*Trichinella* interactions should be viewed one host and one *Trichinella* species at a time since the genetically variable combinations are not necessarily comparable (Wakelin; Goyal, 1996).

Trichinella antigens can be divided into three groups based on their location: surface-, excretory/secretory (ES)- and residual somatic antigens (Dea-Ayuela; Bolas-Fernandez, 1999). Antigenic material in muscle larvae has been localized in the stichosome, body cuticle, hindgut cuticle, hypodermis, haemolymph, glycogen aggregates, oesophagus-occupying substance, midgut-occupying substance, brush-border, cytoplasmic granules in the cord, intestinal gland and some areas in genital primordial cells (Takahashi et al., 1990) (Fig. 2). In immunochemical analysis of cuticular components, they did, however, turn out to be relatively non-immunogenic (Maizels et al., 1982). In adults, antigens have also been found in reproductive organs (Takahashi et al., 1994).

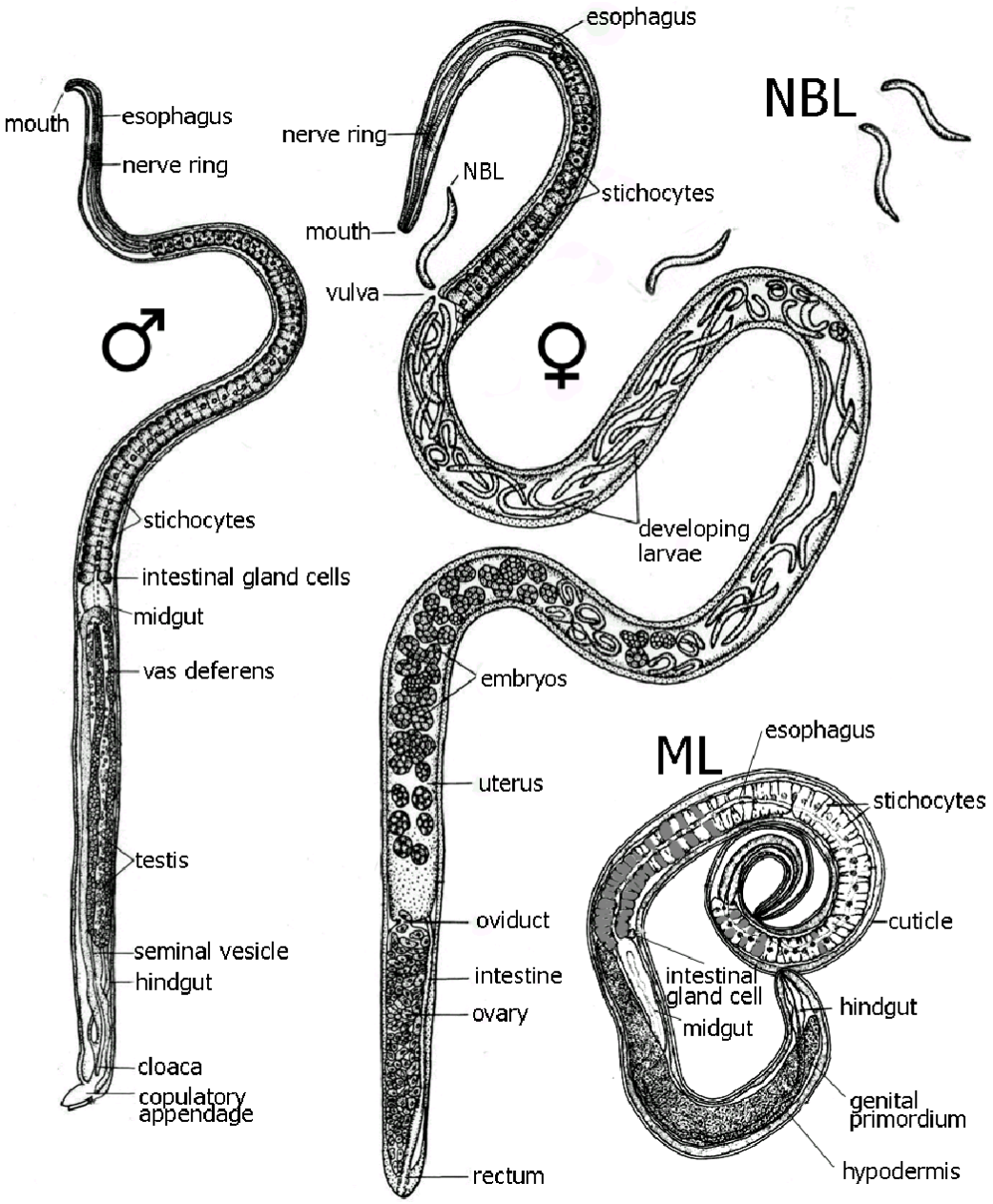

Figure 2. Morphology of *Trichinella* male (♂), female (♀), muscle larvae (ML) and newborn larvae (NBL). Modified by S. Saari from: http://www.trichinella.org/ with the permission of Dr. D. Despommier.

Different larval stages have different antigens. This is believed to be one of the mechanisms that prevent the host's defence system from killing the larvae (Ortega-Pierres et al., 1996). Two groups of *Trichinella* antigens induce the response in two

waves: the first at two weeks after infection (at the end of the intestinal phase) and the second at 4-5 weeks after infection (muscle phase). The latter group of antigens is called group II or TSL-1 antigens (Appleton; Romaris, 2001). TSL-1 antigens have an apparent molecular weight of 40-50kDa in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Appleton et al., 1991). TSL-1 antigens share a carbohydrate epitope, which carries an unusual dideoxysugar known as tyvelose (Appleton; Romaris, 2001). Even though tyvelose is also present in some bacteria and in *Ascaris* eggs, it is stage-specific in *Trichinella*: anti-tyvelose antibodies are not protective against adult stages (Appleton; Romaris, 2001). Despite the strong antibody response against tyvelose, it has neither host nor parasite protective function during the intestinal phase of infection (Goyal et al., 2002). Also non-secretory phosphorylcholine, although shown to be immunogenic, does not act as a target for protective immunity in *Trichinella* infection (Peters et al., 1999).

NBL have major antigens with a molecular weight of 20, 30, 58 and 64 kDa, whereas major antigens of muscle larvae are 47, 55, 90 and 105 kDa (Dea-Ayuela; Bolas-Fernandez, 1999).

The main source of ES antigens is the stichosome, which is composed of stichocytes. One of the most intensely studied ES antigens is a 43-kDa glycoprotein (Dea-Ayuela; Bolas-Fernandez, 1999), which has also been used as a diagnostic antigen in a commercial *Trichinella* Western blot kit (Yera et al., 2003).

Somatic antigens are conserved and less specific than other antigens. Despite cross-reactions, somatic antigens can be very useful, especially in the detection of early stages of *Trichinella* infection. They might also be of diagnostic importance after parasite death and destruction (Dea-Ayuela; Bolas-Fernandez, 1999), when they are released to a great extent.

Cross-reactivity between *Trichinella* species and several monoclonal antibodies (mAbs) has been tested (Boireau et al., 1997). Among 40 mAbs tested, 23 cross-reacted with all five species studied and T5. Some of the mAbs were specific to only a few of the species.

After a primary infection, partially protective immunity develops. Rapid expulsion of infective larvae from the intestine in secondary infection is well defined in rats, and is considered an IgE-mediated reaction. The intestinal IgE response appears earlier, has a higher titre and is more specific than the serum IgE response after *T. spiralis* infection in rats (Negrao-Correa, 2001). Mast cells are, however, not likely to be involved (Watanabe et al., 2005). IgA involvement in the intestinal expulsion has also been described in mice (Inaba et al., 2003). In the challenge infection of swine, the fecundity of females was diminished by up to 51%, and the adults were expelled from the intestine within three weeks (Marti; Murrell, 1986). In rats, the challenge immunity is mainly directed towards the pre-adult stages, and the expulsion occurs within hours (Wakelin; Denham, 1983). Passive immunization with anti-tyvelose mAbs in neonatal rats induces a rapid and almost complete expulsion of *Trichinella* larvae in a challenge infection (Appleton et al., 1988).

Partial immunity for trichinellosis can also be produced by vaccination. Vaccination with TSL-1 antigens stimulates protective reactions. Of these antigens, the 43 kDa molecular weight antigen is especially potent (Goyal et al., 2002). Vaccination of mice with 10 μg of a 48 K (probably corresponding to a 43 kDa) protein has been shown to reduce the larval burden by 81% compared with controls (Silberstein; Despommier, 1984). Vaccination against *T. spiralis* by intranasal administration of a larval homogenate or peptide antigen in the presence of cholera toxin B subunit as an adjuvant has also resulted in a significant reduction of adults on day eight post-challenge infection (McGuire et al., 2002). Also a DNA vaccine preparation has been made (Cui et al., 2004). Its efficacy testing *in vivo* in mice showed humoral and cellular immune responses and a significant reduction in larval burden in muscles (Wang et al., 2005).

4.6.5 Complement system

An innate immunological defence mechanism, the complement system, consists of plasma proteins, complement factors C1-C9, which become activated in the presence of foreign cell surfaces in a cascade-like manner (Fig. 3). The system is strongly regulated to prevent the lysis of normal host cells. It is activated by antibodies bound to antigens (classical pathway) or directly by cell surfaces without antibodies (alternative pathway) (Abbas, 2000). Mannose-binding lectin (MBL) or ficolins, structurally similar to C1q, bind carbohydrates, which typically are surface structures of microbes. MBL-associated

serine protease 2 (MASP-2) cleaves C2 and C4, thus activating the classical pathway without antibodies or C1 (lectin pathway) (Dahl et al., 2001).

The membrane attack complex (MAC) is the final, pore-forming part of the complement system. It consists of complement factors C5b, C6, C7, C8 and C9. Only small amounts of C5 and C9 have been found to bind to *T. spiralis* (Kennedy; Kuo, 1988), and the larvae survive despite complement consumption (Hong et al., 1992). The small amount and the monomeric nature of bound C9 may indicate that MAC inhibitors are present on the surface of *T. spiralis* (Kennedy; Kuo, 1988; Hong et al., 1992).

Complement inhibitors are fluid-phase or membrane-bound proteins that are able to regulate complement activation. The alternative pathway is constantly slowly activated, and therefore requires control. Factor H inhibits C3 convertases in the fluid phase and on cell surfaces. Complement receptor type 1 (CR1), membrane co-factor protein (MCP) and decay accelerating factor (DAF) inhibit complement on cell surfaces (Jokiranta et al., 1995). C4 binding protein (C4bp) competitively inhibits the binding of other components to C4b (Abbas, 2000). Protection against MAC on membranes is mediated by CD59, which inhibits the assembly of the lytic pore (Cole; Morgan, 2003). In addition, physical barriers, such as double membranes and dense lipopolysaccharide structures of bacterial surfaces, can prevent the function of MAC. MAC already formed can be exocytosed from the membrane (Jokiranta et al., 1995). In plasma, S protein binds to soluble precursors of MAC, thereby preventing their insertion into membranes (Abbas, 2000).

All stages of *Trichinella* are able to activate the complement system, mainly through the alternative pathway. NBL appear to be the most potent activators when parasite number and size are taken into consideration (Hong et al., 1992). *In vivo*, they are readily exposed to blood; muscle larvae are more sheltered. There is evidence of complement involvement in newborn larval death in the presence of antibodies (Mackenzie et al., 1978). This suggests that the classical pathway may play a role in defence against *Trichinellae*. *Trichinella spiralis* is known to be able to activate complement also through the lectin pathway, but this activation does not lead to inactivation of the parasite (Gruden-Movsesijan et al., 2003).

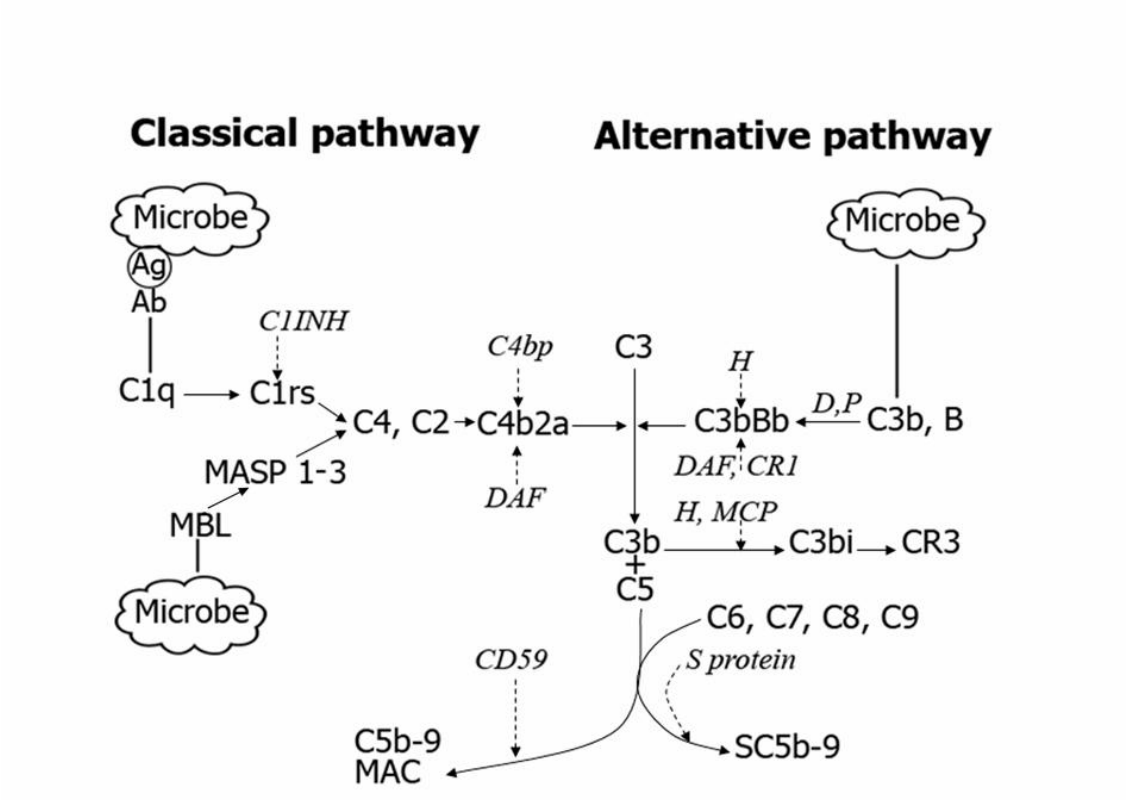

Figure 3. Schematic illustration of the complement cascade. Complement inhibitors are written in italics, and the site of action is indicated with -->. Figure by Professor S. Meri (modified by A. Näreaho & S. Saari).

4.7 Tools in diagnostics and research

4.7.1 Detection of the parasite in muscle samples

The definitive diagnosis of trichinellosis can be made microscopically from muscle samples. In weak infections, this could, however, be complicated because of the small number of parasites in the muscle. Earlier, meat inspection involved compression of pieces of muscle between glass plates and mircroscopic examination, but today digestion of muscle is the method of choice (Gamble et al., 2000). This method is also used to isolate muscle larvae for research purposes. In digestion, the muscle is treated with an artificial “stomach fluid” consisting of hydrochloride and pepsin, to free larvae from their capsules. Larvae sediment to the bottom of the tube or container, and can then be counted under a microscope. Because of the low prevalence of larvae in meat inspection, pooled samples are analysed. When evaluating the diagnostic methods used at slaughterhouses, the detection level should be at least 1 larva per gram (lpg), since

clinical trichinellosis is assumed to occur after ingestion of 100 larvae (assuming consumption of 100g of infected meat) (Geerts et al., 2002).

4.7.2 Species differentiation

Trichinella species are biologically defined by cross-breeding experiments (Pozio; La Rosa, 2000). In addition to eight verified species, three *Trichinella* genotypes of unspecified status exist (Pozio et al., 2002). Identification requires isolation of infective larvae from muscle samples. Species were earlier separated by isoenzymate typing methods, but the current differential diagnostics is PCR-based. Several PCR primers have been published for *Trichinella* diagnostics; initially for random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) (Bandi et al., 1993; 1995), and later for multiplex-PCR (Zarlenga et al., 1999; 2001). In *Trichinella* multiplex-PCR, five primer pairs are added to a single reaction mixture. This enables the identification of seven genotypes by known molecular weight band or band patterns typical for a specific species or a genotype.

4.7.3 Serological methods

Serological methods are valuable in the diagnostics of human disease since taking representative biopsies is uncomfortable for patients. Indirect haemagglutination, bentonite flocculation, indirect immunofluorescence microscopy, latex agglutination and enzyme-linked immunosorbent assay (ELISA) are the most commonly used serological tests (Bruschi; Murrell, 2002).

General problems in *Trichinella* serology are cross-reactions with other parasites (Abdel-Rahman et al., 2003; Yera et al., 2003; Abdel-Megeed et al., 2005; Abdel-Rahman; Abdel-Megeed, 2005). Cross-reactive antibodies are also found in patients with certain autoimmune diseases (Robert et al., 1996). Animals are often exposed to other nematode parasites, and this might affect results if the test is not designed properly. Crude larval extract, in particular, should not be used for testing because of cross-reactions. Serology is not recommended in meat inspection (Gamble et al., 2004). Serology in human diagnostics has, however, turned out to be quite reliable when combined with other parameters. At least two tests should be performed in a diagnostic laboratory to confirm a diagnosis (Bruschi; Murrell, 2002). Serological methods today lack *Trichinella* species diagnostics (Gamble et al., 2004). In addition to blood samples,

muscle fluid can be used in the diagnostics (Haralabidis et al., 1989; Beck et al., 2005; Møller et al., 2005).

ELISA can be used to measure host-produced antibodies or *Trichinella* antigens in a sample. They are detected with antibodies linked to an enzyme that reacts with a substrate to generate a colour reaction. The optical density (OD) of the reaction is then spectrophotometrically measured. When possible, paired serum samples taken with a two-week interval are recommended to demonstrate the increase in antibodies. The cut-off value for positive diagnosis is test-specific. In the literature, several ways have been introduced for setting the cut-off for *Trichinella* ELISA: e.g. 4 X mean value of uninfected serum (Gamble, 1996), [positive control OD – negative control OD] x 0.15 + negative control OD (Kapel; Gamble, 2000), negative mean plus 2-3 standard deviations (La Rosa et al., 1998; Oivanen et al., 2000; Pozio et al., 2002). Detailed instructions for validation of diagnostic assays can be found on the internet site of the World Organization of Animal Health (2004). Applications of ELISA have been used for the detection of antibodies against *Trichinella* with crude (Kapel et al., 1998b; Sukura et al., 2001), ES (Murrell et al., 1986; Van der Leek et al., 1992; Kapel et al., 1998b) and synthetic tyvelose antigens (Pozio et al., 2002; Forbes et al., 2004). Poly- and monoclonal antibodies can be utilized to demonstrate circulating *Trichinella* antigens (Li; Ko, 2001a). The latest application of ELISA in *Trichinella* diagnostics is a dipstick method; nitrocellulose filter paper is cut into strips and dotted with antigen or specific antibody (Al-Sherbiny et al., 2004). This test is fast and more field-applicable than the conventional ELISA.

The Western blot technique can also be applied to detect antibodies in serum or to define antigens with the aid of immune sera. With this technique, the antigens are recognized by their molecular weight. Industrially produced Western blot strips, which seem to work without major cross-reactions, are available for human *Trichinella* diagnostics (Yera et al., 2003). However, differences exist in Western blot patterns observed between human and animal sera infected with the same *Trichinella* species (Dupouy-Camet et al., 1988) – caution should therefore be taken when extrapolating methods to animal diagnostics.

4.7.4 Two-dimensional electrophoresis

Two-dimensional polyacrylamide gel electrophoresis, (2D-PAGE or 2DE) is a method for separating and constructing a gel map of the proteins of whole organisms. By purifying fractions and analysing them separately or by enrichment of certain fractions, the power of the method can be increased (Barrett et al., 2000).

The first dimension in 2DE is isoelectric focusing (IEF). Proteins are separated by their isoelectric point (pI) in a pH gradient with increasing voltage. Nowadays, immobilized pH gradients are commercially available in pre-cast thin gel strips (IPG strips). In the second dimension the strip is placed on an ordinary SDS-acrylamide gel, which is then run as usual. Proteins are thus separated by their molecular weight. As a result, peptide spots are spread all over the gel according to their individual isoelectric points and molecular weights. Spots are visualized with staining, usually with silver or Coomassie blue. Silver staining is more sensitive, detecting 2-5 ng of protein. Fluorescent stains are, however, even more sensitive, the detection limit being as low as 50 fmol (Barrett et al., 2000).

If a two-dimensional Western blot is desired, the 2DE gel is left unstained, and the proteins are transferred onto a nitrocellulose membrane. The membrane is then handled as in a conventional Western blot; the membrane is blocked and incubated with antibodies, and the binding reaction is then visualized.

Early experiments of two-dimensional protein analyses of *Trichinella* were conducted in 1981 by Despommier and Laccetti. The strip of the first dimension was cut into pieces and eluted after electrophoresis, and pH was measured. The proteins at a predetermined pH were then analysed in electrophoresis in the second dimension. Even earlier, two-dimensional immunoelectrophoresis was performed with arctic (*T. nativa*), north-temperate (*T. spiralis*) and tropical (*T. nelsoni*) isolates with homological and heterological hyperimmune serum (Sukhdeo; Meerovitch 1979). The arctic isolate differed the most by antigens from the other two isolates. The results are not, however, directly comparable with those of more advanced methods (Jasmer, 1990; Homan et al., 1992; Wu et al., 1999; Dea-Ayuela et al., 2001). In 1987, the larval fractions, enriched in stichocytes, of four different *Trichinella* species were compared in 2DE (Garate;

Rivas, 1987). In this study, *T. nativa* was also analysed and found to have a pattern similar to *T. spiralis.*

4.7.5 MALDI-TOF mass spectrometry

Two-dimensional electrophoresis can be complemented with analysis of single spots by matrix assisted laser desorption / ionization – time of flight (MALDI-TOF) mass spectrometry and database searches. A specific peptide mass fingerprint is generated after proteinase digestion of the spot, followed by mass spectrometry. The peptide masses are then compared with databases of theoretically calculated sequence-based masses to identify the protein. The identifications nowadays are extremely reliable, but the searches can be supplemented by adding partial sequence information from tandem mass spectrometry (MS/MS) of selected peaks (Barrett et al., 2000).

5. AIMS OF THE STUDY

The aims of the studies I-IV were as follows:

1. to compare *T. spiralis* and *T. nativa* infections in an adaptive natural host model (I, II),
2. to analyse *T. spiralis* and *T. nativa* infections in a selective laboratory animal model with a deficiency in the terminal complement pathway (III),
3. to compare soluble protein patterns of *T. spiralis* and *T. nativa* (IV).

6. MATERIALS AND METHODS

6.1 Animals and experimental designs

All the study protocols were approved by the relevant authorities (Committee on Animal Experiments, Faculty of Veterinary Medicine, University of Helsinki, and National Veterinary and Food Research Institute 28th May 1998, 5/1999, 9/2002 and State Provincial Office of Southern Finland STU 425A ESHL-2003-02673/Ym-23).

6.1.1 Mice and *Trichinellae*

Trichinella-infected mice were used in all studies (I-IV) as a source of infective larvae. *Trichinella* strains were maintained in Swiss mice at the National Veterinary and Food Research Institute (EELA). First-stage larvae were digested from the muscle samples with a magnetic stirrer method (Gamble et al., 2000). *Trichinella spiralis* (ISS 559) is a Finnish strain, originally from swine. *Trichinella nativa* (ISS 558) is also from Finland, from a raccoon dog. Both strains were isolated from natural infections in 1996 and later passaged in laboratory mice. The species were identified at a *Trichinella* Reference Centre (Istituto Superiore di Sanita, Rome, Italy). *Trichinella* species were also confirmed with multiplex-PCR (Zarlenga et al., 1999).

For experiments on resistance to the complement system (III), newborn larvae (NBL) and adult *Trichinellae* were derived from mice infected with 100-200 muscle larvae of either *T. spiralis* or *T. nativa*. After six days, the mice were euthanized and the small intestines were removed immediately, cut longitudinally and rinsed with physiological saline solution. Adult fertile female larvae were picked and cultured overnight *in vitro* (Marti; Murrell, 1986) to obtain the NBL.

To test survival and infectivity in frozen mouse muscle for the protein analysis (IV), we infected four mice with *T. spiralis* and another four with *T. nativa*. Larvae were digested from either fresh mouse meat or meat frozen at -20ºC for one week. The infection dose, 200 digested muscle larvae, was administered with a gastric tube. After two months of infection, the mice were sedated with carbon dioxide and cervically dislocated. The mice were then eviscerated, the skin and tail removed, and the carcass minced and carefully mixed to yield a homogeneous mass. After digestion of a 3-g sample of the

well-mixed meat, the reproductive capacity index (RCI) was calculated by dividing the total number of larvae per mouse by the number of inoculated larvae.

6.1.2 Raccoon dogs (I , II)

Eighteen male raccoon dogs were divided into three equal groups (*n*=6 in each group). Animals were kept in individual cages at the Faculty of Veterinary Medicine, University of Helsinki. They were infected with *Trichinella*-containing minced mouse meat *per os:* six with *Trichinella spiralis*, six with *Trichinella nativa* and six without inoculum (controls). The infection dose was 1000 *Trichinella* muscle-stage larvae/kg body weight.

Rectal temperatures were recorded at each blood sampling, and the animals were weighed weekly. Animals were observed daily for systemic signs of diseases; their alertness, the consistency of their stools and their consumption of feed were monitored.

Blood samples were collected for up to 12 weeks post-infection. One sample pre-infection and 21 samples post-infection were collected; first daily and then three times a week, followed by sampling once a week. At three, six and nine weeks post-infection, surgical biopsies were taken from leg muscles.

6.1.3 Rats (III)

Eighteen male PVG laboratory rats with genetically deficient complement factor C6 (C6-), and 18 male PVG rats with normal complement system (C6+) were included in the experiment. The rats were at least two months old at the beginning of the experiment, but the normal-rat group was a few weeks younger than the C6-deficient group. In both groups, C6+ and C6-, six rats were infected with *T. spiralis* and six with *T. nativa* and the six remaining were left without infection to serve as controls; thus, altogether 36 rats were examined. The infection dose, 2000 *Trichinella* muscle larvae/rat, was artificially digested from mouse muscle and then fed to rats with a gastric tube.

The condition of the rats was followed daily, and they were weighed weekly. Faecal samples for direct microscopy were collected daily for two weeks after the infection, and after this one sample at three and another at four weeks of the infection. A sample

comprised two droppings per day per rat. The total time of the experiment was two months, after which the rats were sedated with carbon dioxide and euthanized with cervical dislocation.

6.2 Histological and immunofluorescence methods

Every third week, the raccoon dogs were sedated and muscle biopsy specimens were surgically taken (II). Pieces of muscles were fixed in buffered formalin and then embedded in paraffin in both longitudinal and transverse orientation. In addition, some samples were frozen in isopenthane, pre-cooled with liquid nitrogen, for cryomicrotomy and adenosinetriphosphatase (ATPase) staining.

Raccoon dogs (II) and rats (III) were studied for gross findings immediately after euthanasia. Histological samples of rats were taken from the duodenum, tongue and left hind leg (*M. semitendineus*). The muscles of the right hind leg were removed and stored at -20ºC until analysed for infection intensity.

Paraffin sections (5µm thick) were stained for histopathological analyses. Haematoxylin and eosin (HE) staining was performed on all samples. Masson-trichrome, Periodic Acid Schiff (PAS) with and without diastase, and van Giesson stainings were also used (II). For muscle fibre typing (II), frozen specimens were stained with ATPase at pH 4.3, 4.6 and 10.3.

6.2.1 Quantitative analysis (II)

In this study, we used the term “cyst” to differentiate the entire *Trichinella* complex i.e. nurse cell, capsule and related cells, from the surrounding collagen capsule. The following histopathological characteristics were recorded for HE-stained raccoon dog muscle tissue sections (II): number of cysts per microscope field, shape of cyst, basophilic layer of nurse cell, number of nuclei in each nurse cell, maturation/thickness of the capsules collagen layer, number of mononuclear cells around the capsule and the exact number of eosinophils around the capsule. Quantitative image analysis was also performed. The area of the cyst, area of the nurse cell, area of the capsule and width and length of the cyst were measured from the digital, microscopic photograph. To avoid

variation in section level of the cylinder-shaped object, only cysts showing larvae and muscle fibres with longitudinal orientation were included in morphometric analyses.

6.2.2 Immunohistochemistry (II, III)

The binding of antibodies to antigenic structures can be observed by immunohistochemistry: histological sections were first treated with serum from immune animals, the bound primary antibodies were detected with labelled secondary antibodies, and the result was then visualized. By this method, also the binding of antibodies to internal structures of the worm could be studied. Sections of *Trichinella* cysts from raccoon dogs, sampled at 12 weeks post-infection (II), were incubated with homologous and heterologous *Trichinella* species-infected raccoon dog sera. Serum from a control animal served as a negative control, and normal goat serum was used for blocking. The sections were incubated with horseradish peroxidase (HRP) -labelled secondary anti-IgG antibody. Aminoethylcarbazole (AEC) was used as a chromogenic substrate to visualize the reaction.

Study III, adults and NBL, cultured overnight, were collected into a test tube and fixed with formalin, and melted agarose was then poured on them. The agarose pellet containing larvae was then embedded in paraffin and cut into sections. Rat muscle samples with encapsulated *Trichinellae* were also sectioned. As a positive control, we used yeast (*Saccharomyces cerevisiae*), which is known to activate complement. The slides were incubated in normal human serum, which acted as a complement source. For the immunohistochemical staining, we had four different primary antibodies for complement factors: rabbit anti-human C1q, rabbit anti-human C3, goat anti-human C8 and goat anti-human C9. We used commercial kits for stainings according to manufacturers'instructions (III). To control the staining procedure, slides without normal human serum treatment and slides without primary antibody were also included.

6.2.3 Immunofluorescence microscopy (III)

The antigenicity of the surfaces of *Trichinellae* can be examined with immunofluorescence methods. The worms were incubated with antibodies and then visualized by microscopy with fluorescent reagents conjugated to secondary antibodies. The binding to internal structures cannot be observed since the reagents do not penetrate an intact cuticle. Immunofluorescence staining reactions were performed in Eppendorf

tubes to study the binding of complement components on the worm surfaces. As controls, samples without human serum treatment, samples treated with inactivated human serum, samples without primary antibody and samples without secondary antibody were included. The protocol was also applied to tubes containing yeast (*Saccharomyces cerevisiae*) as a positive control. Normal human serum acted as a complement source. Primary antibodies against human complement factors C1q, C3, C8 and C9 were used, followed by secondary antibodies conjugated to fluorescein isothiocyanate (FITC). Samples were analysed with a microscope using a bright field filter and a fluorescein-specific filter.

6.3 Haematology and serum biochemistry

Blood was collected from the *vena cephalica* of raccoon dogs (I, II). Serum samples of rats (III) were collected before infection and three weeks post-infection from the tail vein or the *v. saphena* (lateral), and at the time of euthanasia with cardiac puncture. We also collected EDTA-treated blood from rats at the last sampling.

Blood smears of the raccoon dogs were observed for up to nine weeks post-infection to recover circulating first-stage larvae (I). Modified Knott's solution (Jain, 1986) was used to lyse the red blood cells (RBCs). The sample was centrifuged and the pellet was smeared on an object glass and stained with May-Grünwald-Giemsa.

Haematological and clinical chemistry analyses (I, III) were performed at the Central Laboratory of the Department of Clinical Veterinary Medicine, University of Helsinki. Blood haemoglobin (Hb), packed cell volume (PCV), erythrocyte (RBC) and leucocyte (WBC) counts, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) and platelet count were determined from EDTA blood by an automatic cell-counter adjusted for animal cell counting. For raccoon dogs, the mean cell values were calculated. May-Grünwald-Giemsa–stained smears were used for a differential cell count. The heat precipitation method was applied for the determination of fibrinogen from EDTA blood. The concentrations of serum total protein and albumin and the activities of alanine aminotransferase, (ALAT) were determined. Aspartate aminotransferase (ASAT) and creatine kinase (CK) were investigated as muscle-specific enzymes. As acute-phase

reactants, haptoglobin, ceruloplasmin, serum iron and total iron-binding capacity (TIBC) were measured.

6.4 Immunology and proteomics

6.4.1 Larval lysis and protein extraction

Larvae were lysed in sample buffer by heating (II) or mechanical homogenization (IV) and ultrasonication. The lysis solution was centrifuged and the supernatant collected. The protein concentrations of supernatants were measured by a commercial BCA Protein Assay Reagent kit (Pierce, Rockford, IL, USA).

6.4.2 Basis for serological assays

For developing serological assays, we first confirmed the cross-reactivity of commercial anti-dog IgG antibody (rabbit anti-dog, Serotec, Oxford, UK) with raccoon dog antibodies. The animals had been vaccinated against canine distemper at the place of origin. In Western blot assays, antigen from canine distemper-infected Vero cells confirmed the cross-reactivity between anti-canine IgG antibody and raccoon dog immunoglobulins, enabling the use of dog-specific reagents also for raccoon dogs.

6.4.3 ELISA

Microtitre plate wells were coated with *Trichinella* crude antigen protein (II) or *T. spiralis* excretory-secretory (ES) protein (III). Twofold serial dilutions of test sera (raccoon dog or rat) were applied. Peroxidase-conjugated antibody was placed into the wells, and tetramethyl benzidine substrate was added for the colour reaction. The reaction was terminated with sulphuric acid according to colour development, and absorbances were spectrophotometrically measured at a wavelength of 450 nm.

6.4.4 SDS-PAGE and Western blot (II)

Proteins were separated in SDS-PAGE under reducing conditions and then transferred to a nitrocellulose membrane. To reduce unspecific binding, strips were incubated in blocking buffer before incubation with dilutions of raccoon dog serum samples. Secondary antibody was HRP-labelled and diaminobenzidine (DAB) was used as a chromogen. Intensity of immunoreaction was categorized visually into four classes.

6.4.5 Two-dimensional electrophoresis (IV)

In the first dimension, isoelectric focusing, *Trichinella* protein was dissolved with rehydration buffer and the sample was loaded into the strip. Focusing was started according to the manufacturer's instructions. The focusing program for the 18 cm strip was 500 V for 1h, 1000 V for 2h, 4000 V for 2h and 8000 V for 4h. For the 7-cm strip, the focusing times were cut in half. The current during isoelectric focusing was 50 mA / strip. For the second dimension, the strips were loaded onto an SDS-PAGE gel, run and silver-stained with a modification suitable for MALDI-TOF, or used for Western blotting. Analysis of silver-stained 2DE gels was performed by visual comparison of the gels and with computer assistance.

6.4.6 Two-dimensional Western blot (IV)

Proteins were transferred from 2DE gels to nitrocellulose filters by fast semi-dry blotting. The filters were incubated in blocking buffer and then with primary antibody, *Trichinella*-infected diluted raccoon dog sera (from Studies I and II). Sera of uninfected raccoon dogs served as a control. The filters were incubated with diluted horseradish peroxidase-conjugated anti-canine IgG. The spots were visualized by enhanced chemiluminescence by exposing the blot to an X-ray film.

6.4.7 MALDI-TOF mass spectrometry (IV)

Spots of interest were cut out of the silver-stained gels and digested with trypsin. The recovered peptides were, after concentration and desalting with commercial chromatography columns, subjected to MALDI-TOF mass spectrometric analysis. Protein identification with the generated data was performed using public search programs and databases.

6.4.8 Testing for complement sensitivity (III)

The NBL were collected after a one-night *in vitro* culture of adults in Dulbeccos's modified medium and exposed to C6-deficient and normal rat sera. NBL were also incubated in sera from *T. spiralis*- or *T. nativa*-infected rats (with antibodies in sera). The sera were pooled from samples of several rats to standardize individual variation. Larval survival, *i.e.* movement, was observed. The follow-up period was up to 24 h.

6.5 Statistical analysis

Repeated analyses of variance (ANOVA) were used to test the effect of infection on parameters in raccoon dogs (I). Greenhouse-Geisser-adjusted *p*-values were used to evaluate the significance of day-effect and its interactions (I).

To analyse information from each of the tissue cysts (II), differences in each sampling time between groups were tested by a mixed-models ANOVA with group as a fixed factor and animal as a random factor nested within the group. The effect of time was analysed separately in each experimental group, with time as a fixed factor.

In Study III, differences between group means in the number of *Trichinellae* recovered from muscle samples were compared using Student's t-test. Comparisons were made between both parasitic species and complement status of animals. Haematological values were determined by one-way ANOVA followed by comparison of means between groups with the Bonferroni method.

P-values of less than 0.05 were considered to indicate statistically significant differences.

7. RESULTS

7.1 *T. spiralis* and *T. nativa* infection in raccoon dogs (I, II)

Most of the raccoon dogs were asymptomatic during the experiment. The most prominent signs of *Trichinella* infection were appetite loss and diarrhoea. Fever did not seem to be associated with the infection. All animals initially lost weight. After two weeks, control animals started to gain weight, reaching a plateau at the ninth week post-infection. Weight loss in infected animals was more noticeable and lasted longer than in controls.

Three raccoon dogs were excluded from statistical analysis because of a prolonged infection at the muscle biopsy sites. *Trichinella nativa* and *T. spiralis* groups did not differ significantly in the course of infection. They were thus pooled as a *Trichinella* infected group for comparison with the control group.

7.1.1 Haematology and serum biochemistry

Attributes of erythrocytes (RBC, PCV, and Hb) decreased in infected animals and in control groups during the intense sampling phase at the beginning of the experiment (four weeks post-infection), and then increased back to baseline levels. Erythrocyte values in infected animals were significantly lower than in controls. Both infected and control animals showed microcytic hypochromic anaemia.

No difference existed between the groups in total leucocyte counts, but *Trichinella* infection caused evident peripheral eosinophilia starting at the end of the first week post-infection and lasting until the end of the third week. This increase was statistically significant for a two-day period.

After the third week, platelets were more numerous in animals suffering from *Trichinella* infection than in controls, but this difference was not significant. Nevertheless, the same difference remained between the groups in all 12 samplings from the second week until the end of the ninth week.

Trichinella infection had no influence on the levels of muscle enzymes CK and ASAT. The biopsy procedure, by contrast, had a temporary elevating effect on these enzymes in all groups.

Serum total protein levels decreased more in the infected group than in controls. Significant differences were localized between the third and the seventh week post-infection. Time differed in its influence on albumin values between groups, but this difference could not be statistically localized to a certain time point.

Haptoglobin levels decreased in the infected group compared with control group after two weeks post-infection, then slowly increasing towards the values of the control animals. Haptoglobin and serum iron levels in the infected group were below the control group values in the fifth and sixth weeks post-infection.

No differences between the groups were detected in the values of fibrinogen, ALAT, ceruloplasmin and TIBC.

From the peripheral blood samples, only one circulating larva from a *T. spiralis*-infected raccoon dog was detected.

7.1.2 Infection intensity and capsule morphology

Inoculation with 1000 *Trichinella* larvae per kilogram of body weight resulted in intense infections at the muscular level in all raccoon dogs. The intensity of infection was similar for both parasites, with no statistically significant difference: on average 320 larvae per gram (lpg) of tissue in *T. spiralis*-infected animals and 375 lpg in the *T. nativa* group, in final samples analysed by digestion.

Muscle biopsy samples were also used for the quantitation of infection intensity during the experiment. The intensity increased from three weeks post-infection to six weeks post-infection, but the overall intensity in biopsy samples did not differ between species.

In three-week biopsy specimens, larvae were seen both extracellularly and penetrating into the muscle cells. Infected myofibrils became swollen, lost their cross-striation and

were more basophilic with a foamy cytoplasm. The nuclei of maturing nurse cells were oval, hypertrophic – indicating possible polyploidia – and had 1-2 nucleoli. In six-week samples, mature nurse cells were present, surrounded by newly formed capsules, which were PAS-positive, diastase-resistant and stained positive for collagen with Masson trichrome staining. The capsule thickened during the study period for both parasite species, but was thicker in *T. nativa* than in *T. spiralis.* Nurse cells lost their type I (slow) or type II (fast) fibre specific ATPase staining.

The shape of the cyst was round in transversal muscle sections and elongated in longitudinal orientation. A clear difference existed in the shape of mature cysts between the parasite species. The longitudinal section of the mature *T. spiralis* cyst was more elongated and lemon-shaped than the mature *T. nativa* cyst, which was more spherical (Fig. 4).

At three weeks, cellular inflammation associated with each developing nurse cell was minimal but became more evident at six weeks and even more so at nine weeks. Inflammatory cells seemed to gather at the polar ends in longitudinal sections, a reaction more evident in the *T. nativa* group than in the *T. spiralis* group. The capillary bed around the cyst started to develop early and was very obvious at six weeks. Calcifying cysts were not detected in these animals during the 12 weeks of follow-up.

The capsule became thicker during the study period in both groups. The shape of the *T. spiralis* cysts showed no change from the initial elliptical form, but the shape of the *T. nativa* cysts changed over time, becoming more spherical. Moreover, the numbers of mononuclear cells and eosinophils were similar at the beginning in both groups, but *T. nativa* seemed to evoke more intense inflammatory infiltration until 12 weeks post-infection. The inflammatory cell reaction in the *T. spiralis* group seemed to fade after six weeks.

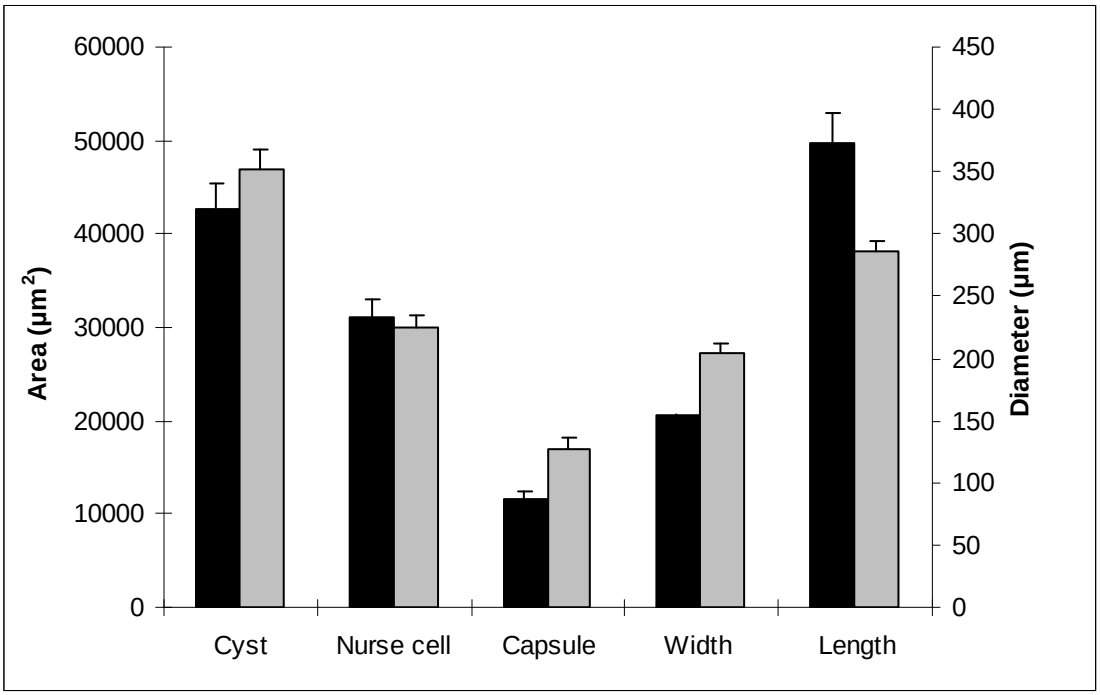

Figure 4. Mean image analysis values of *T. spiralis* (■) and *T. nativa* (■) capsules show a difference between the species in capsule thickness and shape. Left-side y-axis: area (µm^2) for cyst, nurse cell and capsule; right-side y-axis: width and length diameter (µm) of the capsule.

7.1.3 Detection of the antibodies by Western blot

Sera of infected animals reacted with a number of bands when crude antigen of the larval lysate was used. Major bands were around 100 kDa, with a set of bands between 76 and 40 kDa, many of them doublets. Sera of *T. spiralis*-infected animals reacted more strongly with *T. spiralis* than *T. nativa* antigens. In addition, antibodies of *T. native*-infected animals detected the homologous antigens more avidly, but some cross-reactivity was obvious.

7.1.4 Detection of the antibodies by ELISA

ELISA was used to detect the rise in antibody levels during the infection. The cut-off value for positive samples was set at the mean absorbance plus two times the standard deviation of the absorbance of negative animals at the end of the experiment. A clear homologous tendency was present, i.e. stronger reactions occurred when the coating antigen was from the same *Trichinella* species as that used in infecting the animal. With *T. spiralis* antigens, animals infected with the same species showed their first signs of

seroconversion as early as one week post-infection; this was confirmed with the sampling done at two weeks post-infection. Cross-reactive antibodies in *T. nativa*-infected animals could also be demonstrated, but with lower absorbance and later in the course of the infection.

7.1.5 Immunocytochemical localization of the antigens

Sera of the non-immune control animals showed no reactivity against any *Trichinella* structures. Immune sera from both *T. spiralis*- and *T. nativa*-infected raccoon dogs reacted against the cuticle and the internal structures of L1 larvae, especially against stichocytes. No reactivity was identified on the capsule or in the nurse cell structures.

7.2 *T. spiralis* and *T. nativa* infection in C6- and C6+ rats (III)

7.2.1 Clinical findings

Analyses of infection intensity (lpg) in rat muscles confirmed that *T. spiralis* was clearly more infective than *T. nativa*. No significant differences between C6- and C6+ groups were observed. The rats showed no clinical signs of trichinellosis. When stool samples of the infected rats were analysed, only four samples were positive for *Trichinella,* with one larva in each sample.

In both C6+ and C6- groups, *T. spiralis*-infected animals gained less weight than *T. nativa*-infected rats or controls. The total number of leucocytes was higher in both infected C6- groups than in the C6+ groups. Interestingly, this was mainly due to an increase in lymphocyte levels.

All animals had a normal appearance at autopsy, except for being overweight and having steatosis of the liver. In histological sections, *T. spiralis*-infected animals had several *Trichinella* capsules in their muscles and tongue. In the muscles of *T. nativa*-infected rats, the capsules were rare, indicating a difference in infection level. There was no detectable difference between C6+ and C6- groups in the cellular inflammation around the cysts. Duodenal samples taken eight weeks post-infection showed no histological changes due to *Trichinella* infection.

7.2.2 Anti-*Trichinella* antibodies

The levels of anti-*Trichinella* antibodies studied by ELISA increased faster during the first three weeks of infection in the *T. spiralis* groups than in the *T. nativa* groups, regardless of C6 deficiency. The higher infection intensity (lpg) in the *T. spiralis* groups explains the difference between *Trichinella* species in antibody levels at three-week sampling point. The levels of antibodies balanced by the eight-week sampling point.

7.2.3 Susceptibility to complement

To study whether the larvae of *Trichinella* can be killed by serum complement, NBL of both *T. spiralis* and *T. nativa* were exposed to sera from C6+ and C6– rats with and without *Trichinella* antibodies. The larvae survived, as evidenced by their movement, in all tested sera for the whole 24-h follow-up period. The presence or absence of antibodies in the serum did not seem to affect the NBL. The adult females also survived the 24-h serum treatment. These results suggest that *Trichinellae* can efficiently resist complement killing.

To investigate whether the resistance of *Trichinellae* to complement lysis was due to lack of complement activation, we treated histological sections of *Trichinellae* with serum and analysed binding of individual components. Neither the adult, newborn, nor muscle larvae bound complement factor C1q from human serum. Strongly positive staining for complement factor C3 was seen in certain stichocyte types of muscle larvae and in the musculature of adults. The outermost layer of the cuticle was always unstained, as were the unborn and newborn larvae. Factors C8 and C9 showed a similar but weaker staining intensity. No difference was observed between *T. spiralis* and *T. nativa* in binding of complement factors.

We also tested fresh *Trichinella* female and male adults and NBL for their ability to bind complement components. Neither adults nor NBL showed any binding of C1q, C3, C8 or C9 from human serum to the cuticle surface. Clear fluorescence was present in the intestinal glands of adult worms, but the structure was distinguishable in IF also in samples without any complement source, indicating unspecific staining. Weaker finger-like fluorescent invaginations were observed throughout the adult worms. Very strong diffuse binding of C3 was present on the surface of the *Saccharomyces cerevisiae* yeast control samples. Punctate immunofluorescence was observed in the yeast control

samples also with antibodies against C8 or C9. No C1q binding was detected in the control samples. In control cells, the alternative pathway became activated, but *Trichinellae* did not activate the classical or the alternative pathway.

7.3 *T. spiralis* and *T. nativa* in two-dimensional electrophoresis (IV)

To compare protein patterns of *T. spiralis* and *T. nativa*, soluble proteins were separated in two-dimensional electrophoresis. The effect of freezing on these patterns was also investigated.

7.3.1 Effect of freezing on the reproductive capacity index

The reproductive capacity index (RCI) is the final muscle larval count in relation to the amount of ingested larvae. The RCI of mice was higher with *T. spiralis* than with *T. nativa*. Mice infected with frozen *Trichinella*-containing muscle showed no larvae after two months, not even mice infected with *T. nativa*. *Trichinella nativa* was not able to reproduce after one week at -20ºC in mouse muscle. We, however, have an earlier unpublished result of low reproduction (0.67 lpg) of *T. nativa* in a mouse infected with 160 larvae that has been stored frozen at -20ºC for one week.

7.3.2 Two-dimensional electrophoresis and protein identification

To study differences between protein patterns, *T. spiralis* and *T. nativa* were analysed with two-dimensional electrophoresis. In silver-stained gels, the protein patterns of *T. spiralis* and *T. nativa* were clearly different. Especially the areas around and under 20 kDa were the variable. The differences were the most evident at a low molecular weight, where spots were distinct and separate from each other. Some temperature-related changes in protein patterns within a species were observed.

In MALDI-TOF analysis of eight *Trichinella* species-specific spots, peptide mass fingerprints successfully showed mass fragments with minimal background. This enabled a search in protein mass databases, but no matches were found. This is probably due to the limited amount of *Trichinella* data available.

7.3.3 Antigen analysis with two-dimensional Western blot

Western blotting was performed on both species with and without freezing treatment of the parasite. The reaction was performed with homologous and heterologous primary antisera (from Studies I and II), providing a total of eight combinations of blots. At a higher molecular weight area, over 30 kDa, strong signals were always seen, regardless of the sample, treatment or primary antibody. In *T. spiralis* samples, a three-spot signal was present at about 22 kDa and isoelectic point 5.8-6 when using homologous *T. spiralis*-infected raccoon dog serum. The signal was also observed in a few of the frozen *T. nativa* blots when heterologous *T. spiralis* serum was used.

8. DISCUSSION

8.1 *T. spiralis* and *T. nativa* infection

The raccoon dog can be considered an excellent host for *Trichinella*; both *T. spiralis* and *T. nativa* infected this host intensely and almost equally well. Rats, however, favour *T. spiralis*. In *T. nativa* infection, the parasite burden is low in the rat. In other rodents, *T. nativa* also does not reproduce as well as *T. spiralis*, nor does it tolerate freezing as well as in its natural host, the fox (Pozio et al., 1992b). The reproduction capacity index (RCI) of *T. spiralis* is higher than of *T. nativa*; the three-day average of NBL released per female *in vitro* is 110 with *T. spiralis* and about 30 with *T. nativa* (Pozio et al., 1992b). This difference was also observed in our studies. Nevertheless, the total reproduction capacities appeared to be lower than those mentioned in Pozio et al. (1992b), likely due to the different infection techniques used.

Clinical signs of trichinellosis were minimal in both raccoon dogs and rats. Weight loss or slower weight gain was noted in infected animals, but otherwise *Trichinella* infection caused no symptoms. In mice, instead of fever, which is a common symptom of human trichinellosis, hypothermia has been reported to be associated with *Trichinella* infection (Nishina; Suzuki, 2002). In our raccoon dogs, body temperature was slightly lower in infected animals than in controls. The general decrease in body temperature can be explained by adjustment for handling stress and seasonal variation, but the difference between groups is probably due to *Trichinella* infection. The body temperatures of rats were not determined.

In raccoon dogs, microcytic, hypochromic anaemia was present in the infected group. Frequent sampling and anorexia were probably the initial reasons for the decrease in the RBC values, but the slow recovery was caused by the *Trichinella* infection.

Eosinophilia, commonly associated with invasive helminth infections – including trichinellosis – peaked in raccoon dogs during the larval migratory phase. The most prominent peak was short term, but slightly elevated eosinophil values were detected for a two-week period, after which they remained within the normal range.

Lymphocyte counts were higher in complement factor C6-deficient rats than in controls. Lymphocyte counts typically decrease as rats age (Thrall et al., 2004). This does not, however, explain the difference since C6- rats were older than their C6+ peers. Diurnal variation also affects values, and stress during handling can decrease the amount of circulating lymphocytes (Thrall et al., 2004). Because these factors were standardized between the groups, the difference in lymphocyte counts likely arose from the different complement status. In the absence of C6, more material may become exposed to the immune system, leading to a stronger lymphocyte response.

Muscle enzymes CK and ASAT have been reported to react to the penetration of larvae into muscle cells by leaking into the blood stream. The levels of these enzymes are elevated in human trichinellosis (Capo; Despommier, 1996). In raccoon dogs, we did not notice any increase in enzyme levels due to *Trichinella* infection. Total protein and albumin values, however, differed between the raccoon dog groups. This can be explained by anorexia but also by *Trichinella*'s consumption of some of the host's nutrition.

We found only one circulating larva in the concentrated samples. NBL production starts on day 5-6 of infection. More than 75% of larvae are released during the first three days of production, and about 40% of circulating larvae are removed per hour (Bell; Wang, 1987). Thus, the circulation period is short.

A practical additional result of Study I was obtaining suggestive reference values for the haematological parameters of raccoon dogs. When we started the experiment, we found no such values for raccoon dogs to compare with our results. Our results are from an insufficiently large population to serve as actual reference values, but they do offer a basis for raccoon dog haematology and serum biochemistry.

Study II, *T. spiralis* and *T. nativa* were found to have different appearances in muscle biopsies; the cyst of *T. nativa* was more rounded and the inflammation more intense. Similar morphological observations have been made in Russia (Artemenko; Artemenko, 1997). These results are different from a previous study, which had showen no difference in capsule formation of these species (Evensen et al., 1989). Through the

morphological difference, it is possible to estimate of the infective species, even though definitive determination of species must be based on molecular biological methods.

To detect anti-*Trichinella* antibodies, we used the ELISA method and a crude larval extract or an ES antigen. Especially when using a crude larval extract, there is a risk for cross-reactions with other nematode infections. Therefore, its use in diagnostics is not recommended (Gamble et al., 2004). In autopsy, no findings suggestive of other nematode infections were detected. The animals were raised in a controlled environment. The possibility of cross-reactions can thus be ruled out. The absence of *Trichinella* antibodies in control animals and in pre-infection samples supports this assumption.

Trichinella spiralis infection led to formation of antibodies earlier and to higher levels than *T. nativa* infection in raccoon dogs. The same was seen in rats, regardless of C6 deficiency. The higher infection intensity (lpg) and RCI in the *T. spiralis* group explained the titre difference. This is in accordance with other ELISA results in rats (Malakauskas et al., 2001). Similar patterns have also been reported in other animals with different detecting antigens in ELISA (Møller et al., 2005). We also measured the antibody levels in heterologous sera, i.e. by using *T. spiralis* antigen against *T. nativa* antibody, and *vice versa*. Homologous combinations gave higher absorbances than heterologous combinations. Designing a serological method for species-specific diagnostics may therefore be possible. A similar homologous tendency of antibody reactions with *T. nativa* is mentioned by Kapel and Gamble (2000). The ES antigen in ELISA for rat sera was of *T. spiralis* origin, which might also have resulted in lower titres in *T. nativa*-infected rats.

8.2 *T. spiralis, T. nativa* and complement membrane attack complex

Trichinellae are invasive nematodes that are inevitably exposed to complement attack in blood and tissues. Thus, the ability to evade complement is vital for their survival in the host. The rat is known to resist *T. nativa* infection well. We compared rats with and without C6 for their sensitivity to *T. spiralis* and *T. nativa* infection. Studies were performed both *in vivo* and *in vitro*. In the *in vivo* study, no differences in *Trichinella*

infection intensities were noted between C6- and C6+ rats. This suggests that blocking of the complement occurs prior to the C6 stage of the complement cascade.

All stages of *Trichinella* are known to be able to activate the complement. Activation takes place mainly via the alternative pathway, the NBL being the most potent activators (Hong et al., 1992). Despite activation and consumption of complement, the larvae can evade complement attack and survive. *Trichinella spiralis* is able to activate complement through the lectin pathway as well. This activation route also does not lead to inactivation of the parasite (Gruden-Movsesijan et al., 2003).

We were unable to demonstrate significant larval or adult expulsion in faeces of rats with either a C6-deficient or a normal complement system. However, the amount of faeces analysed was only a small fraction of the day's production. Thus, we may have missed the expulsion.

In the *in vitro* assays, we followed NBL and adult females in human sera with and without *Trichinella* antibodies for several hours and observed no changes in their viability. Thus, both *in vivo* and *in vitro* tests showed that MAC is unable to destroy NBL. *Trichinellae* are well adapted to their hosts since they are able to reach the muscles despite the host's defence mechanisms.

Immunohistochemical and immunofluorescence analyses of serum-treated sections revealed that the outermost surface of *Trichinellae* remaines unstained for complement components. NBL, which are the development stage that must survive in the serum environment, were also devoid of bound complement factors also inside. Thus, complement activation appeared to be inhibited by *Trichinellae*. This could be due to a lack of complement activation on the *Trichinella* surface or to binding of complement inhibitors. Binding of complement control factor H has been studied earlier in *Trichinella* muscle larvae (Kennedy; Kuo, 1988). As there was no notable binding of factor H, this can not explain the resistance to MAC - at least not in muscle larvae. *Trichinella* may acquire a protective shield containing CD59-protein, an inhibitor, that prevents polymerization of MAC. This would, however, not inhibit the earlier stages of complement activation. Alternatively, *Trichinellae* may, like schistosoma, synthesize complement inhibitors or enzymes that cleave complement factors (Jokiranta et al.,

1995). *Trichinellae* are covered by a cuticle that forms a strong physical barrier against harmful events, including possible complement attack. Shedding of the outer membrane during maturation of larvae from L1 to L5 or scaling of surface material can also liberate the worm from bound complement components.

In conclusion, *T. spiralis* and *T. nativa* seem to control equally well the formation of MAC both *in vivo* and *in vitro*. This property obviously plays an important role in the survival of the *Trichinella* adults, NBL and muscle larvae in their hosts. Study III proved that the difference in infectivity between *T. spiralis* and *T. nativa* in rats is not MAC-related.

8.3 *T. spiralis vs. T. nativa* protein patterns

The differences between *T. spiralis* and *T. nativa* were also analysed by two-dimensional gel electrophoresis (2DE). Clearly distinct patterns were observed. In an earlier study, four different *Trichinella* species were compared by 2DE (Garate; Rivas, 1987). In that study, the larval fraction was enriched in stichocytes. We used crude soluble muscle larval proteins instead. Our data would have been easier to analyse had the samples been fractionated; the differences in intensity might also have been more prominent. Gel-to-gel variation could have been controlled by fluorescent difference gel electrophoresis (DIGE) methods (Unlu et al., 1997) or just simply by cutting the strips in half and running the comparable halves in the same gel (Wang et al., 2003). The low molecular weight spots described in the study by Garate and Rivas (1987) were also recognizable in our gels.

The 2DE method could be used to distinguish of peptides differing between the species. A species-specific serological method could thus be devised. We found such differing peptides and attempted to recognize them by MALDI-TOF mass spectrometry. However, the database searches gave no identification of the respective proteins. The molecular weights of the proteins might have been too small to provide sufficient peptides to ensure a reliable match in the database searches. The possibility of proteolysis causing these spots of relatively small molecular weight should also be taken into consideration. The main reason for the lack of identification was likely, however, that the databases are still imperfect with respect to *Trichinella*. By using

protein sequencing techniques, the peptides could, however, be identified. A recently published article showed identification of some *T. britovi* and T8 proteins using techniques similar to ours with partial sequencing (Dea-Ayuela; Bolas-Fernandez, 2005).

In two-dimensional Western blot analysis, the earlier mentioned homologous tendency of antibody reactivity was also observed. Antibodies from immune sera recognized more antigens from the membrane within the species. *Trichinella spiralis* produced a stronger antibody response than the other species. The reason for this is its higher reproductive capacity (Gamble et al., 2004), which leads to higher serum antibody levels and stronger reactions in Western blot.

Serum from *T. spiralis*-infected raccoon dogs recognized a specific set of proteins in the two-dimensional Western blot. Interestingly, the same pattern was seen in an earlier study with serum from an experimentally infected pig (Homan et al., 1992). Thus, these proteins could probably be used to separate *T. spiralis* and *T. nativa* infections and to design *Trichinella* species-specific serological diagnostics (Gamble et al., 2004).

Serology is not recommended to replace direct methods in meat inspection (Gamble et al., 2000), but it can be valuable in screening and follow-up diagnostics on farms. We believe that localizing differences between other *Trichinella* species is also possible with the present experimental design. Moreover, the differing proteins may explain how *T. nativa* is protected against freezing temperatures. A recrystallization-inhibiting protein belonging to a family of nematode-specific proteins has been introduced with terrestrial nematodes in the Antarctic (Wharton, 2003). Similar proteins might be present in *T. nativa*. Thus, closer examination is warranted.

9. CONCLUSIONS

1. In raccoon dogs, which are the adaptive natural hosts of *Trichinella spiralis* and *T. nativa*, these two *Trichinella* species caused equal infection intensities. Significant eosinophilia in peripheral blood was detected from the end of the first infection week to the end of the third week. Mild anaemia and weight loss also occurred. In histological sections of muscle larvae inside the nurse cells, morphological differences were noted between *T. spiralis* and *T. nativa*. At nine weeks post-infection, *T. spiralis* capsules were lemon-shaped, whereas *T. nativa* capsules were more spherical. The cellular inflammation response was more intense around *T. nativa* capsules. Seroconversion was apparent at two weeks post-infection by ELISA. Cross-reactions between species were common, but within species antigen-antibody reactions were more evident (homologous tendency) in ELISA and Western blot analyses.

2. Complement factor C6 deficiency, and consequent inactivity of the membrane attack complex, did not complicate the course of infection with *T. spiralis* or *T. nativa* in laboratory rats. The infection intensities and antibody responses were at the same levels regardless of the presence or absence of the C6 factor. Newborn larvae did not die during incubation in serum in the presence of complement, and the binding of complement components on the surface or the internal parts of adults or newborn or muscle larvae was minimal, suggesting that both species can evade complement, probably with the aid of complement inhibitors. Thus, the membrane attack complex of the complement system is not markedly involved in defence against *Trichinella* infection.

3. *Trichinella spiralis* and *T. nativa* differed in their soluble protein patterns in two-dimensional gel electrophoresis. Differences in intensities of some protein spots were also apparent when comparing frozen *T. nativa* samples with non-frozen ones. In two-dimensional Western blots stained with antisera from infected animals, the antigenic patterns differed as well, indicating different immune responses between species.

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