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Prevalence of *Toxoplasma gondii* Antibodies in Barren-Ground Caribou (*Rangifer tarandus groenlandicus*) From the Canadian Arctic

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ABSTRACT: Prevalence of antibodies to *Toxoplasma gondii* was determined in 147 barren-ground caribou (*Rangifer tarandus groenlandicus*) from 5 herds in the Northwest Territories and Nunavut, northern Canada, by the modified agglutination test (MAT). In the mainland herds (Bluenose, Bathurst, and Beverly), antibodies were found in 43 (37%) of 117 caribou, and MAT titers were 1:25 in 10, 1:50 in 24, and 1:500 in 9. In the island herds, only 1 (4.3%) of 23 animals sampled from the North Baffin Island herd was positive (titer = 1:25) and no antibodies were detected in 7 caribou from the Dolphin and Union herd. The high prevalence of antibodies to *T. gondii* in the mainland caribou herds indicates that caribou meat may contain viable *T. gondii.*

Toxoplasma gondii occurs worldwide in numerous species of warm-blooded animals (Dubey and Beattie, 1988), and natural infections have been described in at least 19 species of captive and free-ranging ungulates (Ratcliffe and Worth, 1951; Riemann et al., 1974; Kapperud 1978; Gorman et al., 1986; Stover et al., 1990). In wild ungulates of northern Canada, antibodies to *T. gondii* have been reported in muskoxen (*Ovibos moschatus*) in the Northwest Territories (Kutz et al., 2000) and in caribou (*Rangifer tarandus groenlandicus*) from the George River herd in northern Quebec (McDonald et al., 1990). In other circumpolar countries, antibodies to *T. gondii* have been found in moose (*Alces alces*) in Alaska (Kocan et al., 1986) and in reindeer (*R. t. tarandus*) in Norway, Finland, and Komi A.S.S.R. of the former U.S.S.R. (Oksanen et al., 1996; Oksanen et al., 1997). Recently, Zarnke et al. (2000) reported *T. gondii* antibodies in 7% of 319 Dall sheep (*Ovis dalli*), 6% of 241 caribou, 1% of 240 moose, and 1% of 241 bison (*Bison bison*) from Alaska.

In the Canadian Arctic and Subarctic, caribou are widely distributed and are the most abundant large herbivore. They are preyed on by wolves (*Canis lupus*) and grizzly bears (*Ursus arctos*) and are an important subsistence food source for communities across northern Canada (Kuhnlein et al., 1999). We report for the first time a serologic survey for antibodies to *T. gondii* in barren-ground caribou from the Northwest Territories and Nunavut.

Sera were collected during 1993 and 1994 from a total of 147 caribou from 5 different herds (Fig. 1). The Bathurst herd was sampled near Napaktulik Lake (66°22′N, 113°00′W) from 26 to 31 March 1993 during a commercial meat harvest conducted by the Kugluktuk Hunters' and Trappers' Association southeast of Kugluktuk. The remaining caribou collections were done as a component of a wildlife health study conducted by the Department of Resources, Wildlife, and Economic Development, Government of the Northwest Territories, Canada, in collaboration with local hunters' and trappers' organizations. The Bluenose herd was sampled at Sitidgi Lake (68°33′N, 132°42′W) from 16 to 20 March 1994. The Beverly herd was sampled near Doran Lake (61°13′N, 108°06′W) from 11 to 15 April 1994. The north Baffin Island herd was sampled near Pond Inlet (72°34′N, 78°23′W) from 26 to 30 April 1993. The Dolphin and Union herd was sampled on the Kent Peninsula (68°30′N, 107°00′W) from 17 to 22 November 1993.

Caribou were shot by local hunters and blood samples were collected, handled, and stored as described by Kutz et al. (2000). In 1998, at the United States Department of Agriculture Parasite Biology and Epidemiology Laboratory, Beltsville, Maryland, sera were screened for antibodies to *T. gondii* at 1:25, 1:50, and 1:500 dilutions using the modified agglutination test (MAT) as described by Dubey and Desmonts (1987). Although there is no information on the sensitivity and specificity of the MAT for the diagnosis of toxoplasmosis in caribou, on the basis of a validation study of MAT in pigs naturally and experimentally infected with *T. gondii* (Dubey, Thulliez, and Powell, 1995; Dubey, Thulliez, Weigel, Andrews, Lind, and Powell, 1995; Dubey, 1997), a MAT titer of 1:25 was considered an indicator of *T. gondii* exposure in caribou.

Antibodies to *T. gondii* were found in 44 (29.1%) of 147 sera (Table I). On the mainland the titers were 1:25 (10 sera), 1:50 (24 sera), and ≥1:500 (9 sera). A single positive animal from the North Baffin herd had a titer of 1:25.

Seroprevalence is summarized (Table I) and was analyzed using a χ^2-test with the level of significance set at $P = 0.05$ (Statview SE and Graphics™ 1988). Seroprevalence in adult animals was compared among herds and between mainland and island herds. For mainland caribou only, seroprevalence was compared among age classes (calves <1 yr old, yearlings 13- to 24 mo old, and adults >24 mo old) and between adult males and females. The low prevalence of *T. gondii* in island herds precluded statistical comparison of age and sex classes. There was no significant difference among mainland herds (Bluenose, Bathurst, and Beverly; $\chi^2 = 0.484$, $P = 0.785$, 2) or between island herds (Dolphin and Union, and North Baffin Island; $\chi^2 = 0.225$, $P = 0.6349$, 1), but seroprevalence in mainland caribou was significantly greater than in island caribou ($\chi^2 = 12.431$, $P = 0.0004$, 1). On the mainland, seroprevalence did not significantly differ between age classes ($\chi^2 = 1.735$, $P = 0.42$, 2), and there was no difference in prevalence between adult males and adult females ($\chi^2 = 0.258$, $P = 0.6116$, 1).

The antibody prevalence in caribou in the present study (29.1%) was considerably higher than that for reindeer in Fennoscandia (0.9% overall, 0.45% in calves, 1.29% in adults) (Oksanen et al., 1997), but the trends for increasing prevalence

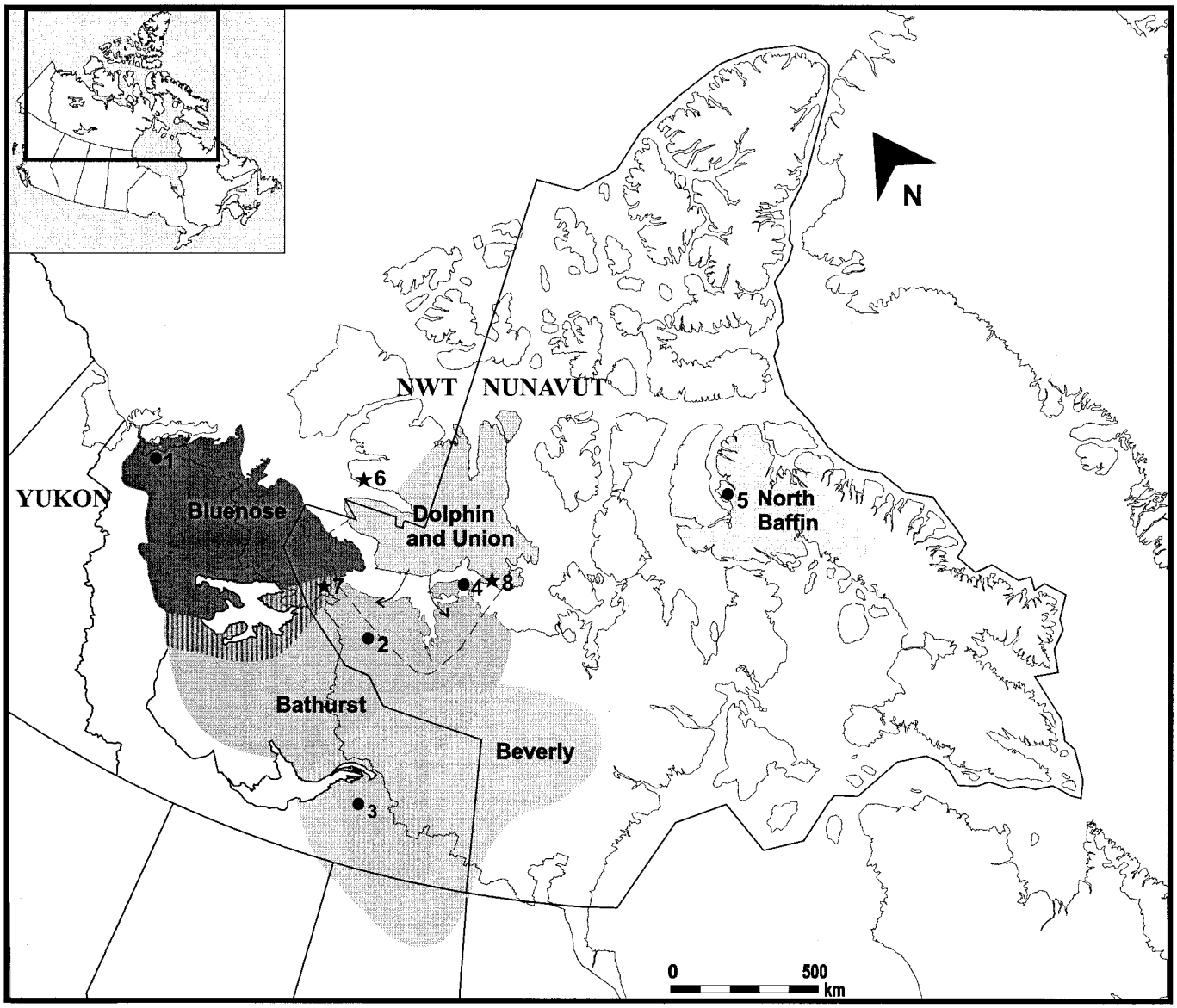

FIGURE 1. Map of the Northwest Territories and Nunavut, Canada showing the range (shaded areas) and sampling locations (numbers) for the Bluenose (1), Bathurst (2), Beverly (3), Dolphin and Union (4), and North Baffin (5) caribou herds. Sampling locations (6, 7, 8) for muskoxen survey (Kutz et al., 2000) are also shown.

with age and no difference between adult males and females were similar. In the present study, the higher prevalence on the mainland compared with the arctic islands is consistent with that observed for muskoxen in Canada (40% prevalence on mainland, 4.7% on Victoria Island; see Fig. 1 of Kutz et al., 2000). The prevalence in the present study was also higher than 6% seroprevalence in caribou from Alaska (Zarnke et al., 2000).

The source of *T. gondii* oocysts for caribou is unknown. The Canadian lynx (*Lynx canadensis*), the only wild felid in the Northwest Territories and Nunavut, generally remains below the tree line and its suitability as a definitive host is unknown. Experimental infections of a related felid *Lynx rufus* (bobcat), however, have resulted in *T. gondii* oocysts in the feces (Miller et al., 1972). If lynx are able to shed oocysts, they may serve as a source of infection for the mainland caribou herds (Bluenose, Beverly, and Bathurst) when these animals move below the tree line during their annual seasonal migrations (Fig. 1). The Dolphin and Union herd has a seasonal migration between Victoria Island and the mainland (Gunn et al., 1997), but does not migrate below the tree line (Fig. 1). A few lynx sitings near northern Bathurst Inlet suggest that there may be minimal opportunities of exposure of these caribou to lynx feces. For the North Baffin Island herd, overlap with lynx is improbable; lynx have not been reported from northern Baffin Island. Domestic cats (*Felis domesticus*) are uncommon in Victoria and Baffin Island communities, and it is unlikely that feral cats would survive outside these communities. Thus, the source of oocysts and exposure for the single positive case in the North Baffin Island herd is unclear and may indicate a false positive reaction.

TABLE I. Occurrence (prevalence in parenthesis) of *Toxoplasma gondii* antibodies in sera from free-ranging, barren-ground caribou from northern Canada.

		Calves		Yearlings		Adults		Unknown age		
Herd	n	M	F	M	F	M	F	M	F	Herd total
Bathurst	80	—	—	—	0/3	4/15	20/51	1/1	3/10	28/80 (35)
Beverly	22	—	—	0/1	0/1	7/10	2/10	—	—	9/22 (41)
Bluenose	15	0/1	—	1/1	—	4/11	1/2	—	—	6/15 (40)
Mainland Total	117	0/1 (0)	—	1/2 (50)	0/4 (0)	15/36 (42)	23/63 (37)	1/1 (100)	3/10 (30)	43/117 (37)
Dolphin and Union	7	0/1	0/1	—	—	0/1	0/4	—	—	0/7 (0)
North Baffin Island	23	—	—	—	—	0/4	1/19	—	—	1/23 (4.3)
Island Total	30	0/1 (0)	0/1 (0)	—	—	0/5 (0)	1/23 (4.3)	—	—	1/30 (3.3)

M = male, F = female.
Dash indicates no animals sampled.

In many species, infection with *T. gondii* may be common, but clinical disease is rare (Garell, 1999). Clinical disease in free-ranging caribou or reindeer has not been reported, but experimental infection of reindeer resulted in acute, severe, fatal hemorrhagic enteritis in 1 animal (Oksanen et al., 1996). *T. gondii* is an important cause of abortion and stillbirth in sheep and goats and has been documented to cause clinical disease in several captive ungulate species (Dubey and Beattie, 1988; Stover et al., 1990). It was suspected as the cause of abortion in a musk ox at the San Francisco Zoo, San Francisco, California (Crawford et al., 2000). Additionally, an outbreak of toxoplasmosis in Stone's sheep (*Ovis dalli stonei*) at the Assiniboine Park Zoo, Winnipeg, Canada resulted in the death of 1 animal and severe anemia in several others (G. Crawshaw, pers. comm.). The epidemiology and clinical significance of toxoplasmosis in free-ranging caribou and other wildlife populations need to be investigated further.

Resource harvesting is important to the Northwest Territories and Nunavut economies, with many northern people still relying on hunting and fishing for food and income. Caribou are currently harvested for subsistence use, commercial meat sales, and big-game hunting. The replacement cost of the 5 million kg of meat and fish harvested domestically each year is estimated at U.S. $40 million, with the estimated value of caribou harvesting at $16.6 million and increasing (Gunn et al., 1991). Factors compromising the health and affecting the viability of caribou populations could have profound socioeconomic impacts.

The potential public health significance of *T. gondii* in caribou should be evaluated. Human exposure appears to be high in northern North American communities: 40% of the population of Kuujjuaq in northern Quebec, Canada were seropositive for *T. gondii* (McDonald et al., 1990) and in Alaska, 28% of 1,572 aboriginal people tested during the 1970s were seropositive (Peterson et al., 1974). *T. gondii* was implicated in an outbreak of abortions in aboriginal women in northern Quebec and a statistical association was observed between seroconversion and both the consumption of raw caribou meat and the skinning of animals for fur (McDonald et al., 1990). *T. gondii* infection in humans has also been documented from the consumption of undercooked venison (Sacks et al., 1983).

Although seroprevalence of *T. gondii* in the mainland caribou herds in the present study was relatively high, serological evidence alone does not accurately assess the public health risks associated with handling or consumption of undercooked meat products (Gajadhar et al., 1998). Further study on the distribution, intensity, and presence of live *T. gondii* in tissues of different game and fur-bearing species is necessary to assess the risk of human infection associated with handling or consumption of these animals.

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Estimating Transmission Potential in Gastrointestinal Nematodes (Order: Strongylida)

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ABSTRACT: Microparasite virulence (the potential to cause harm in the host) is thought to be regulated by a direct trade-off with pathogen transmission potential, but it is unclear whether similar trade-offs occur in macroparasites (helminths). In this analysis, the transmission potentials of 5 nematode species (order Strongylida), known to differ in their virulence, were estimated using an index based on egg production and larval survivability. Virulence estimates were based on the minimum number of worms that cause host death. In nematode species where mature adults cause pathology (trichonematidic development), there is a direct relationship between virulence and transmission, suggesting that high virulence is related to parasite fitness in these worms. However, in nematodes where the juvenile stages produce pathology during migration and development (strongylidic development), virulence is not correlated with transmission. These data suggest that trade-offs between transmission and virulence in nematode parasites are not analogous for all species and may depend on the developmental strategy and mechanism of pathogenicity of the parasites.

A direct relationship between transmission and virulence, defined here as the potential of a parasite to kill its host, has been reported in many bacteria and viruses (microparasites) (Anderson and May, 1991; Bull et al., 1991; Toft, 1991; Antia et al., 1994; Ewald, 1994, 1995; Lenski and May, 1994). In microparasites, the basic reproductive ratio (R_0) is defined as the number of new infections established from a single infected host. Thus, reproductive success of these organisms is thought to be the result of trade-offs between increased parasite multiplication and decreases in transmission due to host mortality or morbidity. Under these conditions, a microparasite with a high transmission rate would be expected to exhibit high virulence.

The relationship between transmission and virulence is sometimes assumed to be similar for all parasites (e.g., Anderson and May, 1982; May and Anderson, 1990). However, the biology of helminths (macroparasites) is very different from that of microparasites, and the relationship between transmission and virulence of macroparasites has not been examined in detail. While microparasite virulence is usually the direct result of the pathogen destroying host cells or tissues during replication, most helminth species do not replicate within an infected host so that virulence is not necessarily a direct result of parasite reproduction. Instead, worm virulence is related to the density of parasites within a host, which is the result of the accumulation of infective stages from the environment (Anderson and May, 1991). In addition, at high densities, worm reproduction can be significantly decreased (Keymer et al., 1983; Fleming, 1988; Anderson and May, 1991), so that high levels of virulence do not appear to have intrinsic benefits to worms. Dobson and Merelender (1991) suggested that R_0 of a macroparasite (defined as the number of female offspring from a sin-

TABLE I. Larval survivability (expressed as % penetration ± SEM), egg volume, and adult female size of nematodes used in this study.

Species (identification)	Development	Larval survivability (S)	Egg volume (mm^3)	Worm volume (mm^3)
Small strongyles (A)	Trichonematidic	76.4 ± 21.1	1.1	2.9
Trichostrongylus colubriformis (B)	Trichonematidic	92.4 ± 22.7	0.7	4.7
Haemonchus contortus (C)	Trichonematidic	74.6 ± 11.6	0.6	17.3
Strongylus equinus (D)	Strongylidic	15.1 ± 4.8	0.9	100.2
Strongylus vulgaris (E)	Strongylidic	50.5 ± 4.7	0.7	27.6

gle female parasite that survive to reproduction, see Anderson and May [1991]) may be regulated by its transmission potential. The present study was designed to test the hypothesis that virulence is directly related to transmission in macroparasites by comparing estimates of transmission potential in closely related nematode species in the order Strongylida that differ in their virulence.

Infection with parasitic nematodes can result in host death but more often causes various degrees of host morbidity (Craig, 1988; Anderson and May, 1991). Morbidity as a result of worm infection results in great economic losses to the livestock industry. Heavy worm infections can cause significant reductions in weight gain and meat quality in beef cattle and in the growth and quality of wool in sheep (Craig, 1988; Albers et al., 1989; Maclean et al., 1992). As a result, the large variation in the harm that parasitic nematodes cause to their hosts has been well documented. For example, in the approximately 40 species of sheep nematodes (Strongylida), pathology ranges from mild malaise, e.g., *Ostertagia circumcincta,* to severe anemia and death due to gastric ulcers that develop as a result of the feeding behavior of *H. contortus* (Soulsby, 1965, 1982). This variation in virulence among strongylid nematodes makes them particularly suited to testing hypotheses on the evolution of virulence in worms.

The strongylid nematodes examined here have similar direct life cycles; adults live in the gut of the host and produce eggs that develop to infective larvae (L_3) in the host feces. The L_3 then migrate onto grass and are ingested when the host grazes (Soulsby, 1965; Olsen, 1974; Anderson, 1992). The species included in this analysis are *H. contortus* and *Trichostrongylus colubriformis* from sheep, and large (*Strongylus vulgaris* and *Strongylus equinus*) and small strongyles (a mixed culture of *Triodontophorus brevicauda, Triodontophorus serratus,* and *Cyliocyclus elongatus*) from horses. These species belong to a single monophyletic group based on phylogenetic reconstruction from both morphological and molecular data (Anderson, 1984; Adamson, 1986; Sukhdeo et al., 1997).

Despite the basic similarities in the life cycles of these nematodes, developmental differences within the host determine the parasites' mode of pathogenicity and may influence the evolution of virulence. The sheep parasites *H. contortus* and *T. colubriformis* and the small strongyles of horses undergo a trichonematidic form of development, whereas *S. vulgaris* and *S. equinus,* the large strongyles of horses, develop via a strongylidic pathway (Popova, 1964). In trichonematidic worms, the juvenile stages develop in the wall of the intestine and emerge as adults, with no extraintestinal migration. The majority of the pathology in these infections is associated with the feeding habits of reproductively mature adults in the lumen of the intestine (Soulsby, 1965). The strongylidic development of *S. equinus* and *S. vulgaris* includes a period of extraintestinal migration and development of juvenile worms, and the majority of pathology is associated with this phase of infection. Larvae of *S. equinus* migrating through the abdominal viscera and larvae of *S. vulgaris* migrating through the vasculature surrounding the host gut during development can cause peritonitis, verminous arteritis, colic, and death, even at low parasite densities (Duncan, 1972; White, 1981; Austin, 1994).

The complex life history stages of parasitic nematodes, as well as density-dependent effects on their growth and reproduction within the host, make it difficult to measure the transmission of nematodes directly (Toft, 1991). In addition, transmission potential can vary greatly due to environmental and biological conditions and thus is normally expressed as an estimate based on the biology of the parasite (Anderson and May, 1991). Nematode parasites employ 2 basic strategies to increase their transmission, i.e., they either produce large numbers of short-lived offspring or produce few long-lived offspring (Rea and Irwin, 1994). Our estimate of transmission potential is an index derived from the product of offspring production (V) and larval survivability (S).

$$\text{Transmission index} = V \cdot S.$$

Linear increases in egg production with female size have been reported both within a species and between related species in a number of nematodes and other parasites (Sukhdeo and Meerovich, 1977; Anderson and May, 1991; Poulin, 1995; Morand, 1996). The eggs of the nematodes in this study are comparable in size (Table I), and differential egg production potential was estimated by determining the volume of adult female worms for each species (V). Worm volume was calculated from the average length and width of adult female worms (data from Popova, 1964; Soulsby, 1965; Noble and Noble, 1971; Olsen, 1974; Anderson, 1992) (see Table I).

Infective L_3 larvae do not feed, and survive on stored energy, primarily in the form of lipids (Rogers, 1939; Clark, 1969; Croll, 1972; Barrett, 1976; Lee and Atkinson, 1977; Medica and Sukhdeo, 1997). Survivability of larvae (S) is dependent on larval energy reserves. In this standardized assay, the worm's ability to infect gut tissue was determined following depletion of larval energy reserves. For each species, 4 replicate tubes, each containing approximately 100 infective larvae, were exposed to host body temperature (38 C) for 8 days to stimulate sustained muscular activity and exhaust the lipid stores of the L_3 larvae. Controls for each species were incubated at 4 C for 8 days. At the end of the incubation period, the infectivity of the larvae was estimated using an in vitro penetration assay.

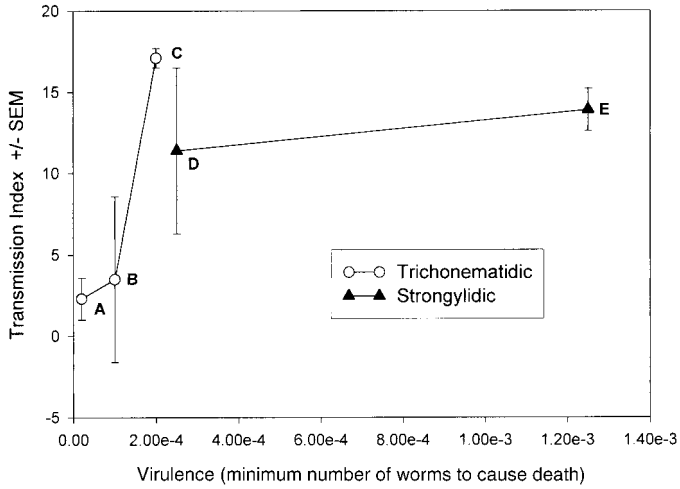

FIGURE 1. A direct relationship between transmission and virulence occurs in trichonematidic (○) nematode species that cause pathology as reproductively mature adults (correlation coefficient = 0.746, $P < 0.05$). Virulence is not correlated with transmission potential in strongylidic (▲) nematodes where pathology is caused by migrating juvenile stages (correlation coefficient = 0.194, $P = 0.644$). Species identification: (**A**) small strongyles, (**B**) *Trichostrongylus colubriformis,* (**C**) *Haemonchus contortus,* (**D**) *Strongylus equinus,* and (**E**) *S. vulgaris.*

Survivability was expressed as the percentage change from controls following the method of Medica and Sukhdeo (1997).

The definition and appropriate measurement of parasite virulence are controversial (Poulin and Combes, 1999). To allow comparison between nematode species, we utilized a pathogenicity-based estimate of virulence that incorporates both parasite actions and host responses.

$$\text{Virulence} = (\text{minimum number of worms that cause death})^{-1}.$$

It is difficult to obtain quantifiable estimates of virulence in nematode parasites, because their pathology is often expressed as morbidity (decreased weight gain and wool production), and comparisons across host species and between experiments conducted by different laboratories are problematic. Mortality-based measures of virulence are the most consistent, and the mortality data that we used provide estimates that correspond to the generalized ranking of virulence, in both the sheep and the horse nematodes, given by clinical practitioners. The data that form the basis of our estimates of virulence (minimum number of worms to cause death) come from a synthesis of a number of studies that were conducted prior to the advent of current drug treatment and pasture management strategies (Soulsby, 1965, 1982).

When the strongylid nematodes in this study are treated as a single group, the relationship between transmission and virulence is positive but not linear, nor significant (correlation coefficient = 0.356, $P = 0.0958$) (Fig. 1), suggesting that virulence is not correlated with transmission potential. When trichonematidic and strongylidic nematodes are analyzed separately, transmission potential is directly related to virulence in the trichonematidic worms (correlation coefficient = 0.746, $P < 0.05$) (Fig. 1), but there is no significant correlation between virulence and transmission potential in the strongylidic worms (correlation coefficient = 0.194, $P = 0.644$) (Fig. 1).

Theoretical models suggest that nematodes in which virulence is decoupled from fecundity and transmission (as in strongylidic development) should exhibit minimal virulence, but in species where transmission and pathogenicity are not independent of each other because increased use of host resources increases egg production (trichonematidic worms), increased transmission leads to high virulence (Dobson and Merelender, 1991). The results of our study only partially confirm this model. The data suggest that the trichonematidic parasites, *H. contortus, T. colubriformis,* and the small strongyles of horses, may experience a trade-off between virulence and transmission similar to that of microparasites. However, in *S. equinus* and *S. vulgaris,* which develop via the strongylidic pathway and cause most of their pathology during the migration of juvenile stages, there is no correlation between transmission potential and virulence. In addition, virulence is not minimized in this parasite as the theoretical models suggest (Dobson and Merelender, 1991), and *S. vulgaris* is, in fact, considered the most pathogenic of the horse nematodes. Thus, some selective force other than transmission potential could be acting on the evolution of virulence in these worms. In summary, this analysis suggests that the relationship between macroparasite virulence and transmission is not analogous for all nematode species but may vary with the parasite's developmental strategy and mechanism of pathogenicity in the host.

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Antibody Levels in Goats Fed *Toxoplasma gondii* Oocysts

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ABSTRACT: Outbred goats were fed 10^5 *Toxoplasma gondii* oocysts and were monitored twice weekly for 8 wk for rectal temperature, clinical signs, parasitemia, and antibody levels by indirect fluorescence antibody test (IFAT), latex agglutination test (LAT), and indirect enzyme-linked immunosorbent assay (ELISA). After 8 wk, all goats were killed, and samples of heart, skeletal muscle, brain, lymph nodes, kidneys, and liver were bioassayed in mice. Anorexia, fever, and lethargy were observed from day 3 to day 7 postinfection (PI). Parasitemia was detected by bioassay in 50% of infected goats from day 7 to day 14 PI. Viable *T. gondii* organisms were isolated from all infected goats. Antibodies to *T. gondii* were detected in some animals on day 10 PI by IFAT and LAT and on day 14 PI by ELISA. The infected goats were seropositive on day 17 PI.

Toxoplasmosis is a widespread zoonosis, causing large losses in production animals. Clinical signs in infected adult goats range from no signs to fever, abortion, stillbirth, and perinatal mortality. Brazil has approximately 13 millions goats, and little is known of caprine toxoplasmosis in this country. In previously reported studies from the United States (Dubey et al., 1980; Dubey, 1989), goats were fed oocysts of a highly virulent strain (GT-1) of *Toxoplasma gondii* during pregnancy. The AS28 strain, used in the present experiment, was initially isolated in wild mice (Deane et al., 1971) and has been used in cattle where tissue cysts where isolated (Costa et al., 1977). The purpose of the present study was to detect, using different methods, antibody response in goats fed *T. gondii* oocysts.

Six 6–9-mo-old outbred goats were fed 10^5 *T. gondii* oocysts (AS28 strain); 2 unexposed goats served as controls. None of the goats had detectable antibodies to *T. gondii* prior to the experiment, as determined by the indirect fluorescent antibody test (IFAT) (Camargo, 1964), the latex agglutination test (LAT; Toxotest®, Eiken Chemical Co., Tokyo, Japan), and the indirect enzyme-linked immunosorbent assay (ELISA) (Voller et al., 1976). The goats were maintained in metabolic cages and were provided with hay and water ad libitum.

Oocysts were obtained from cats fed *T. gondii*-infected mouse brain. After sporulation in 2% sulfuric acid, oocysts were washed in 0.15 M saline. The number of sporulated oocysts was determined by counting in a hemacytometer. Oocysts were maintained in the saline solution at 4 C for 4–8 mo. A

TABLE I. Presence of fever, anorexia, and parasitemia and first detection of antibody in goats fed 10^5 *Toxoplasma gondii* oocysts.

	Days postinfection			
Goat no.	Fever	Anorexia	Parasitemia	Antibody detection
1	3, 7	3, 7	—	10
2	3, 7	3, 7	—	10
3	3, 7	3, 7	—	10
4	7	3, 7	7, 10, 14	14
5	7	3, 7	7, 10, 14	17
6	7	3, 7	7	10

viability test was conducted by intraperitoneal injection in mice before inoculation in goats.

Twice weekly, rectal temperatures were measured and blood samples were collected from a jugular vein into tubes with ethylenediaminetetraacetic acid for *T. gondii* detection and into tubes without anticoagulant for serological assays. Sera were stored at −20 C until tested. Antibodies against *T. gondii* were detected using the IFAT, the LAT, and the ELISA.

The IFAT antigen was prepared from whole formalin-inactivated *T. gondii* tachyzoites, using the standard RH strain, and antigen–antibody reaction was detected by fluorescein-labeled anti-goat antibodies (A50-104F-2, Bethyl Laboratories, Montgomery, Texas). The LAT was performed according to the instructions of the manufacturer. All sera were tested in duplicate using 2-fold dilutions from 1:16 until the last reactive dilution. Titers are given as the reciprocal of highest reactive dilution. The cutoff values were 64 for the IFAT and LAT and 0.24 for the ELISA.

For the ELISA, soluble antigen obtained from sonicated RH tachyzoites was diluted in carbonate-bicarbonate buffer. Skim milk was used as blocking solution, and sera were diluted to 1: 100. Presence of specific antibody was detected by horseradish peroxidase–labeled rabbit anti-goat antibody (A5420, Sigma Chemical Co., St. Louis, Missouri). Optical densities (O.D.), using a 492-nm filter, were converted into indices by using negative and positive control sera. The indices (S/P ratio) were determined by the following formula: [(O.D. from the sample) − (O.D. from the negative control)] ÷ [(O.D. from the positive control) − (O.D. from the negative control)].

At 8 wk postinfection (PI), all goats were slaughtered, and samples of heart, skeletal muscle, brain, lymph nodes, kidneys, and liver were collected for bioassay in mice. Samples of muscle, lymph nodes, and kidneys were digested in acid-pepsin (Jacobs and Melton, 1957), whereas liver and brain were bioassayed without digestion. Homogenates of each sample were inoculated intraperitoneally into 5 Swiss white mice. The mice were observed for 6 wk and then examined for *T. gondii* infection as described by Dubey and Beattie (1988). After 6 wk, survivors were exsanguinated and examined for presence of cysts in cerebral tissue or specific antibody in serum by IFAT using 1:16 and 1:64 dilutions.

Goats fed *T. gondii* oocysts became anorexic, developed fevers (40.4–41.2 C), and were lethargic from day 3 to day 7 PI. Only 1 animal developed more severe symptoms, with anorexia, weakness, and lethargy, but it recovered after the second week and no other physical signs were observed. All infected goats showed clinical signs from day 3 to day 7 PI, similar to the observations recorded by Dubey et al. (1980) and Chhabra et al. (1982) in goats fed 10^4 oocysts. Dubey (1989) also recorded acute disease, lesions, and death in oocyst-infected kids (2–3 mo old) but not in infected females.

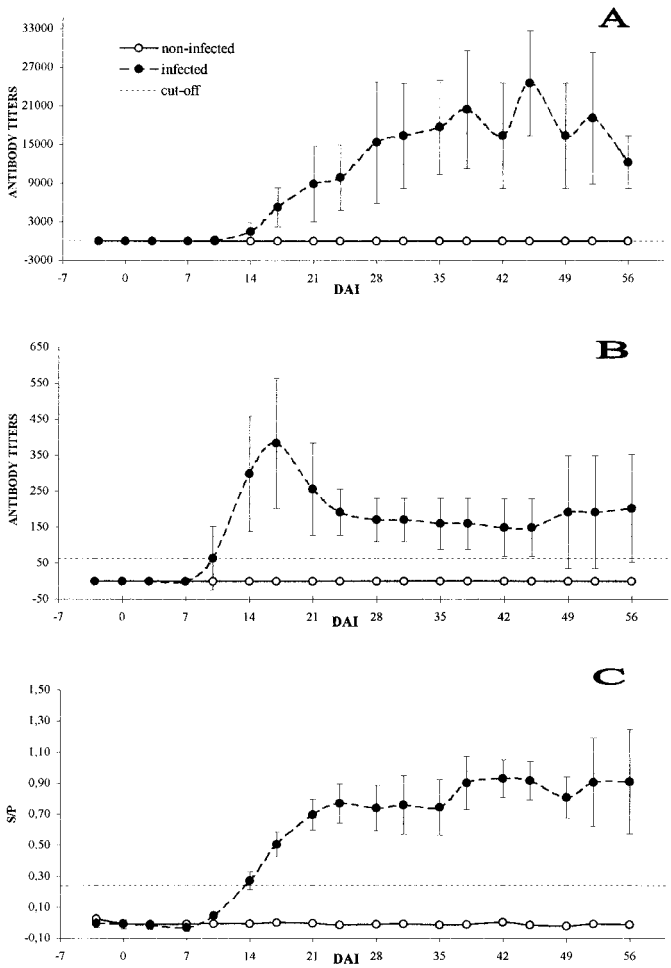

FIGURE 1. Antibody levels ($\bar{x} \pm$ SE) in serum of noninfected control goats and in serum of goats orally infected with 10^5 oocysts of *T. gondii* as detected by IFAT (**A**), LAT (**B**), and ELISA (**C**).

Parasitemia was detected in 3 of the 6 infected goats from day 7 to day 14 PI by bioassay in mice (Table I). A positive bioassay result was determined by the presence of *T. gondii* organisms. The serum IFATs for these mice were negative.

TABLE II. Detection of *Toxoplasma gondii* by bioassay in mice.

		Tissue assayed					
Goat no.	Day killed	Brain	Liver	Kidney	Skeletal muscle	Heart	Lymph nodes
1	56	−	−	−	+	+	−
2	56	+	+	+	+	+	+
3	58	+	−	+	+	+	−
4	63	+	+	−	+	+	+
5	71	+	+	+	+	+	−
6	72	+	+	+	+	+	+

Chhabra et al. (1982) found circulating tachyzoites between day 5 and day 12 PI in oocyst-inoculated goats. However, in goats infected intravenously, parasitemia was detected earlier (day 1 to day 12 PI). After the onset of antibody response, *T. gondii* was not detected in blood.

Antibody response was first detected by the IFAT and LAT on day 10 PI and by ELISA on day 14 PI (Fig. 1). All infected goats were seropositive at the day 17 PI, and antibody levels increased until day 21 PI and remained high until the end of the experiment. Samples from the uninfected controls, tested by IFAT, LAT, and ELISA, remained negative throughout the experimental period. Chhabra et al. (1982) detected circulating antibody in oocyst-infected goats from day 15 to day 22 PI by the indirect hemagglutination (IH) test and observed peak titers from day 28 to day 35 PI. The tachyzoite-infected group developed acute toxoplasmosis and died before the onset of the antibody production. By inoculating *T. gondii* tachyzoites subcutaneously, Vitor et al. (1999) detected antibodies in infected goats from day 5 to day 12 PI using ELISA and IH. Differences in the type of inoculum and the manner of infection also influence the dynamics of the disease and of the host's humoral response. However, infection by oocyst ingestion, as used in the present experiment, mimics more closely the field situation.

The LAT detected intensive production of antibodies on day 17 PI, which decreased by day 21. This late finding can be explained by the capacity of the IFAT and ELISA as used in the present assay to detect only specific IgG class antibodies. After this initial period, the 3 methods showed similar detection patterns.

IFAT, LAT, and ELISA were suitable to detect humoral response against *T. gondii* in experimentally infected goats; IFAT and LAT detected the antibody response before it was detected by ELISA. Diagnosis of *T. gondii* infection is most difficult during the first 2 wk. The choice of test will depend on the equipment availability, laboratory conditions, and quantity of samples to be tested.

Viable tissue cysts were isolated from all infected goats in cardiac and skeletal muscle samples. *Toxoplasma gondii* was present in 5 of 6 samples of brain and in 4 of 6 samples of liver, kidneys, and lymph nodes (Table II). No visible lesions were observed macroscopically in those tissues. The presence of *T. gondii* in different tissues of goats emphasizes the potential for transmission of toxoplamosis to the human population in Brazil.

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Organ Infectivity of *Toxoplasma gondii* in Interferon-γ Knockout Mice

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ABSTRACT: To determine the influence of interferon (IFN)-γ on the organ infectivity and on the genetic susceptibility of susceptible (C57BL/6) and resistant (BALB/c) strains after peroral infection with cysts of *Toxoplasma gondii,* IFN-γ knockout (KO) mice in C57BL/6 and BALB/c backgrounds were utilized. The kinetics of the changes in *T. gondii* abundance were evaluated with a quantitative competitive polymerase chain reaction assay in various organs at different times after peroral infection. In IFN-γ KO mice, a *T. gondii*-specific gene, *SAG1,* was detected in all organs examined, and the protozoan proliferated much more actively than in wild-type mice. The abundance of *T. gondii* was much higher in mesenteric lymph nodes and the heart than in other organs. In contrast, in the nervous system organs and kidneys, only a weakly detectable reaction was observed. *Toxoplasma gondii* grew at a more rapid rate in the organs of IFN-γ KO C57BL/6 mice than in the organs of IFN-γ KO BALB/c mice during the course of infection. Destruction of the IFN-γ gene showed remarkable effects on the infectivity in both susceptible and resistant mice.

Cell-mediated immunity is crucial in host defenses against *Toxoplasma gondii,* an intracellular protozoan parasite that frequently causes severe congenital defects (Dunn et al., 1999)

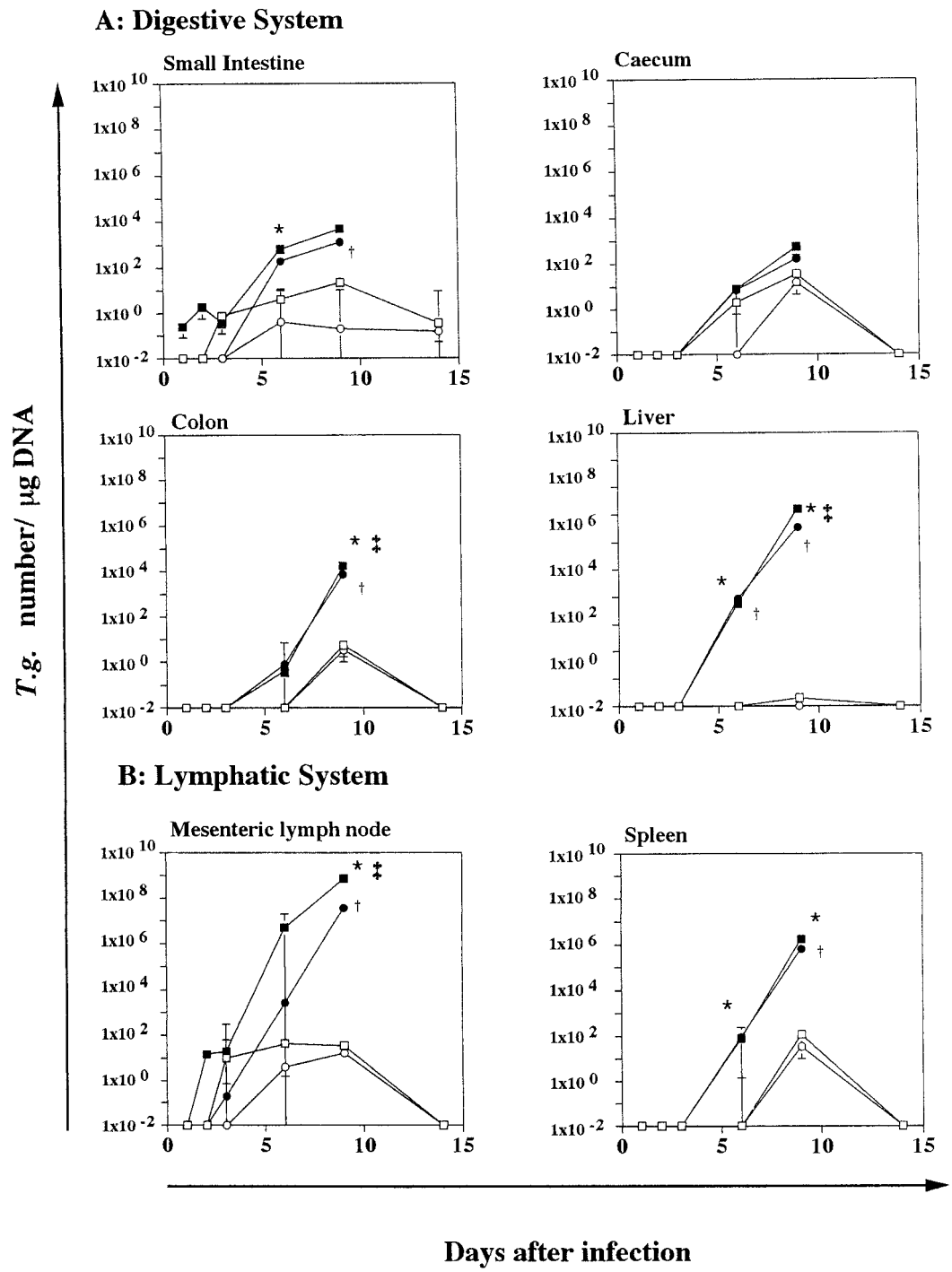

FIGURE 1. Kinetics of *Toxoplasma gondii* abundance in WT and IFN-γ KO mice. Comparison of the number of *T. gondii* in organs at various times after infection of IFN-γ KO C57BL/6 (■), IFN-γ KO BALB/c (●) mice, WT C57BL/6 (□), and WT BALB/c (○) mice. Data are expressed as number of parasites per μg of specimen DNA. Results were obtained from 2 mice each from WT and IFN-γ KO strains. The experiments were performed 3 times, with similar results. *Significant difference at $P < 0.05$ between IFN-γ KO and WT C57BL/6 mice; †significant difference at $P < 0.05$ between IFN-γ KO and WT BALB/c mice; ‡significant difference at $P < 0.05$ between IFN-γ KO C57BL/6 and IFN-γ KO BALB/c mice; §significant difference at $P < 0.05$ between WT C57BL/6 and WT BALB/c mice.

and life-threatening disease in immunocompromised individuals (Luft and Remington, 1988). Interferon (IFN)-γ, a cytokine secreted by activated T cells (Schreiber and Celada, 1985), natural killer cells (Trinchieri and Perussia, 1985), and the more recently defined dendritic cells (Ohteki et al., 1999), is the pivotal mediator inducing anti-*T. gondii* effector mechanisms (Suzuki et al., 1989; Scharton-Kersten et al., 1996). There have been numerous reports concerning the critical roles of IFN-γ in the modulation and inflammation of *T. gondii* infection (McCabe et al., 1984; Suzuki et al., 1988; Gazzinelli et al., 1992; Suzuki

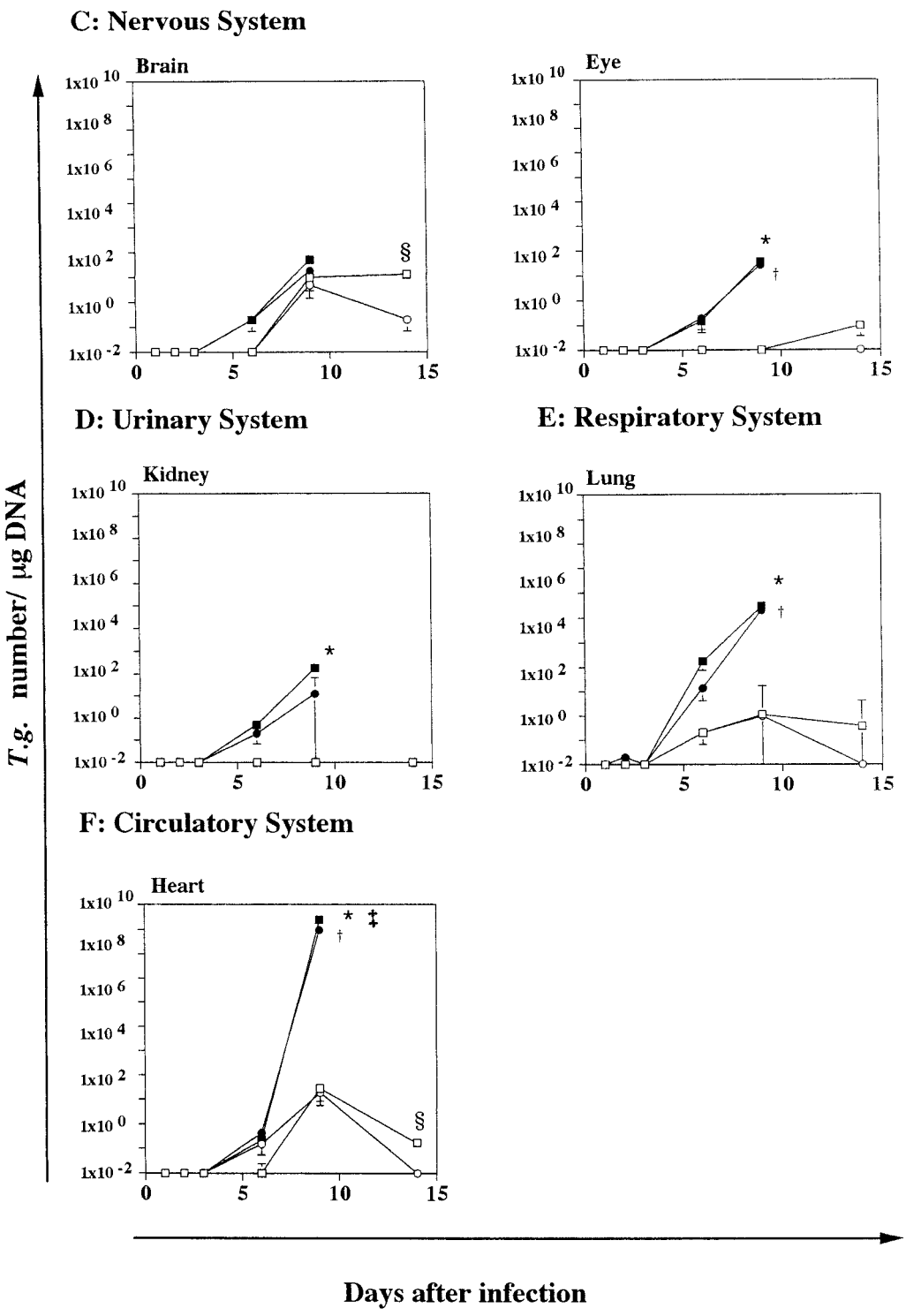

FIGURE 1. Continued.

et al., 1993; Yang et al., 1995; Deckert-Schlüter et al., 1996; Liesenfeld et al., 1996; Yang et al., 1996; He, Aosai, Mun et al., 1997).

It is well known that there is a significant difference in susceptibility to acute and chronic infections of *T. gondii* among inbred mouse strains with different genetic backgrounds (Araujo et al., 1976; Luo et al., 1997; Kobayashi et al., 1999). Mortality and cyst development, which are implicated in the susceptibility of mice infected with *T. gondii*, have been shown to be influenced by genes within the major histocompatibility complex (MHC)(McLeod et al., 1989; Yano et al., 1989; Freund et al., 1992; Blackwell et al., 1993; Suzuki et al., 1993; Aosai

et al., 1994; Yang et al., 1995, 1996; He, Aosai, Luo et al., 1997; Luo et al., 1997), as well as non-MHC-linked genes (Brown and McLeod, 1990). We previously reported that destruction of the IFN-γ gene showed less effect on the resistance to infection in BALB/c mice, but remarkable augmentation of infectivity of *T. gondii* in the digestive tract in C57BL/6 mice at 24 hr after ingestion of *T. gondii* (Kobayashi et al., 1999). The purpose of the present paper is to clarify the effects of IFN-γ at early and subacute stages on the infectivity of *T. gondii* in various organs and on genetic susceptibility against *T. gondii* infection in susceptible (C57BL/6) versus resistant (BALB/c) mice using IFN-γ knockout (KO) mice.

Cysts of an avirulent Fukaya strain (Kamei et al., 1976) of *T. gondii* were obtained as previously described (Luo et al., 1995). Six- to 8-wk-old inbred wild-type (WT) C57BL/6 and WT BALB/c mice were purchased (SLC, Hamamatsu, Japan). The IFN-γ KO mice in both the C57BL/6 and BALB/c backgrounds were used at the same ages. IFN-γ KO mice were genotyped by polymerase chain reaction (PCR) (Tagawa et al., 1997). Mice were killed at 1, 2, 3, 6, 9, or 14 days after peroral infection with 10 cysts of Fukaya strain administered using a syringe fitted with a 19-gauge needle with a round end. Genomic DNA from the small intestine, cecum, colon, liver, mesenteric lymph nodes, spleen, brain, eyes, kidneys, lungs, and heart was prepared using a Quiagen DNA tissue kit (Quiagen K.K., Tokyo, Japan). Using 1 μg of genomic DNA from these organs, quantitative competitive PCR (QC-PCR) was carried out to determine the distribution of *T. gondii* as described previously (Luo et al., 1995; He, Aosai, Luo et al., 1997; Kobayashi et al., 1999). Briefly, genomic DNA (1 μg) extracted from these organs was coamplified with a constant concentration of truncated *SAG1* DNA that competitively binds oligo primers with WT *SAG1*. The amplified cDNAs were electrophoretically separated on 1% agarose gels containing ethidium bromide, and the ratio to competitor (T/C) *SAG1* DNA subsequently amplified was measured with an IPLab Gel densitometer (Signal Analytical Corp., Vienna, Virginia). The number of *T. gondii* was calculated as described previously (Luo et al., 1995, 1997). Data were considered significant at a value of $P < 0.05$ using Student's *t*-test.

In WT C57BL/6 mice, *SAG1* genes, indicating the presence of *T. gondii*, were detected in the small intestine and mesenteric lymph nodes 3 days after infection and in the cecum and lungs 6 days after infection (Fig. 1). Nine days after infection, amplified bands were seen in the colon, liver, spleen, brain, and heart. Finally, protozoans were detected in the eyes 14 days after infection. The numbers of protozoans in these organs, with the exception of the brain and eyes, reached a plateau 9 days after infection and decreased thereafter. The *SAG1* gene in the brain was maintained at an intermediate level until day 14 after infection. No detectable PCR product targeting the *SAG1* gene was observed from the kidneys at any time during infection.

In WT BALB/c mice, the patterns of the kinetics of the abundance of protozoans in organs except the liver, brain, and eyes were almost the same as those in WT C57BL/6 mice. The abundance of *T. gondii* in the organs of WT BALB/c mice was less than that in WT C57BL/6 mice at day 9 after infection. In the brains of WT BALB/c mice, the abundance of *T. gondii* reached a peak 9 days after infection and decreased thereafter, as in other organs. The number of protozoans in the brain was markedly higher ($P < 0.05$) in WT C57BL/6 mice than in WT BALB/c mice 14 days after infection, as previously reported (Luo et al., 1997). No *SAG1* gene was detectable by QC-PCR in the liver, eyes, or kidneys in WT BALB/c mice throughout the course of infection.

On the other hand, in IFN-γ KO mice from both genetic backgrounds disseminated infection was clearly evident (Fig. 1). Amplified bands were obtained from every organ examined. The time course showed that in IFN-γ KO C57BL/6 mice, the *SAG1* gene was detected first in the small intestine, second in mesenteric lymph nodes, and third in the colon, liver, spleen, brain, eyes, kidneys, lungs, and heart, 1, 2, and 6 days after infection, respectively. The number of protozoans estimated by the T/C ratio increased dramatically and kept on increasing. The band intensity of the PCR product was highest in the mesenteric lymph nodes and in heart. On the other hand, the abundance of protozoans in the brain, eyes, and kidneys was lower than that in other organs. The levels of *T. gondii* loads in the organs of IFN-γ KO were much higher than those in the organs of WT C57BL/6 mice at 6 and 9 days after infection.

In IFN-γ KO BALB/c mice, the patterns of the kinetics of the abundance of protozoans in various organs were similar to those in IFN-γ KO C57BL/6 mice. Although protozoans grew at a slower rate in the organs of IFN-γ KO BALB/c mice than in those of IFN-γ KO C57BL/6 mice during the early phase of infection, the numbers of *T. gondii* in the organs of IFN-γ KO BALB/c mice increased thereafter. The levels of *T. gondii* loads in the organs of IFN-γ KO were much higher than those in the organs of WT BALB/c mice at 6 and 9 days after infection.

The present study revealed that once the IFN-γ KO mice were infected with *T. gondii*, the number of protozoans in both the IFN-γ KO mice with susceptible and resistant backgrounds increased markedly in every organ examined, although there were some significant differences between the abundance of *T. gondii* in the 2 types of IFN-γ KO mouse.

We have previously reported that the *SAG1* gene was detected in the small intestine and the cecum in WT C57BL/6 mice and in the whole digestive tract in IFN-γ KO C57BL/6 mice at 24 hr after infection with 100 cysts of Fukaya strain, and that the genetically controlled susceptibility to peroral infection of *T. gondii* cysts is apparently predetermined at the stage when the protozoans and digestive tract first come into contact (Kobayashi et al., 1999). As shown in the present study, the *SAG1* gene was detectable in the small intestine and in the cecum at 3 and 6 days after infection, respectively, in WT C57BL/6 mice. It took 1–6 days to detect the *SAG1* gene in the digestive tract even in IFN-γ KO mice. The differences between the results obtained in our previous study and the present study were caused by the different doses of infection. In our previous study, the *SAG1* gene was not amplified in any tissues of either WT or IFN-γ KO BALB/c up to 24 hr post-infection (Kobayashi et al., 1999). The present study showed that the *SAG1* gene was detected in every organ of IFN-γ KO and WT BALB/c mice except the liver, eyes, and kidneys of WT BALB/c mice at 6 or 9 days after infection. Destruction of the IFN-γ gene thus clearly affected the resistance to infection in BALB/c mice in subacute stages.

The numbers of protozoans in the organs of IFN-γ KO mice continued to increase in all organs examined until the time of

death, whereas the abundance of protozoans in the organs of the WT mice from both genetic backgrounds, with the exception of the brain and eyes of WT C57BL/6 mice, reached a peak at 9 days after infection and decreased thereafter. There were differences in the proliferative rate of *T. gondii*, especially in various organs in IFN-γ KO mice. The number of *T. gondii* was highest in the mesenteric lymph nodes, heart, and spleen in IFN-γ KO mice. The small intestine, cecum, colon, liver, kidneys, and lungs contained intermediate numbers of protozoans. Nervous system organs contained lower numbers of protozoans even in IFN-γ KO mice. The small intestine was rapidly infected after oral intubation in both WT and IFN-γ KO mice. Lymphoid organs were rapidly and heavily invaded after oral infection of an avirulent Fukaya strain in IFN-γ KO mice, as Sumyuen et al. (1995) reported in WT mice infected with 20 cysts of the avirulent C strain of *T. gondii*. We previously reported precise assessments of the numbers of *T. gondii* in the peripheral blood of WT and IFN-γ KO mice (Luo et al., 1997; Kobayashi et al., 1999). Thus, the pattern of the kinetics of tissue invasion of IFN-γ KO mice after peroral infection suggests 2 possible routes for parasite dissemination, i.e., mesenteric circulation and general blood circulation (Luo et al., 1995; Sumyuen et al., 1995).

Both types of IFN-γ KO mice died 9–10 days after peroral infection, whereas both types of WT mice survived more than 3 mo after infection. There have been some reports about the factors associated with death. Liesenfeld et al. (1999) reported that treatment of *T. gondii*-infected mice with anti-tumor necrosis factor (TNF)-α monoclonal antibody or an inducible nitric oxide synthase (iNOS) inhibitor, prevented necrosis of the small intestine and prolonged the time until death in genetically susceptible mice. Suzuki et al. (1989) reported that neutralization of IFN-γ in chronically *T. gondii*-infected mice reactivated the disease, and the animals subsequently died of overwhelming necrotizing encephalitis. We speculate that dysfunction of multiple organs was the leading cause of death in both types of IFN-γ KO mice because disseminated infection of *T. gondii* was clearly apparent. Pathological investigations will provide additional information on this issue.

The abundance of *T. gondii* in organs other than the brain of WT C57BL/6 mice was relatively low or disappeared at 14 days after infection in both types of WT mice, suggesting that some other factors in addition to IFN-γ are required to inhibit the proliferation of *T. gondii* in the brain of WT C57BL/6 mice (Chao et al., 1994). The numbers of *T. gondii* in the brains of IFN-γ KO mice in susceptible (C57BL/6) and resistant (BALB/c) backgrounds differed only slightly from those in WT C57BL/6 and BALB/c strains, suggesting that proliferation of *T. gondii* in the brain might be relatively uninfluenced by IFN-γ. Scharton-Kersten et al. (1997) postulated that the induction of reactive nitrogen intermediates by IFN-γ may be a major mechanism of host resistance to intracellular pathogens, but that nevertheless in the central nervous system, iNOS-independent pathways for parasite control exist and are important. Using mice genetically deficient for iNOS showed that iNOS-derived nitric oxide (NO) is required mainly for effector cell activity in the central nervous system during the chronic phase of infection. At that stage, parasite expansion and pathology were evident in the central nervous system but not the periphery, suggesting that the protective role of NO against this intracellular infection is tissue specific rather than systemic.

Persistence of *T. gondii* was observed in the small intestine and the brain in both WT C57BL/6 and BALB/c mice, and in the eyes, lungs, and heart in susceptible WT C57BL/6 mice. This observation suggests that iatrogenic toxoplasmosis may occur in human recipients of transplants of these organs. Actually, toxoplasmosis after transplantation of *T. gondii*-infected heart has been reviewed (Delmonico and Snydman, 1998). The present study may be useful in the establishment of screening for organs from *T. gondii*-infected donors in organ transplantation.

Further studies on IFN-γ KO mice infected with *T. gondii*, including analysis of stage conversion, may help define the role of IFN-γ in vivo in the host response to *T. gondii* infection and suggest rational strategies for the therapeutic use of this immunoregulatory cytokine.

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Effect of Refrigeration on the Antinematodal Efficacy of Ivermectin

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ABSTRACT: Several observations have suggested that the anthelmintic ivermectin can affect nematodes by non-oral entry into the nematode body. To investigate this possibility further, we refrigerated *Caenorhabditis elegans* at 5 C to prevent its locomotion and to block the pharyngeal pumping that is so prominent a feature of its feeding. Worms were exposed to ivermectin (1–25 μg/ml) at that temperature for 1 hr, after which the medium was replaced by unmedicated medium at room temperature. After 1 hr at room temperature the worms were examined and counted to determine the degree to which irreversible immobilization had occurred. The drug was significantly less effective at 5 C than at room temperature. This reduction in potency could be attributed to a general cold-induced decline in the rate of the biochemical processes involved in drug action. Alternatively, the reduction could be attributed to the cold-induced blockade of pharyngeal pumping, which would suggest that the efficacy of ivermectin is partially the result of oral intake of drug. The fact that antinematodal efficacy was not entirely abrogated and reached a significant level despite blockade of pharyngeal pumping supports the former interpretation and is in accord with earlier indications that ivermectin can enter by non-oral routes. This conclusion is further supported by the observation that ivermectin is active against the nonfeeding third-stage larva of *Haemonchus contortus.*

The nematode pharynx appears to be particularly sensitive to the paralyzing effect of the avermectin class of antiparasitic agents (Avery and Horvitz, 1990; Geary et al., 1993). Cully et al. (1994) have isolated 2 subunits of an avermectin receptor (a glutamate-gated chloride-ion channel), and there is evidence that, in *Caenorhabditis elegans,* 1 of them is confined to the

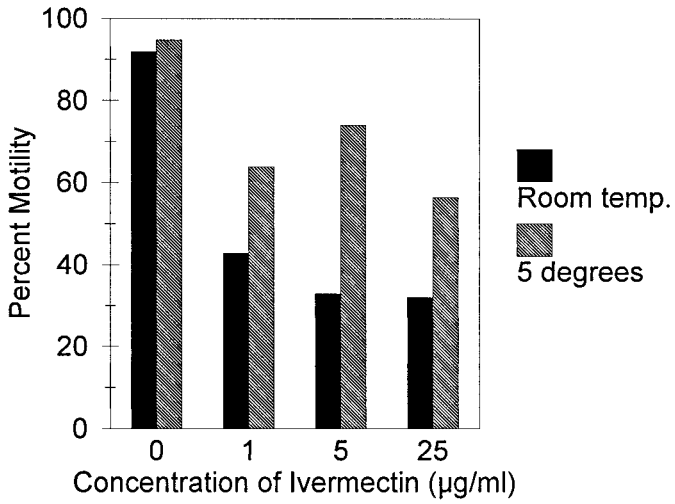

FIGURE 1. Percentage motility of worms exposed to various concentrations of ivermectin at room temperature or at 5 C (results of experiment 1).

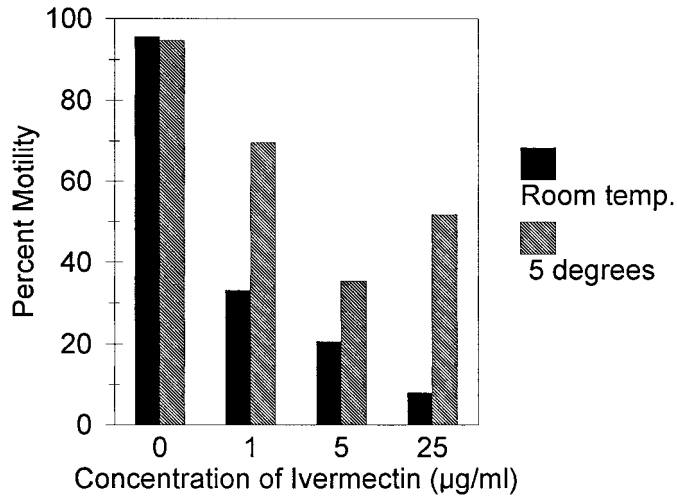

FIGURE 2. Percentage motility of worms exposed to various concentrations of ivermectin at room temperature or at 5 C (results of experiment 2).

muscle cells of the pharynx (Laughton et al., 1997). Furthermore, the gene *arv-15* has been shown to encode a subunit of a chloride-ion channel that is glutamate sensitive and ivermectin sensitive, and that is necessary for the proper functioning of neuromuscular synapses in the pharynx of *C. elegans* (Dent et al., 1997). Nevertheless, previous studies have suggested that some antiparasitic drugs, including ivermectin, may gain entry to nematodes by penetration of the cuticle or cuticular openings, rather than (or as well as) by means of ingestion (Geary et al., 1993; Ho et al., 1994; Smith and Campbell, 1996). Presumably, ingestion is not involved in the efficacy of ivermectin against the nonfeeding (ensheathed) third-stage larva of *Haemonchus contortus* (Patel and Campbell, 1997) but that could be a special case, and uncertainty remains about the mode of entry into nematodes in general. We here report an attempt to examine the question in a simpler manner than those used previously. The free-living species *C. elegans* was used as the test nematode, and the objective was to chill the worms enough to stop muscular activity but not enough to block all chemical and biochemical reactions. It was thought that cold-induced cessation of muscular activity would include cessation of pharyngeal pumping; and that, if the drug normally enters the worm by mouth, this blockade of the ingestion mechanism would result in a blockade of drug efficacy. If, on the other hand, the drug normally enters by a non-oral route, blockade of pharyngeal pumping should not affect drug efficacy. It was recognized, however, that the suppressive effect of cold on other body functions might complicate the interpretation of results.

Direct microscopic observation showed that exposure to 5 C for 1 hr resulted in cessation of motility (whole-body movement) and of pharyngeal pumping. Motility was resumed 1–2 min after restoration to room temperature (approximately 20 C), but resumption of pharyngeal pumping required at least 5 min.

Populations of *C. elegans* were raised on an agar medium (Avery and Horvitz, 1990) seeded with the OP50 strain of *Escherichia coli*. Worms were washed off the agar surface, using room-temperature water, and 1-ml aliquots were dispensed into petri dishes (35 × 10 mm). A parallel series of dishes was prepared using chilled 5 C water for collection and dispensing of worms. Aqueous solutions of ivermectin (or water, as control) were added to the dishes so as to give a concentration of 1, 5, or 25 µg/ml in a final volume of 4 ml. These high concentrations had been found necessary, under our experimental conditions, to achieve the desired degree of immobilization within a period of 1 hr. For the room-temperature dishes, test solutions were added at room temperature (approximately 20 C) and held at that temperature for 1 hr. For the 5 C dishes, prechilled solutions were added, and the dishes were refrigerated at 5 C for 1 hr. At the end of the 1-hr test period, the worms were rinsed in drug-free water to remove whatever ivermectin remained in solution despite its tendency to bind to plastic when in aqueous solution. Most of the fluid was removed by aspiration, the dishes were filled with water at room temperature, and this rinsing procedure was carried out at least 3 times. All dishes were allowed to stand at room temperature for a further 1 hr, after which the motile and nonmotile adult worms were counted. The underside of the dishes had previously been scored to aid counting.

In experiment 1, the mean number of worms in the test wells was 164.9 (range 91–241). In experiment 2, the mean was 153.5 (range 92–205). The percentage of worms remaining motile at the end of the test period is shown in Figure 1 (results of experiment 1) and Figure 2 (results of experiment 2). The data indicate that exposure of worms to ivermectin at 5 C was less efficacious than exposure at 20 C; that is, fewer worms were immobilized when the treatment was conducted under refrigeration. A chi-square test indicated that in each experiment the difference was highly significant ($P < 0.0005$).

These results would support the conclusion that the efficacy of ivermectin against *C. elegans* is partly dependent on oral ingestion, so that blockade of pharyngeal pumping results in reduced efficacy. It is also possible, however, that exposure to 5 C merely suppresses, to some degree, the biochemical processes involved in ivermectin efficacy. These processes could include transport of drug molecules across membranes to reach

receptor sites, the binding of molecules to those sites, or biochemical events consequent to binding.

The numbers of worms remaining motile after exposure to ivermectin at 5 C were lower than in the untreated control dishes held at the same temperature. The difference was significant ($P < 0.005$). Thus, the data also indicate that ivermectin remained effective (though less effective) at 5 C. Because motility and pharyngeal pumping ceased at this temperature, it is evident that efficacy was not totally dependent on, and may not have been at all dependent on, active oral intake of drug. The possibility of passive oral entry, unaided by pharyngeal pumping, cannot be discounted.

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Susceptibility of Avian Hosts to Experimental *Gymnophalloides seoi* Infection

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ABSTRACT: To determine whether avian species are susceptible to infection with *Gymnophalloides seoi* (a human-infecting intestinal trematode), we exposed 7 species of birds with metacercariae obtained from oysters. The birds were necropsied at days 2, 4, and 6 postinfection (PI). The highest worm recovery at day 6 PI was obtained from the Kentish plover (*Charadrius alexandrinus*; $\bar{x} = 56.0\%$), followed by the Mongolian plover (*C. mongolus*; 49.3%), and the grey plover (*Pluvialis squatarola*; 32.3%). In contrast, no mature worms were recovered from the great knot (*Calidris tenuirostris*), dunlin (*C. alpina*), black-tailed gull (*Larus crassirostris*), and mallard (*Anas platyrhynchos*). Among the plovers, the worms attained the greatest size at day 6 PI (254.1 × 190.4 μm) in the Kentish plover, with a significantly higher number of eggs in the uterus. The 3 species of plovers are highly susceptible to experimental *G. seoi* infection, suggesting that they could play a role as definitive hosts for these worms in nature.

Gymnophallid trematodes (Gymnophallidae) are intestinal, gall bladder, or bursal parasites of rice rats and birds, including waders, shorebirds, gulls, diving ducks, and willets (James, 1964; Yamaguti, 1971; Ching, 1973, 1995; Schell, 1985). In 1988, however, *Gymnophalloides seoi* Lee, Chai, and Hong, 1993 was identified from a 66-yr-old Korean woman who suffered from severe epigastric discomfort (Lee et al., 1993). Subsequent studies revealed that *G. seoi* is highly prevalent among the human inhabitants of Shinan-gun, Chollanam-do (Province), a southwestern island of the Republic of Korea (Lee et al., 1994). Oysters (*Crassostrea gigas*) harvested naturally near the village were verified to be the second intermediate host and the source of human infection (Lee et al., 1995). The Palearctic oystercatcher *Haematopus ostralegus* has been found to be a natural definitive host (Ryang et al., 2000).

To understand host–parasite relationships, including the pathogenicity and host responses, appropriate animal models are needed. Dogs, ducks, guinea pigs, and chicks showed very low susceptibility to *G. seoi* infection, with worm recovery at day 7 postinfection (PI) of <2% (Lee et al., 1997). Gerbils, cats, hamsters, mice, and rats showed comparatively higher susceptibility, with worm recovery at day 7 PI of 4–28.0% (Lee et al., 1997). Despite low susceptibility, C3H/HeN mice were suggested as a laboratory host because immunosuppression in these mice markedly elevated worm recovery (Lee et al., 1997) and prolonged the worm survival period (Chai et al., 1999). However, these mice are not satisfactory for studies of host–parasite relationships, and better experimental hosts are needed. The present study was conducted to evaluate the susceptibility of 7 species of birds to experimental *G. seoi* infection.

Oysters (*C. gigas*) infected with *G. seoi* metacercariae were collected from Aphae Island, Shinan-gun, Chollanam-do, Republic of Korea, the known endemic area of *G. seoi* (Lee et al., 1994). After the oyster shell was opened with a dull knife, the mantle surface was examined for *G. seoi* metacercariae at magnifications of ×10 to ×40 with a stereomicroscope. Pieces of oyster tissues were cut by scissors and loaded on top of a Baer-

TABLE I. Recovery rate of *Gymnophalloides seoi* from bird species by postinfection (PI) day.

Bird	Total no. birds exposed	Dosage of metacercariae	Worm recovery (%)*		
			Day 2 PI	Day 4 PI	Day 6 PI
Kentish plover	6	300	61.0 ± 9.0	67.0 ± 6.7	56.0 ± 9.0
Mongolian plover	6	300	55.0 ± 5.4	58.7 ± 4.1	49.3 ± 3.7
Grey plover	3	1,000	19.0 ± 0.9	24.1 ± 5.2	32.3 ± 2.6
Great knot	6	300	7.4 ± 4.0	1.4 ± 0.8	0.0
Dunlin	6	300	7.0 ± 2.2	2.0 ± 0.4	0.0
Black-tailed gull	12	1,000	0.0	0.0	0.0
Mallard	6	1,000	0.4 ± 0.4	0.2 ± 0.2	0.0

* Mean ± SD.

mann's apparatus (Beaver et al., 1984), and the metacercariae were collected from the sediment 2 hr later.

Wading birds, including the Kentish plover (*Charadrius alexandrinus*), Mongolian plover (*C. mongolus*), grey plover (*Plurialis squatarola*), dunlin (*Calidris alpina*), and great knot (*C. tenuirostris*), were caught with mist nets at the Tae-An Coast National Park and Ganghwa Island located on the western coast of the Republic of Korea. Young black-tailed gulls (*Larus crassirostris*) were caught on the Hong Islet, Kyongsangnam-do (Province). Mallards (*Anas platyrhynchos*) 1 wk of age were obtained from a mallard breeding farm in Wonju-city, Kangwon-do (Province). Unfortunately, it was impossible to catch the Palearctic oystercatchers alive for use in the present study. All captured birds were sedated by intramuscular injection with 10–20 mg/kg ketamine HCl (Yuhan Corp., Seoul, Korea) and brought to the laboratory. They were fed at least twice daily with the flesh of bivalves, which was gamma irradiated at 200 Gy to ensure sterility.

Praziquantel (Shinpoong Pharmaceutical Co., Seoul, Korea), at a dose of 10 mg/kg, and albendazole (Shinpoong), at a dose of 6 mg/kg, were administered orally to each bird to eliminate the possibility of preinfection with intestinal helminths. Two weeks later, after confirming absence of helminth eggs by fecal examination, each bird was orally inoculated with 300 or 1,000 metacercariae of *G. seoi*. The birds were euthanized at days 2, 4, and 6 PI, and worms were recovered from the small intestine. The small intestine was divided into anterior and posterior sections. Each was opened longitudinally in a Petri dish containing saline. The gut contents were loaded on a Baermann's apparatus, and adult flukes were collected after 2 hr from the sediment and counted.

Ten specimens recovered from each bird species were fixed in 10% formalin under coverslip pressure and stained with Semichon's acetocarmine. Worms were measured and their developmental status was observed with a light microscope. Measurements are given in micrometers. Student's *t*-test and the chi-square test were used to evaluate differences in data between groups. Values of $P < 0.05$ were regarded as significant.

The recovery of *G. seoi* varied considerably among different species of birds (Table I). Through the experimental period of 6 days, the 3 species of plovers exhibited significantly higher ($P < 0.05$) worm recovery than did the other 4 species of birds. Among the plovers, the greatest number of worms was recovered from the Kentish plover, followed by the Mongolian and grey plovers. The great knot, dunlin, and mallard provided much lower recovery at days 2 and 4 PI, and no worms were recovered at day 6 PI. No worms were recovered from the black-tailed gull throughout the experimental period. In the Kentish plover and Mongolian plover, 70–80% of worms were harvested from the anterior half of the small intestine, and in the grey plover, 65–70% of worms came from the posterior half.

Among the 3 plover species, the growth and development of *G. seoi* was best in the Kentish plover and fairly good in the other 2 species of plovers. At day 2 PI, the mean (±SD) length × width of the worms recovered from the Kentish plover was 229.6 ± 8.5 × 165.7 ± 5.7, and at day 4 PI it was 244.3 ± 15.8 × 178.6 ± 2.9. At day 6 PI, worms from these birds were still larger (Table II). In the Mongolian plover and grey plover, the worms were significantly smaller ($P < 0.05$): 176.7 ± 7.1 × 152.5 ± 5.3 and 167.9 ± 8.0 × 153.4 ± 5.3, respectively, at day 2 PI and 181.5 ± 8.8 × 173.5 ± 4.5 and 184.3 ± 7.8 × 160.1 ± 3.5, respectively, at day 4 PI. Worms from these 2 plover species were still larger by day 6 PI but not as large as the worms recovered from the Kentish plover (Table II). The ovary, testes, and vitellaria were larger in worms recovered from the Kentish plover than in those from the other 2 species of plovers.

Eggs were present in the uterus of 2-day-old worms (8–10 eggs/worm), with little difference in number of worm eggs

TABLE II. Measurements* of 6-day-old adult *Gymnophalloides seio* recovered from plovers.

Worm measurements	Kentish plover (n = 10)	Mongolian plover (n = 10)	Grey plover (n = 10)
Body length (μm)	254.2 ± 10.9	193.0 ± 5.7	191.8 ± 5.5
Width (μm)	190.4 ± 4.3	172.7 ± 4.1	161.5 ± 3.8
Oral sucker (μm)†	86.7 ± 1.6	94.7 ± 3.0	91.2 ± 2.6
Ventral sucker (μm)†	53.1 ± 0.7	45.6 ± 1.1	50.1 ± 1.2
Ovary (μm)†	37.7 ± 1.5	30.7 ± 2.2	30.0 ± 1.5
Testis† right (μm)	56.0 ± 1.2	28.0 ± 2.9	36.2 ± 2.2
Left (μm)	45.8 ± 1.1	29.5 ± 2.3	28.5 ± 2.5
Vitellaria† right (μm)	30.3 ± 1.0	27.9 ± 1.4	25.7 ± 1.3
Left (μm)	30.4 ± 1.0	26.5 ± 1.0	25.7 ± 1.0
Eggs (uterine)			
Length (μm)	21.4 ± 1.0	21.3 ± 1.1	21.3 ± 1.2
Width (μm)	14.2 ± 0.9	14.2 ± 0.9	14.1 ± 1.0

* Mean ± SD.
† Measurements of longest axis.

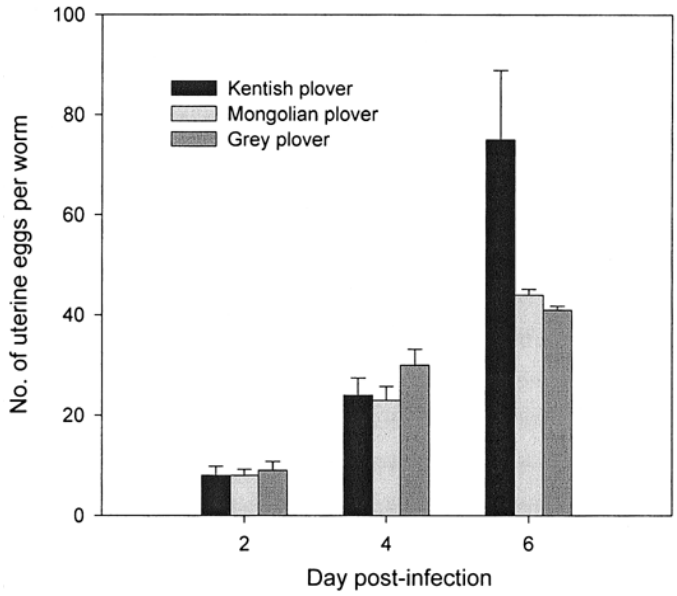

FIGURE 1. Number of uterine eggs per specimen of *Gymnophalloides seoi* from the 3 species of plovers by postinfection (PI) day. Data represent the mean (bars) and standard deviation (lines) of 10 specimens in each host species. The value for the Kentish plover at day 6 PI was significantly higher ($P < 0.05$) than that of the other 2 species.

among the plover species (Fig. 1). By day 4 PI, the number of eggs increased to 23–30/worm, also without significant differences ($P > 0.05$) among the plover species. At day 6 PI, however, the number of eggs was greater in worms recovered from the Kentish plover than in those from the other 2 species.

The 3 species of plovers were highly susceptible to experimental infection with *G. seoi*. The great knot and dunlin were less susceptible, and the black-tailed gull and mallard were not susceptible. Among the plovers, the Kentish plover has the highest worm recovery, and the worms from this plover were more developed and had more eggs. These results suggested that plovers may play a role as definitive hosts of *G. seoi* in nature.

The average number of uterine eggs in worms recovered from the Kentish plover at day 6 PI was about 80, much lower than the 200 in fully gravid specimens obtained from humans with natural infections (Lee et al., 1993, 1994). The number is also lower than the 120 in worms obtained from immunosuppressed C3H/HeN mice at day 14 PI (Chai et al., 1999). One reason for the reduced number of worm eggs obtained may be the shorter infection period in these experimentally infected birds. If we could maintain the plovers (and worms) longer than 7 days, the worm fecundity may have been higher.

Palearctic oystercatchers, the known natural host (Ryang et al., 2000), were not included in this study because of the difficulty in catching these birds alive. In a previous study, most oystercatchers were collected after being shot (Ryang et al., 2000). However, we speculate that the susceptibility of the Palearctic oystercatchers to experimental *G. seoi* infection would be as high as that of the 3 species of plovers examined.

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