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Biological Characterization of *Trichinella* Isolates with the Emphasis of the Use of the Drug Thiabendazole

by

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BIOOOGICAL CHARACTERIZATION OF TRICHINELLA ISOLATES WITH THE EMPHASIS OF THE USE OF THE DRUG THIABENDAZOLE

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TO WHOM IT MAY CONCERN

The late Dr. George Lubinsky served on Mr. Chadee's M.Sc. Committee and contributed to the thesis up to the time of his death. Dr. Lubinsky had accepted the thesis and his suggestions for its improvement have been incorporated into the final draft, by Mr. Chadee. Dr. Lubinsky's name therefore remains in the list of examiners who participated in Mr. Chadee's oral examination.

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ABSTRACT

A system was proposed to designate and define northern isolates of Trichinella spiralis which gives the host the the isolate was recovered from, geographic origin (latitude and longitude), and year of recovery. The ability to withstand low temperature by some isolates of Trichinella is well known. This study extends our knowledge of geographical locations and hosts harbouring low temperature resistant forms of Trichinella. Biological characteristics of a Trichinella isolate (wolverine) were studied in detail and compared with a previously well-defined isolate (polar bear) originally caught near the site of capture of the wolverine isolate. Both isolates (wolverine and polar bear) shared common features but differed in others. Comparison of biological characteristics for the wolverine isolate in different host species revealed differences in intestinal position, 24-hr in vitro larval release and reproductive capacity indices (RCI). Single-pair-interbreeding experiments between wolverine and raccoon or between polar bear and pig isolates showed reproductive compatibility except between pig and raccoon isolate combinations which did not breed. No hybrid breakdown developed from all multiple-pair crosses and host influenced RCI-values of the hybrids. Thiabendazole (anthelmintic) treatment against pig, wolverine, polar bear and raccoon isolates was effective during the intestinal phase, dissemination phase and against early muscle larvae.

Trichinella sensitivity to thiabendazole treatment varied. The wolverine and polar bear isolates were most susceptible in the intestinal phase while disseminating larvae were highly susceptible to thiabendazole regardless of *Trichinella* isolate or drug dosages. High dosages were effective against early muscle larvae of the polar bear, wolverine and raccoon isolates but less so against the pig isolate. Low drug dosages were ineffective during the late muscle phase of all *Trichinella* isolates. Susceptibility to thiabendazole treatment is related not only to the strain or isolate of *Trichinella* but also to the stage in the parasite's life cycle. Geographical isolates of *Trichinella* have consistent and predictable differences suggesting some degree of genetic isolation. This is probably part of the normal biological variability of *T. spiralis*.

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GENERAL INTRODUCTION

Trichinella spiralis (Owen, 1835) Railliet, 1895 is a nematode parasite producing the disease trichinosis in man and many other domestic and wild mammals. It has for decades remained the only species in the family Trichinellidae, Ward 1907. In recent years, several different strains or varieties of this parasite have been studied (Nelson *et al.* 1961; Read and Schiller 1969; Britov 1969; Ozeretskovskaya *et al.* 1969; Dick and Belosevic 1978; Belosevic and Dick 1979, 1980a). Presently, workers have elevated varieties of *Trichinella* to species: *T. nativa* (Britov and Boev, 1972; arctic form), *T. nelsoni* (Britov and Boev, 1972; tropical form), and *T. spiralis* (north-temperate form). Their reasons were based on reproductive and genetic isolation, geographical location, host specificity and adaptation to wild hosts and ability to survive freezing even though there were limited biomorphological differences.

The description of another species *T. pseudospiralis*, Garkavi 1972 was based on absence of a cyst in the muscle stage, ability to complete its life cycle in birds and dimensions of the infective larvae. The separation of *T. spiralis* into distinct species has been questioned (Machnicka 1979) and the suggestion made that all newly erected species of *Trichinella* should be synonymized (Madsen 1975). Work on North American isolates of *Trichinella* (Sukhdeo and Meerovtich 1977; Dick and Belosevic 1978; Belosevic and Dick 1979, 1980a) has resulted in different conclusions.

Due to the controversy over speciation in the genus Trichinella, a system to designate isolates is outlined and defined. The designation should include host species, latitude, longitude, and year parasite recovered. The Trichinella isolates reported in this study are designated as follows:

Trichinella (pig, Sus scrofa (Linnaeus); 43°00'N, 81°00'W; 1952)

Trichinella (polar bear, Ursus maritimus (Phipps); 58°00'N, 95°00'W; 1976)

Trichinella (wolverine, Gulo gulo (Linnaeus);55°00'N, 100°00'W; 1979)

Trichinella (marten, Martes americana (Turton); 56°00'N, 99°00'W; 1980)

Trichinella (arctic fox, Alopex lagopus (Linnaeus); 69°15'N, 105°00'W; 1980)

Trichinella spiralis var. pseudospiralis (raccoon, Procyon lotor (Linnaeus); 1972)

The main objective was to expand our knowledge on the biological characteristics of Trichinella from isolates of known history. The specific objectives were:

(1) To report on and determine viability of Trichinella larvae from previously frozen carnivore muscles;

(2) To establish criteria for designating isolates;

(3) To determine and compare biological characteristics of the wolverine isolate in various hosts;

(4) To establish if these isolates interbreed and

(5) To investigate the action of thiabendazole on four geographical isolates and to compare drug sensitivity among the isolates as a possible biological characteristic.

CHAPTER I

DESIGNATION OF AND OBSERVATIONS ON SOME ISOLATES OF *TRICHINELLA SPIRALIS* FROM WILD CARNIVORES

Trichinella spiralis (Owen, 1835) Railliet, 1895 from the northern hemisphere was recognized as both a distinct species (Britov and Boev, 1972), and as a northern or arctic strain (Dick and Belosevic 1978; Rausch 1969; Dies 1980; Dick and Chadee 1980). Although geographical isolates of Trichinella are known to differ in their biological characteristics (Belosevic and Dick 1979), the taxonomic rank of isolates is still unresolved. Work on North American isolates of Trichinella (Sukhdeo and Meerovitch 1977; Dick and Belosevic 1978; Belosevic and Dick 1979, 1980a) suggests that these isolates are variants or strains of Trichinella and not distinct species as they interbreed and produce viable F_1 hybrids (Belosevic and Dick 1980a).

As early as 1950 it was suggested that northern isolates of Trichinella may be resistant to low temperatures (Brandly and Rausch 1950). Anecdotal evidence supports this in that viable Trichinella larvae were recovered from previously frozen wild carnivore muscle (Eaton 1979). There are, however, relatively few documented cases which clearly demonstrate the recovery of viable Trichinella larvae from frozen tissue (Clark et al. 1972; Dick and Belosevic 1978; Dies 1980). This study reports on Trichinella larvae recovered from previously frozen carnivore muscles, their viability after various times post-freezing, and proposes a system to designate and define these northern isolates of Trichinella.

MATERIALS AND METHODS

Hosts and locations of carnivores

Wild carnivore carcasses frozen from 1 to 2 months, were obtained from trappers and wildlife personnel. Hosts and locations are outlined in Table I.

Carcass treatment, isolation of larvae and infection procedures

Carcasses were thawed at room temperature (21C) and examined for Trichinella cysts by trichinoscope and for larvae by HCL-pepsin digestion. Muscle was separated from fat and bones and ground in a meat grinder. The ground muscle was weighed and placed in flasks containing a 1% pepsin - HCL solution (ratio of meat (g) to pepsin - HCL solution (ml) was 1:20) for 3 hr at 37C with occasional stirring. A digestion period of 3 hr was necessary to free worms from their cysts in carnivore muscle. Digested material was placed in a Baermann apparatus and larvae were allowed to accumulate for 30-45 mins. Larvae were collected in a centrifuge tube from the bottom 15 ml of digest and counted in a Petri dish with the aid of a dissecting microscope. All digested muscle larvae recovered i.e., moving, motionless, tightly coiled and damaged, presumably due to freezing, were tested for infectivity in mice. Outbred white mice [Crl: COBS CFW (SW) Charles River Breeding Laboratories, Wilmington, Mass.] were infected by gastric intubation with 10 to 500

larvae/mouse (Table II) and were given commercial lab chow (Wayne Lab - Blox) and water ad libitum. Portions of carcasses were refrozen at -15C and muscle samples taken from these refrozen carcasses at various times post-freezing (Table II) and tested for the presence of viable Trichinella larvae.

Isolation of muscle larvae and determination of Reproductive capacity index (RCI) in mice

At 40 days postinfection animals were killed by cervical dislocation, weighed, skinned, and eviscerated. A piece of diaphragm of each mouse was compressed and examined for worms. Mice were ground with a meat grinder, and placed in a flask containing a 1% pepsin - HCL solution for 1 hr at 37C with stirring [ratio of meat (g) to pepsin - HCL solution (ml) was 1:7]. Digested material was placed in a Baermann apparatus and larvae were allowed to accumulate for 30-40 mins. Larvae were collected in a graduated centrifuge tube from the bottom 15 ml of digest, suspended in 0.16% agar, and 0.85% saline at 37C, and stirred several times to ensure even distribution of worms. Each of ten 0.1 ml aliquots was removed, evenly spread on counting grids, and the worms counted with the aid of a dissecting microscope. If counts varied by more than 10%, the worms in four additional aliquots were counted. Number of larvae/milliliter and total number of larvae in suspension were determined.

RESULTS

Seven isolates of Trichinella are defined by host, geographical location and year of isolation (Table I).

Results on infectivity and recovery from frozen muscles are summarized in Table II. Trichinella recovered from frozen muscle samples from 6 wild carnivores were infective to experimental mice. Viable Trichinella larvae were obtained from frozen wolverine muscles for up to 6 months, and from frozen marten muscles at 5 months. Trichinella larvae recovered from 3 of 4 arctic foxes were viable up to 14 months (Table II). All 6 isolates had low but different infectivities in mice at 40 days postinfection. Larvae/g of muscle from mice for the isolates were as follows: from wolverine 97.80; from polar bear, 64.90; from marten, 10.71 and from 4 foxes 664, 1.42, 9.07 and 3.56, respectively.

The level of infection of Trichinella in carnivore muscle varied from 0.008 to 53.0 larvae/g (Table II). In all cases very few calcified cysts were observed.

Table I. Designation of *Trichinella* isolates.

Wild Hosts	Latitude	Longitude	Year established
Polar bear (*Ursus maritimus*)	58°00'N	95°00'W	1976
Wolverine (*Gulo gulo*)	55°00'N	100°00'W	1979
Marten (*Martes americana*)	56°00'N	99°00'W	1980
Arctic fox (*Alopex lagopus*)	69°15'N	105°00'W	1980[1,2,3.4*]

* Four *Trichinella* isolates.

TABLE II. Recovery of infective Trichinella spiralis from North American mammals frozen at -15C. Reproductive capacity determined on Day 40 postinfection from Crl: COBS CFW (SW) mice.

Host	Muscles examined	Time frozen (months)	Larvae/g of host muscle	Infection Dose larvae/mouse	Larvae recovered from mice	$\bar{x}$ index RCI/mouse
Wolverine	Diaphragm Intercostals[a]	3	1.85	500	3,600	7.20
Wolverine	Random Tissue samples[b]	4	6.60	100	1,500	14.60
Wolverine	Posterior half of carcass[b]	5	-	400	6,800	17.05
Wolverine	Random Tissue samples[b]	6	1.04	71	75	1.05
Wolverine	Random Tissue samples[b]	7	3.20	50	-	-
Wolverine	Random Tissue samples[b]	8	3.42	60	-	-
Polar bear	Diaphragm[c]	12	-	40	2,400	60.00
Marten	Whole carcass (except head)	5	0.008	40	300	7.50
Marten	Tongue Masseters[b]	7	0.70	60	-	-
Arctic fox 1	Half carcass[a]	5	4.81	300	18,600	62.00
Arctic fox 2	Half carcass[a]	5	1.78	200	40	0.20
Arctic fox 3	Diaphragm[a]	5	0.50	10	254	25.40
Arctic fox 4	Diaphragm[a]	5	1.45	29	103	3.55
Arctic fox 1	Random Tissue samples[b]	14	53	200	2,280	11.40
Arctic fox 2	Random Tissue samples[b]	14	5.32	100	2	0.02
Arctic fox 3	Random Tissue samples[b]	14	1.47	15	-	-
Arctic fox 4	Random Tissue samples[b]	14	2.98	40	45	1.12

[a]Carcasses completely thawed at room temperature and refrozen at -15C.

[b]Muscle samples taken from frozen carcasses.

[c]From Dick and Belosevic (1978).

DISCUSSION

Due to the controversy over speciation in the genus Trichinella, it is essential that proper designation of isolates be established. For this reason the designation should include host species, latitude, longitude, and year parasite recovered. A typical designation is written as follows: (wolverine; 55°00'N, 100°00'W; 1979). It is strongly suggested that if experimental work is done on any isolate a detailed history of each isolate should be kept and should include the strain of experimental host, RCI - values, number of generations in experimental hosts and generation time.

Northern isolates of Trichinella were suggested to be resistant to low temperatures (Brandly and Rausch 1950). Since then, viable Trichinella have been reported from black bear meat frozen for 81 days at -18C (Clark et al. 1972), recovered from polar bear meat after storage from 12 months at -15C (Dick and Belosevic 1978) and from wolf tissue frozen for 18 months at -10C (Dies 1980). In this study muscle type did not affect the ability of Trichinella to survive freezing nor did the location of frozen tissue, whether surfacial or deep inside the carcass. Furthermore, tissues that were completely thawed at room temperature and refrozen for extended periods of time (Table II) contained infective

larvae. Although, it is not clear how long worms can survive freezing, or the range of temperatures they can tolerate in the wild host, infective larvae were obtained from wolverine muscle for up to 6 months. A decrease in infectivity was noted for both wolverine and marten isolates and by 7 months all worms were noninfective (Table II). It is known, however, that larvae recovered from a polar bear and from arctic foxes frozen for 12 and 14 months, respectively, were infective. It is possible that high arctic isolates such as those recovered from the polar bear and arctic foxes may survive freezing in the carnivore muscles longer than those isolates from lower latitudes.

All northern isolates in this study had different RCI - values in mice and were generally lower when compared to a RCI - value of 151.27 (Belosevic and Dick 1979) from a pig strain of Trichinella (pig; 43°00'N, 81°00'W; 1952). Although there does not appear to be any initial pattern for level of infection in experimental mice, work in our laboratory shows that isolates do stabilize after several generations (Belosevic and Dick 1979). In fact RCI - values become stable and predictable characteristics of isolates. It is possible that localized pressures such as host and geographical isolation are selecting for certain biological characters which are stable and predictable under laboratory conditions.

Attempts to determine if northern isolates of T. spiralis were resistant to freezing following encystment in the muscles of laboratory mice have been unsuccessful (Dick and Belosevic 1978). Many factors are important in ensuring survival after freezing in the wild host and are listed elsewhere (Dick and Belosevic 1978). It appears that resistance to freezing is a biological characteristic of all northern isolates and additional northern isolates of T. spiralis should be evaluated for low temperature resistance.

CHAPTER II

INFLUENCE OF HOST ON THE BIOLOGICAL CHARACTERISTICS OF A GEOGRAPHICAL ISOLATE OF *TRICHINELLA*

(Wolverine; 55°00'N, 100°00'W; 1979)

INTRODUCTION

Biological characterization of different geographical isolates of Trichinella spiralis has indicated differences but the taxonomic rank of isolates is still unresolved. In recent years, characteristics of several different strains or varieties of Trichinella were examined and indicated low infectivity in laboratory rodents (Nelson et al. 1961; Britov 1969; Ozeretskovskaya et al. 1969; Read and Schiller 1969; Arakawa and Todd 1971; Pereverzeva et al. 1974; Sukhdeo and Meerovitch 1977; Dick and Belosevic 1978; Belosevic and Dick 1979, 1980a); differences in morphology of larvae and adults (Schad et al. 1967; Arakawa and Todd 1971; Garkavi 1972; Sukhdeo and Meerovitch 1977; Boev et al. 1979; Belosevic and Dick 1980a) and interbreeding or lack thereof (Britov 1971; Britov et al. 1971; Komandarev et al. 1975; Bessonov, et al. 1975; Britov 1977; Sukhdeo and Meerovitch 1977; Belosevic and Dick 1980a). Although Belosevic and Dick (1979, 1980a) have listed seven additional biological characteristics to define a relatively well adapted form of T. spiralis (pig; 43°00'N, 81°00'W; 1952) to mice and for a newly isolated strain (polar bear; 58°00'N, 95°00'W; 1976) there is no detailed comparative work on isolates from a similiar geographical location. There is evidence from other work that the host can influence the infectivity of Trichinella (Nelson and Blackie 1966; Arakawa and Todd 1971; Pereverzeva et al. 1974;

Bessonov et al. 1975; Sukhdeo and Meerovitch 1977; Dick and Belosevic 1978; Belosevic and Dick 1980a) and its size (Schad et al. 1967; Belosevic and Dick 1980a). But to date there are no detailed comparative studies on the effect of hosts (laboratory or wild) on a wide variety of biological characteristics of any isolate of Trichinella.

This study reports on the biological characterization of a recently isolated Trichinella (wolverine; 55°00'N, 100°00'W; 1979) in a laboratory strain of mice and comparative work on other laboratory and wild rodents. The primary objective of this study was to determine the amount of variation that exists for Trichinella from closely related geographical areas. The wolverine isolate in this study and the polar bear isolate studied by Dick and Belosevic (1978) and Belosevic and Dick (1979, 1980a) fit this criterion. Specific biological characteristics examined for the wolverine isolate in addition to those studied by Belosevic and Dick (1979, 1980a) include characterization and comparison in a variety of laboratory and wild rodents.

MATERIALS AND METHODS

Parasite isolation and maintenance

Trichinella spiralis larvae were isolated from a wolverine trapped at Snow Lake, Manitoba, Canada (55°00'N, 100°00'W; 1979) frozen for 3 months at -15C prior to isolation. Outbred Swiss Webster mice [Crl:COBS CFW(SW), Charles River Breeding Laboratories, Wilmington, Mass] were used for initial infections and subsequent maintenance of the parasite and for experiments.

Passage experiment

All infections were done with 40-day-old larvae, starting with generation one in mice. Ten passages for the *Trichinella* wolverine isolate (hereafter wolverine isolate) were performed at 40 day intervals using 50 to 60 day old outbred Swiss Webster mice. All mice were routinely infected with 400 larvae/mouse, except passage 1 and 2 where 500 larvae/mouse were used. Worm position in the small intestine was determined for 9 passages 5 days postinfection. Mice were killed by cervical dislocation and the small intestines removed and placed on a graduated dissecting board (Brambell 1965). The small intestine was cut in 20 equal segments and each segment placed in a vial containing 8 ml of 0.85% saline solution. Vials containing these segments were refrigerated for 24 hr to facilitate

breakdown of the intestinal mucosa. After refrigeration, contents of each vial were emptied into a Petri dish, each intestinal segment was slit longitudinally, and the mucosa lining vigorously scraped and stirred. The number of worms per segment was established by examining the contents of the Petri dish with the aid of a dissecting microscope.

The median for each population of Trichinella in the small intestine was determined as follows: that point of the intestine where 50% of the worms were anterior and 50% of the worms were posterior. It was assumed that worms in each 5% (1/20th of the intestine) were evenly distributed. Throughout this Chapter the averaged median value will be referred to as position.

The 24-hr release of newborn larvae during the intestinal phase was determined for 9 passages 7 days postinfection. Isolation of adult females and procedures for in vitro inoculation and counting of newborn larvae are modified procedures of Belosevic and Dick (1979). On Day 7 postinfection mice were killed by cervical dislocation and the entire small intestine removed and placed in 0.85% saline at 37C. Adult females were isolated in 0.85% saline, washed 4 times in α-MEM Eagle, Earle's Base (modified) tissue culture medium containing 10% calf serum (by volume) and then placed one per 2-ml plastic cone-bottom-vial containing 1 ml of tissue culture medium. Vials were capped and stored at 37C in an environmental chamber for 24 hr. At the end of incubation the top 0.75 ml of medium was removed from each vial and the remaining 0.25 ml was

placed on counting grids. All newborn larvae in the sample were counted using a dissecting microscope. Reproductive capacity index (RCI)/mouse was determined 40 days postinfection and larvae used for experimental reinfection. Isolation, determination of RCI-value, infective dose and infection procedures were determined by standardized techniques (see Chapter I, p. 6).

Longevity of the wolverine isolate in the small intestine

Parasite position, 24-hr *in vitro* larval release, and longevity during the intestinal phase was determined using one hundred 50 to 60 day old Swiss Webster mice infected with 400 larvae/mouse. From Day 1 postinfection, thereafter, until worms left the small intestine worm position and *in vitro* larval release were determined (see passage experiment for methods). Number of male and female worms was recorded so that sex ratio, rate of establishment (% Recovery), and rate of expulsion could be determined.

Survival of wolverine isolate muscle larvae in mice

Survival of muscle larvae was studied in male and female Swiss Webster mice from 50 to 600 days postinfection. The percent reduction in muscle larvae assumed that at 40 days postinfection the RCI-value is at a maximum and the difference in the number of larvae recovered between Day 50 and 600 would represent the number of worms that died. Isolation of muscle

larvae and determination of RCI were determined by standardized techniques (see Chapter I, p.6).

Comparison of biological characteristics of the wolverine isolate in other laboratory and wild rodents

Parasite position, establishment, 24-hr *in vitro* larval release and RCI (see passage experiment for methods) were determined in three outbred strains of mice [50 to 60 day old Crl: COBS CD-1 (1C1), Charles River Breeding Laboratories, Wilmington, Mass.; and two species of wild rodents *Peromyscus maniculatus* (Wagner) and *Mus musculus* (Linnaeus) (50 to 60 day old, F1 in laboratory)]; mature outbred golden hamsters [(Hor: (FI)] High Oak Ranch, Toronto, Ontario, Canada); mature outbred Sprague-Dawley rats [(Bbl: (SD)] Biobreeding Laboratories, Ottawa, Ontario, Canada) and one strain of inbred mice (50 to 60 day old SEC-J, Jackson Breeding Laboratories, Bar Harbor, Maine, USA), and compared with data from Crl: COBS CFW(SW) mice, the mouse strain routinely used to establish baseline data for all isolates in our laboratory. Reproductive capacity indices were also determined in two hybrid mice crosses. *M. musculus* ♂ x Crl: COBS CFW (SW) ♀ and in offspring from the cross of ♂ hybrid and Crl: COBS CFW(SW) ♀. Mice and wild rodents were infected by gastric intubation with 400 larvae, golden hamsters with 500, and rats with 2000 larvae each.

Statistics

Parasite position, 24-hr *in vitro* larval release and RCI were compared using Crl: COBS CFW(SW) as our baseline against other laboratory and wild rodents and analyzed using Student's *t*-test (Behrens-Fisher solution). Survival of muscle larvae was subjected to simple linear regression (Sub-program regression, SPSS), and a comparison of slopes of the regression line (Brownlee 1968). A value of < 0.05 probability was considered significant.

RESULTS

Passage experiment

Forty day passages of the wolverine isolate through the same species and strain of host were studied. Results are presented in Table I.

Worm position in the intestine did not change with 9 passages through mice, attaining an average mean position of 24.23±2.27 (Table I). The sex ratio (Female:Male) remained relatively constant with an average value of 1.98. Parasite establishment (% Recovery) was initially low for the first 6 passages but increased from passages 7 to 10, with a mean recovery of 53% (Table I). *In vitro* larval release over 24-hr was lowest in passage 2, thereafter, higher but variable results were obtained from passages 3 to 10 and did not correlate with RCI at Day 40 postinfection (Table I). Initially worms from passages 1 to 5 showed a gradual increase in RCI; increased substantially in passages 6 to 8, declined in passage 9 and increased in passage 10. Mean RCI for 10 passages was 22.23±10.43 and the mean number of larvae/g host muscle was 308 (Table I).

Longevity of the wolverine isolate in the small intestine

To determine worm position, establishment, fecudity of female worms and longevity in the small intestine, mice were examined daily. Worm position was initially more anterior on Day 1 (16.67±2.27), thereafter, mean position of worms

TABLE 1. Distribution, sex ratio, percent recovery, 24-hr. *in vitro* larval release and reproductive capacity of the *Trichinella* wolverine isolate through 10 passages in Outbred Swiss Webster mice [Crl:COBS CFW (SW)].

Passage number	Infection dose larvae/ mouse	$\bar{x}$ position of worms ± SD Day 5 PI	sex ratio ♀ : ♂ Day 5 PI	% Recovery Day 5 PI	$\bar{x}$ 24-hr *in vitro* larval release/♀ ± SD Day 7 PI	$\bar{x}$ index RCI/mouse ± SD Day 40 PI	$\bar{x}$ larvae /g of muscle
1	500	–	–	–	–	7.21 (n=4)[a]	129
2	500	21.00+3.00 (n=4)[a]	2.00	48	16.76+9.98 (n=52)[b]	11.43 (n=3)	197
3	400	26.06+4.08 (n=6)	2.29	45	39.22+11.26 (n=50)	19.58+7.64 (n=35)	253
4	400	28.16+6.03 (n=6)	2.03	43	24.90+11.43 (n=50)	18.64+3.27 (n=22)	257
5	400	23.13+5.38 (n=6)	1.99	41	26.02+11.84 (n=48)	15.51+0.51 (n=10)	214
6	400	24.04+5.12 (n=12)	1.96	51	31.22+14.23 (n=102)	27.64+6.76 (n=38)	357
7	400	23.72±3.22 (n=6)	2.02	56	38.50±13.56 (n=50)	39.22±7.69 (n=10)	541
8	400	22.16+3.00 (n=6)	1.79	72	33.38+11.78 (n=50)	34.37+12.45 (n=10)	491
9	400	23.23+3.29 (n=6)	1.83	66	35.66+15.93 (n=60)	17.36+0.40 (n=10)	224
10	400	26.57+4.32 (n=6)	1.94	56	29.90+8.87 (n=50)	31.39+9.87 (n=10)	419
		24.23+2.27[c] (n=52)	1.98 ±0.14[c]	53±10[c]	30.61+7.21[c] (n=512)	22.23+10.43[c] (n=152)	308 ± 137[c]

[a] n = number of animals

[b] n = number of female worms

[c] Averaged value ± SD

remained relatively constant (Day 2 to 8 postinfection) until the onset of worm expulsion (Day 9) where a posterior position (60.00±28.55) was observed. Adult worms persisted in the small intestine for 12 days (Table II). Male and female worms were expelled from the small intestine at the same rate since the sex ratios did not change significantly during the intestinal phase. Parasite establishment stabilized for the first 8 days and by Day 9, a 52% reduction in worm population was noted with a progressive decrease until the worms left the small intestine. Larval production by fecund female worms in vitro was noted on Day 5 with a peak occurring on Day 7, followed by a gradual decrease in production until females left the intestine (Table II). The majority of newborn larvae (75%) were released by Day 8 postinfection and the average fecundity of a single female for the entire intestinal phase was 128.39.

Survival of wolverine isolate muscle larvae in mice

The percent reduction of muscle larvae in male and female mice was compared from 50 to 600 days postinfection. Simple linear regression showed a significant ($p < 0.05$) loss of muscle larvae with time as indicated by high R^2 coefficients (Fig. 1). Comparison of the slopes of the regression lines

Table II. Daily distribution, sex ratio, percent recovery, 24-hr *in vitro* larval release and longevity of the *Trichinella* wolverine isolate in the small intestine of Crl: CORS CFW (SW) mice[a].

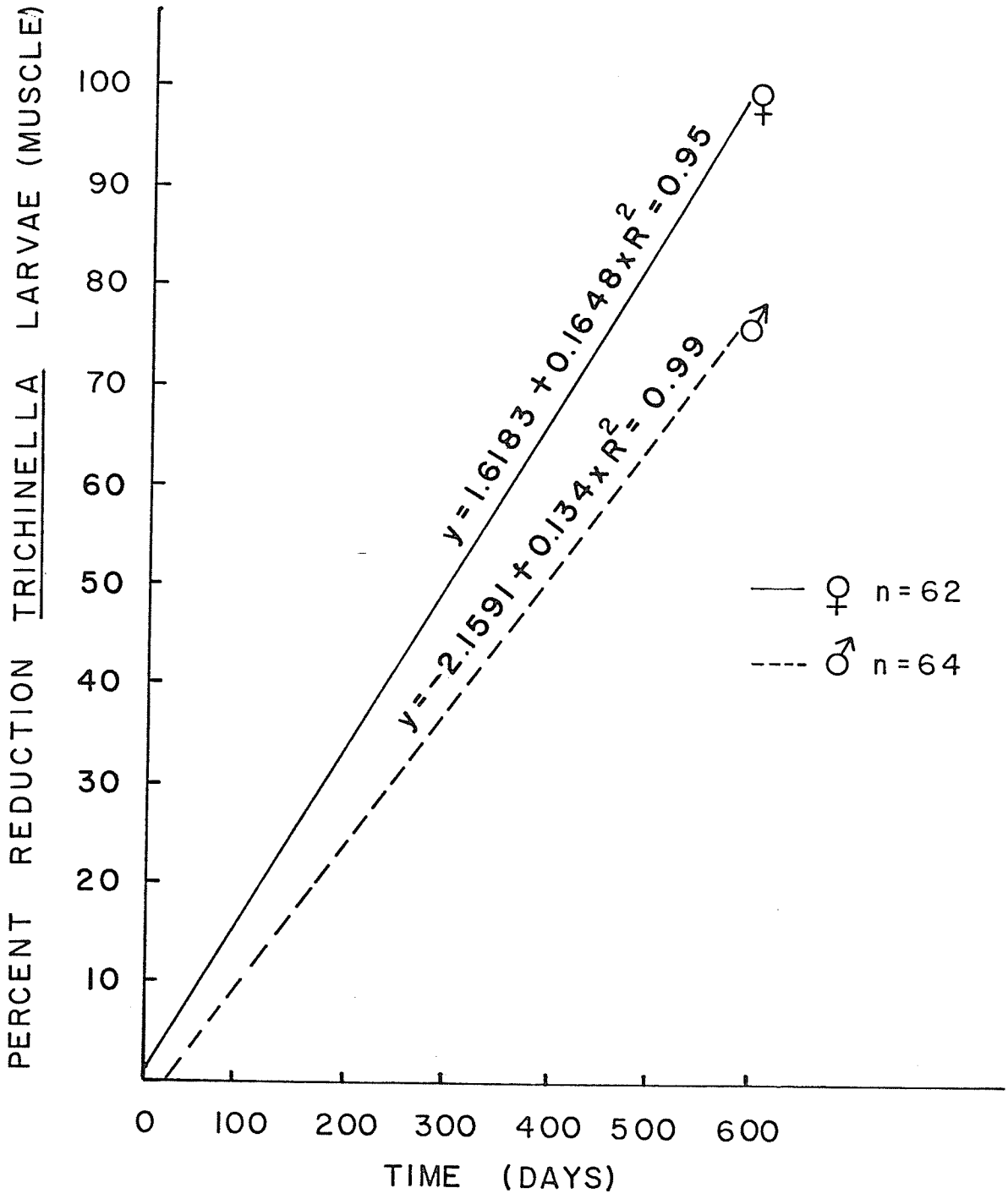
Days PI	$\bar{x}$ position of worms ± SD	Sex ratio ♀ : ♂	% Recovery	$\bar{x}$ 24-hr *in vitro* larval release/♀ ± SD
1	16.67±2.27 (n=6)[b]	1.95	44	- (n=50)[c]
2	23.31±4.37 (n=6)	1.69	38	- (n=50)
3	25.43±3.85 (n=6)	2.08	45	- (n=50)
4	20.97±2.45 (n=6)	1.86	50	- (n=50)
5	27.07±3.72 (n=6)	1.91	50	16.14±11.03 (n=50)
6	23.28±3.85 (n=6)	1.72	53	27.02±12.44 (n=50)
7	25.33±5.47 (n=6)	1.85	56	31.06±13.93 (n=50)
8	20.01±3.67 (n=6)	1.47	46	23.06±10.42 (n=50)
9	60.00±28.55 (n=6)	1.55	22	12.42±8.85 (n=50)
10	- (n=6)	1.56	16	10.22±9.87 (n=50)
11	- (n=6)	2.75	11	4.07±4.07 (n=50)
12	- (n=6)	2.83	6	4.40±6.08 (n=15)
13	- (n=12)	-	-	-

[a] Infection dose 400 larvae/mouse

[b] n = number of animals

[c] n = number of female worms

Fig. 1. Regression between time and percentage reduction *Trichinella* larvae (muscle) in male and female outbred Swiss Webster mice [Crl:COBS CFW(SW)].


PERCENT REDUCTION TRICHINELLA LARVAE (MUSCLE)
100
90
80
70
60
50
40
30
20
10
0
100
200
300
400
500
600
TIME (DAYS)
y = 1.6183 + 0.1648xR² = 0.95
y = -2.1591 + 0.134xR² = 0.99
♀ n = 62
♂ n = 64

indicated that percent reduction muscle larvae was constant, initially, but by 600 days postinfection 78 and 100% reduction muscle larvae was found for male and female mice, respectively. Overall percent reduction in the number of muscle larvae was not significantly different in male and female mice (Fig. 1).

Comparison of wolverine isolate in other laboratory and wild rodents

Worm position, sex ratios, establishment, 24-hr *in vitro* larval release and RCI for the wolverine isolate (Table 1) were compared in other laboratory and wild rodents. Results are summarized in Table III and IV.

Worm position did not differ significantly from Swiss Webster mice (24.23±2.27, Table I) with either SEC-J, CD-1 or *M. musculus* mice (Table III). *Peromyscus maniculatus* and golden hamsters had worms of the wolverine isolate situated in a posterior position while in rats an anterior position was observed. Worm position in *P. maniculatus*, golden hamsters and rats were significantly ($p < 0.05$) different from their position in Swiss Webster mice. Sex ratios (Female:Male) were similar in all rodents except for *P. maniculatus* which harboured significantly more female worms (3.35). Parasite establishment was similar in SEC-J, CD-1 and *M. musculus* mice and golden hamsters (48 to 54%), while significantly less worms, 22 and 4%, were recovered from Sprague-Dawley rats and *P.*

Table III. Distribution, sex ratio, percent recovery and 24-hr *in vitro* larval release of the *Trichinella* wolverine isolate in laboratory and wild rodents.

Host	Infection dose larvae/ animal	$\bar{x}$ position of worms + SD Day 5 PI	Sex ratio ♀ : ♂ Day 5 PI	% Recovery Day 5 PI	$\bar{x}$ 24-hr *in vitro* larval release/♀+SD Day 7 PI
SEC-J mice	400	33.11+11.91 (n=6)[a]	1.56	48	21.04+9.49 (n=50)[b]
Crl: COBS CD-1 (ICR) Mice (outbred)	400	22.73+2.18 (n=16)	1.85	54	29.59+9.51 (n=100)
Golden hamsters (*Mesocricetus auratus*) (outbred)	500	59.71+3.84 (n=6)	2.65	48	62.66+17.30 (n=50)
Sprague-Dawley rats (outbred)	2000	15.49+2.18 (n=8)	1.56	22	28.63+12.28 (n=60)
Deer Mice (*Peromyscus maniculatus*) (outbred)	400	56.66+5.57 (n=6)	3.35	4	16.41+11.48 (n=17)
Wild mice (*Mus musculus*) (outbred)	400	25.27+5.74 (n=6)	1.88	51	28.18+12.09 (n=50)

[a] n = number of animals

[b] n = number of female worms

TABLE IV. Reproductive capacity, mean number of larvae recovered and larvae per gram host muscle of the *Trichinella* wolverine isolate in laboratory and wild rodents 40 days postinfection

Host	Infection dose larvae/animal	n[a]	$\bar{x}$ index RCI/animal ± SD	$\bar{x}$ no. of larvae recovered[b]	$\bar{x}$ larvae /g of muscle[b]
SEC-J mice (inbred)	400	73	31.85±5.79	13,000	550
Crl: COBS CD-1 (ICR) mice (outbred)	400	40	42.51±4.51	17,000	530
Golden Hamster (*Mesocricetus auratus*) (outbred)	500	37	270.00±60.00	135,000	1,250
Sprague-Dawley rats (outbred)	2,000	50	3.05±2.88	6,000	40
Deer mice (*Peromyscus maniculatus*) (outbred)	400	32	9.84±11.59	4,000	250
Wild mice (*Mus musculus*) (outbred)	400	25	30.72±11.96	12,000	730
M. musculus ♂ x Crl: COBS CFW (SW) ♀ #	400	18	10.65±5.66	4,000	200
Cross of hybrid ♀ # x Crl: COBS CFW (SW) ♀	400	18	16.50±6.22	7,000	440

[a] n = number of animals

[b] All figures to nearest whole number

maniculatus, respectively (Table III). In vitro larval release by females recovered from Swiss Webster mice (30.61±7.21, Table I) did not differ from that of CD-1 mice, M. musculus or in Sprague-Dawley rats. In contrast, significantly ($p < 0.05$) less worms were produced in vitro by SEC-J (21.04±9.49) and in P. maniculatus mice (16.41±11.48), and significantly ($p < 0.05$) greater numbers of newborn larvae were released in golden hamsters (62.66±17.30, Table III).

Reproductive capacity indices of all hosts (Table IV) differed significantly ($p < 0.05$) from RCI-values for Swiss Webster mice (Table I). The susceptibility of SEC-J, CD-1 and M. musculus mice and hamsters to the wolverine isolate was significantly ($p < 0.05$) higher, while in P. maniculatus mice, Sprague-Dawley rats and in two hybrid strains of mice susceptibility was significantly ($p < 0.05$) lower. Larvae/g muscle correlated with both body weight and RCI of hosts, the highest in hamsters (1,250 larvae/g of muscle) and the lowest in Sprague-Dawley rats (40 larvae/g of muscle, Table IV).

DISCUSSION

Reproductive capacity indices (RCI) and larvae/g of muscle are common biological characteristics used in the separation of geographical isolates of Trichinella. Although many workers agreed that RCI of isolates from Arctic and/or African regions are lower when compared with a standard laboratory form of T. spiralis (Nelson and Mukundi 1963; Sukhdeo and Meerovtich 1977; Dick and Belosevic 1978; Belosevic and Dick 1979, 1980a) these values should be viewed with caution as prior cycling of the parasite in different hosts can affect RCI in laboratory rodents. The work of Bessonov et al. (1975) clearly shows that after six passages through pigs and five passages through cats and rats RCI of T. nativa (arctic form) equalled that of T. spiralis. Dick and Belosevic (1978) and Belosevic and Dick (1979) reported that RCI-values of a given arctic isolate are predictable after several passages through the same host and Sukhdeo and Meerovitch (1977) suggested that RCI is a genetically fixed characteristic of the parasite.

In the present study 40 day passages of the wolverine isolate through the same species and strain of host showed that worm position in the small intestine did not change suggesting that it is a relatively stable biological characteristic and supports the work of Belosevic and Dick (1979) for two other Trichinella isolates. Differences however, were noted in both in vitro larval release on Day 7 and in RCI at Day 40 postinfection between passages. These differences are difficult to explain

since *in vitro* larval release and RCI were not correlated with passages and wide fluctuations in these values suggested considerably less stability in the wolverine isolate than that reported by Belosevic and Dick (1979) for the polar bear isolate. Nevertheless differences for RCI and *in vitro* larval release were 22.23±10.43 and 30.61±7.21, respectively, for the wolverine isolate in this study and 63.46±19.34 and 20.66±6.50, respectively for the polar bear isolate (Belosevic and Dick 1979). These differences between the two isolates were statistically significant and appeared to be stable and predictable characteristics of these isolates.

Longevity of the wolverine isolate differed from data reported for other isolates (Pawlowski and Rauhut 1971; Belosevic and Dick 1979) and maybe a useful characteristic for the wolverine isolate in Swiss Webster mice. While worm position of the wolverine isolate differed from the pig isolate, it was similar to the polar bear isolate as reported by Belosevic and Dick (1979). A shift in worm position in the mouse intestine from anterior to a more posterior position during the course of infection agrees with that reported by Larsh and Hendricks (1949). Expulsion of male and female worms did not change significantly in the small intestine and differed

from Rappaport (1943) who found a shift in female to male ratio in the later stage of the intestinal phase. Expulsion of worms in the wolverine isolate occurred at Day 12 while in the polar bear isolate expulsion occurred at Day 15 (Belosevic and Dick 1979). The adult phase of the wolverine isolate appears to be more immunogenic than that of the polar bear isolate. This is particularly interesting since the polar bear isolate is more pathogenic than the wolverine isolate (Dick and Chadee 1980) but pathogenicity may be related to the presence of pathogenic microbes (Britov 1980) rather than the immunogenicity of the isolate. Although fecundity of a single female of the intestinal phase was higher for the wolverine isolate (128) than the polar bear isolate (114) (Belosevic and Dick 1979), RCI at Day 40 was considerably lower: wolverine (22.23±10.43), polar bear (63.46±19.34). These differences are probably related to the interaction of newborn larvae and the host immune system and suggest that larvae of the wolverine isolate may elicit a stronger immune response by the host. The interaction of newborn larvae and encysting larvae with the host immune system is still not clear, as the percent reduction of muscle larvae was less for the wolverine isolate in this study than for the

polar bear isolate reported by Belosevic and Dick (1980a). This suggests that the wolverine isolate during the muscle phase is less immunogenic.

The influence of hosts on biological characteristics is not well documented as most workers rely on levels of infectivity (Arakawa and Todd 1971; Pereverzeva et al. 1974. Dick and Belosevic 1978; Sukhdeo and Meerovitch 1977; Belosevic and Dick 1980a). Since baseline data were established in this study for the wolverine isolate in Swiss Webster mice comparisons could be made with other laboratory and wild rodents using standardized procedures. Worm position did not differ in either SEC-J, CD-1 or M. musculus mice, however, a posterior position in P. maniculatus and hamsters was noted. The anterior position of worms in laboratory mice confirmed the findings of Belosevic and Dick (1979) and was also noted in rats by Gursch (1949), Dick and Silver (1980) and Silver et al. (1980). A posterior position of worms in hamsters differed from an anterior position as reported by Boyd and Huston (1954) and Concannon and Ritterson (1965). The posterior position of wolverine isolate worms in P. maniculatus and hamsters is probably related to differences in host digestive processes such as emptying time as T. spiralis does not actively site select within the small intestine (Dick and Silver 1980). A posterior position of

worms was reported in the small intestine in guinea pigs by Roth (1938), in young mice by Larsh and Hendricks (1949), and in chicks by Marty (1966).

Larval production of adult female Trichinella is known to vary widely among different host species and even among individuals of the same species. Adult female worms in CD-1 and M. musculus mice and Sprague-Dawley rats produced the same number of newborn larvae in vitro, while in SEC-J and P. maniculatus mice, significantly lower numbers were produced. Only in hamsters did females produce significantly higher numbers of newborn larvae. Low in vitro larval production in P. maniculatus and high in vitro release in hamsters both correlated with RCI-values in these hosts (Table III and IV). Differences observed here are difficult to explain but clearly showed the influence of host species on in vitro larval production and therefore on the females during the intestinal stage. Differences are even more remarkable if RCI-values are compared. The high in vitro larval production of Day 7 females from the rat intestine is inversely related to the low RCI in rat muscle. It appears that in certain hosts the effect by the host is on larval production by females in the intestine while in other hosts the effect is on the disseminating larvae. The influence of host on RCI-values is again illustrated when

infection levels in hybrids and parental stock are compared. Wild type (M.musculus) in two subsequent crosses with our standard laboratory strain of mice had RCI-values of the wolverine isolate in these hybrid hosts lower than the RCI-values in the two parent hosts. The responses elicited by different hosts, to given Trichinella isolate appears to have a dramatic effect on the genetic expression of that isolate.

How closely related are the wolverine and polar bear isolates? They were geographically closely situated, they shared common biological features such as resistance to freezing, opaqueness of cuticle (Dick and Chadee 1980) and position, but had other characteristics that were significantly different (virulence, 24-hr in vitro larval release, RCI-values, rate of calcification and longevity in the small intestine). In addition, good interbreed success suggests a high degree of reproductive compatibility between these isolates (Dick and Chadee 1980), but both these arctic isolates of Trichinella have consistent and predictable differences suggesting same degree of genetic isolation. Perhaps, their cycling through a given group of host(s) accounts for these consistent differences, but we believe these differences are part of the normal biological variability of T. spiralis.

CHAPTER III

INTERBREEDING EXPERIMENTS BETWEEN ISOLATES OF *TRICHINELLA*

INTRODUCTION

The controversy over speciation in the genus Trichinella continues unabated. Workers like Boev et al. (1979) consider there are four distinct species while Sukhdeo and Meerovitch (1979, 1980) suggest at least two and possibly three species. Belosevic and Dick (1979, 1980a), Dick and Chadee (1980), Machnicka (1979) and Madsen (1975) consider there is insufficient evidence at present to raise any of the isolate or varieties to the species level. Although, Belosevic and Dick (1979, 1980a) showed differences between a lab form of Trichinella and an isolate of Trichinella from polar bears that were predictable and stable after several passages in experimental animals, they also found that these isolates were reproductively compatible in experimental breeding trials. Behavioural studies using sexual attraction as an indicator showed that the lab form and the polar bear isolate were similiar but differed markedly when compared to responses of T. spiralis var. pseudospiralis males and females to target doses of the above isolates (Belosevic and Dick 1980b). T. spiralis var. pseudospiralis has several characteristics that differ from other Trichinella isolates or species, namely (1) lack of a cyst in muscle tissue (2) infectivity to birds (3) differing immunological characteristics (Faubert, pers. comm.) and (4) lack of interbreeding with other isolates or species (Britov 1977, Bessonov et al. 1975).

This study was undertaken to determine if T. spiralis var. pseudospiralis (raccoon, 1972) was reproductively isolated under laboratory conditions from three isolates studied in the laboratory and designated by us as Trichinella (pig; 43°00'N, 81°00'W; 1952), Trichinella (polar bear; 58°00'N, 95°00'W; 1976), and Trichinella (wolverine; 55°00'N, 100°00'W; 1979).

MATERIALS AND METHODS

Parasite isolation and host animals

Infective Trichinella larvae of pig, polar bear and wolverine isolates and of T. spiralis var. pseudospiralis (raccoon isolate) were maintained and isolated in mice by standardized techniques (see Chapter I, p 6). Single and multiple pair interbreeding experiments for the Trichinella isolates were performed using 50 to 60 day old Swiss Webster mice [Crl:COBS CFW (SW)]. F_1 hybrids were passaged either through Swiss Webster mice, golden hamsters [Hor: (FI) High Oak Ranch, Toronto, Ontario, Canada] and/or Japanese Quail (Animal Holding Facilities, Department of Zoology, University of Manitoba).

Sexing of muscle larvae

Muscle larvae were washed several times with 0.85% saline at 37C and were sexed according to the criteria of Belosevic and Dick (1980a). Additional characteristics used were; Males: intestinal bulb close to convex surface; distinct cross-over from convex to concave surface; gonad rounded at the anterior end; long rectum and no genital plate. Females: intestinal bulb close to concave surface and with the intestine continuing close to this surface to the rectum; presence of genital plate; short rectum.

Surgical procedures

Mice were anaesthetized by intraperitoneal injection of sodium pentabarbitol solution (3.3 mg/mouse). The abdominal wall was cut along the linea alba and the duodenal portion of the small intestine elevated with a surgical probe for injection. Single and multiple pairs of sexed larvae were suspended in a 1 ml syringe containing up to 0.05 ml of 0.85% saline solution and injected directly into the lumen of the duodenum. For each inoculation a separate syringe and needle were used. The body wall muscle layer was sutured with catgut chromic (Naila Bayern, West Germany) and the skin closed with black braded 00-silk (Ethicon Inc., Somerville, N. Y.).

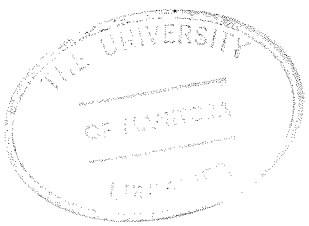
Intestinal controls and 24-hr *in vitro* larval release

Intestinal and muscle controls for the pig and polar bear isolates were reported by Belosevic and Dick (1980a), therefore, only the wolverine isolate and *T. spiralis* var. *pseudospiralis* are reported here. At Day 7 postinfection mice that were injected with either males, females or multiple pairs of larvae were killed by cervical dislocation and the entire small intestine removed and placed in 37C 0.85% saline. The intestine was slit longitudinally, and the mucosa lining scraped and stirred. The mixture was placed in a Baermann apparatus and adult worms were collected after 3-4 hr by removing the bottom

10 ml of saline, and then sexed in preparation for larval release experiments. Female worms were washed twice in 0.85% saline, four times in α-MEM Eagle, Earle's Base (modified) tissue culture medium containing 10% calf serum (by volume) and then placed one per 2-ml plastic cone-bottom-vial containing 1 ml of tissue culture medium. Vials were capped and stored at 37C in an environmental chamber for 24 hr and examined for the presence of newborn larvae.

Muscle controls and determination of reproductive capacity index (RCI)

At Day 40 postinfection mice that were injected with either males, females or single and multiple intra and interisolate combinations were killed and the number of larvae determined by standardized techniques (see Chapter I, p 6). F_1 hybrids were passaged either through mice, hamster, and/or quails and RCI determined Day 40 postinfection. Isolation of infective larvae, determination of infection dose and RCI have been described previously (see Chapter I, p 6).



RESULTS

Single pair Intra- and Interbreeding between *Trichinella* isolates

Single pair interbreeding did not occur in breeding trials between wolverine and raccoon isolates or between polar bear and pig isolates. However, 70% mating success occurred between the polar bear and wolverine isolate using single pairs. Intrabreeding trials had less than 50% mating success within a given *Trichinella* isolate (Table I).

Multiple pair Intra- and Interbreeding between *Trichinella* isolates

Multiple pair interbreeding gave positive results for most breeding trials with the exception of pig and raccoon isolates combinations (Table II). The highest mating success occurred when males of polar bear or wolverine isolates were crossed with females of pig, polar bear or wolverine isolates. Males of pig or the raccoon isolates gave the lowest success ranging from 13-21% of trials. Mating occurred when the raccoon isolate was crossed with either the polar bear or wolverine isolate but was less than 10% successful. Only when 20 individuals/isolate/cross between isolates of polar bear males and raccoon females was 100% successful mating noted (Table II). Isolates of the polar bear and raccoon hybrids backcrossed with both parents produced viable and infective progeny but

Table I. Single Pair Intra- and Interbreeding between *Trichinella* isolates. p = pig, pb = polar bear, w = wolverine, r = raccoon.

Cross	N[a]	No. mice infected at Day 40 postinfection	Percent positive breeding trials
w (1♂) x r(1 ♀)[b]	125	0	0
pb (1♂) x w(1♀)	20	14	70
pb (1♂) x p(1♀)	10	0	0
w (1♂) x w(1♀)	10	3	30
r (1♂) x r(1♀)	15	6	40
pb (1♂) x pb(1♀)	23	9	39
p (1♂) x p(1♀)	26	11	42

[a] Number of mice

[b] Number of worms inoculated/mouse and sex of worms

Table II. Multiple Pair Intra- and Interbreeding between Trichinella isolates. p = pig, pb = polar bear, w = wolverine, r = raccoon.

Cross	N[b]	No. mice infected at Day 40 postinfection	Percent positive breeding trials
p(5 ♂) x w(5 ♀)[a]	7	1	14
p(5 ♂) x pb(5 ♀)	24	5	21
p(5 ♂) x r(5 ♀)	18	0	0
p(5 ♂) x p(5 ♀)	15	14	93
pb(5 ♂) x p(5 ♀)	21	18	86
pb(5 ♂) x w(5 ♀)	7	7	100
pb(5 ♂) x r(5 ♀)	18	2	11
pb(10 ♂) x r (10 ♀)	10	0	0
pb(20 ♂) x r(20 ♀)	3	3	100
pb(10 ♂) x pb(10 ♀)	15	15	100
w(5 ♂) x p(5 ♀)	9	3	33
w(5 ♂) x pb(5 ♀)	9	6	67
w(5 ♂) x r(5 ♀)	20	3	15
w(5 ♂) x w(5 ♀)	13	12	92
r(5 ♂) x p(5 ♀)	9	0	0
r(5 ♂) x pb(5 ♀)	8	1	13
r(5 ♂) x w(5 ♀)	10	2	20
r(5 ♂) x r(5 ♀)	15	13	87
pb(5 ♂) x pb x r Hybrid (5 ♀)	10	0	0
pb x r Hybrid (5 ♂) x pb(5 ♀)	9	1	11
r(5 ♂) x pb x r Hybrid (5 ♀)	9	7	78
pb x r Hybrid (5 ♂) x r(5 ♀)	9	8	89

[a] Number of worms inoculated/mouse and sex of worms

[b] Number of mice

crosses were always higher with hybrid and raccoon isolate combination (Table II). Multiple pair breeding trials within each of the Trichinella isolates gave 85-100% mating success (Table II).

In vitro larval production by female worms

Recovery of males or females from the intestine, absence of larvae following in vitro incubation of Day 7 females, and absence of muscle larvae showed sexes were accurately determined. No larvae were produced in vitro by females of the raccoon isolate recovered from multiple-cross experiments using either polar bear or wolverine isolate males (Table III).

Reproductive capacity indices in F_1 hybrids

In general, RCI-values of the hybrids were much lower than either parent in the same host. The exception was wolverine males crossed with polar bear females where RCI-values were initially about the same as wolverine,but increased by generation two so that it surpassed that of the parent polar bear isolate in the same strain of experimental host. Hybrid infectivity was highest by generation three when wolverine males were crossed with either pig or polar bear isolate females but,decreased in crosses of polar bear males against pig of wolverine isolate females (Fig. 1).

Table III. Sexing accuracy controls, 24-hr *in vitro* larval release and multiple pair crosses between *Trichinella* isolates. pb = polar bear, w = wolverine, r = raccoon.

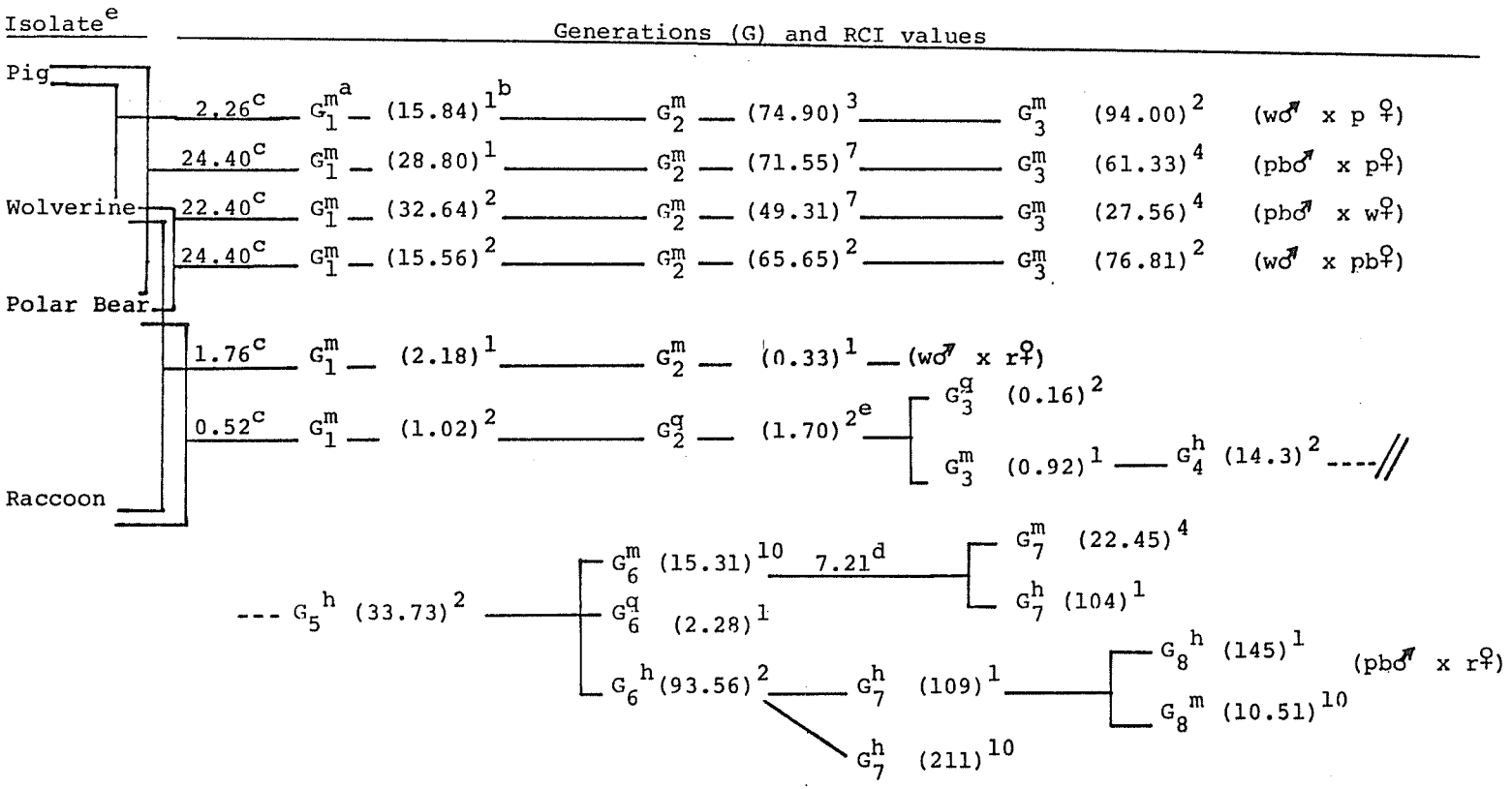
Inoculum/ Crosses	N[a]	Control[c] Intestine: No. worms recovered ♂	Control[c] Intestine: No. worms recovered ♀	Control[c] Intestine: 24-hr *in vitro* larval release	Control Muscle: No. mice infected at Day 40 postinfection
w (10 ♂)[b]	2	6	0	-	
w (10 ♀)	2	0	8	0	
r (20♀)	4	0	35	0	
r (10 ♂)	4	12	0	-	
w (20 ♂) x r (20 ♀)	10	80	86	0	
pb (20 ♂) x r (20 ♀)	3	15	24	0	
r (10 ♀)	3				0
r (20 ♀)	3				0
r (10 ♂)	6				0
w (10 ♂)	4				0
w (10 ♀)	4				0

[a] Number of animals

[b] Number of worms inoculated/mouse and sex of worms

[c] Examined at 7 days postinfection

Fig. 1. Reproductive capacity Day 40 postinfection in F_1 hybrids from *Trichinella* isolate crosses. w = wolverine, pb = polar bear, p = pig, r = raccoon.



[a] Indicate host animals m = mouse
h = hamster
q = quail

[b] Indicate sample size

[c] RCI from original cross (Table II)

[d] RCI Day 90 in mice

[e] Pig control mice: 151.27±27.30
Polar bear controls: mice 63.46±19.34
hamster 235±82.65

Wolverine controls mice: 22.23±10.43

Raccoon controls mice: 23.29±1.99
hamster: 168±26.30
quail: 3.00±0.16

Progeny of wolverine - raccoon isolate crosses were low but viable and infective in mice. Infectivity was 90% less than either parental controls (Fig. 1). Similarly, hybrids of polar bear and raccoon isolates were initially very low in mice but when transferred through mice-quail-hamster for several passages RCI-values increased reaching a value of 211 in hamsters and 22.45 in mice by generation 7 (Fig. 1). All hybrids of the raccoon isolate lacked a cyst in the muscle stage regardless of host.

DISCUSSION

The question of speciation in the genus Trichinella is still in a state of flux and many workers agree there are four distinct species, namely: T. spiralis (Owen, 1835; north-temperate form); T. nativa (Britov and Boev, 1972; arctic form); T. nelsoni (Britov and Boev, 1972; tropical form) and T. pseudospiralis (Garkavi, 1972; North Caucasus). Species criteria are based primarily on reproductive and genetic isolation and geographical location.

Using single- and multiple-pair(s) interbreeding trials for Trichinella raises interesting questions concerning the methods used and evaluating reproductive compatibility between isolates or species. The major problem is whether negative results with one breeding pair is clear-cut evidence of reproductive isolation especially if multiple-pair-breeding trials give positive results. To overcome this problem sexing accuracy controls must be rigorously assessed and RCI-values of hybrids and parental stock determined and followed for 2-3 generations. Backcrossing hybrids to parental stock determines if hybrid males and females are fertile or sterile and whether or not hybrid breakdown will occur. Our results showed reproductive isolation in single-pair interbreeding experiments between wolverine and raccoon isolates (125 breeding trials), yet reproductive compatibility was observed with multiple-pair-crosses.

Reproductive compatibility was observed for most isolates using multiple-pair-crosses but reproductive isolation was noted between pig and raccoon isolates. A given Trichinella isolate was less than 50% successful when single-pair intrabreeding trials were compared with a 85-100% mating success for multiple-pairs. Work by Campbell and Yakstis (1969) showed that a 19% mating success with single-pairs of worms administered orally was due to an inability of males and females to find each other, yet many workers prefer to use this route of infection (Komandarev et al. 1975; Britov 1977; Sukhdeo and Meerovitch 1977; Shaikenov 1980). Dick and Chadee (1980) suggested that a minimum number of individuals is essential to ensure sexual attraction and contact between isolates of Trichinella and that surgical transplants enhance the possibility of contact. Likewise, Britov (1971, 1977) proposed that multiple-pair-breeding trials would improve efficiency of experiments.

High mating success between polar bear and wolverine isolates even with single-pair-crosses suggested a high degree of reproductive compatibility between these two isolates. This is understandable as they were recovered from animals approximately 500 km apart at approximately $55^{\circ}00'$-$58^{\circ}00'$N latitude. Lack of breeding of either the wolverine or polar bear isolates to the pig isolate in single-pair-crosses but with successful mating in multiple-pairs indicates some

reproductive compatibility though at a lower level than between wolverine and polar bear. The raccoon isolate is probably furthest removed genetically as it did not interbreed with the pig, but interbred reasonably well with both wolverine and polar bear isolates. Hybrids of polar bear and raccoon isolates backcrossed successfully with both parents and there were no hybrid breakdown. Numerous attempts by various workers using both single and multiple-pair breeding trials showed complete reproductive isolation between T. spiralis, T. nativa, T. nelsoni and T. pseudosprialis (Britov 1971, 1977; Komandarev 1975; Sukhdeo and Meerovitch 1977; Shaikenov 1980), while others (Bessonov et al. 1975) have shown reproductive compatibility between T. spiralis and T. nativa and concluded there was insufficient evidence to consider them as separate species. Bessonov et al. (1975), showed that T. pseudospiralis was reproductively isolated from T. spiralis and T. nativa. Britov (1977) also found reproductive compatibility between T. nelsoni and T. spiralis but male hybrids were sterile while females were fertile. Work on North American isolates of Trichinella (Sukhdeo and Meerovitch 1977) demonstrated complete reproductive isolation between arctic and north-temperate forms, while Belosevic and Dick (1980a) showed reproductive compatibility between the polar bear and pig isolates. Britov (1977) using multiple-pair-breeding trials concluded that the degree of closeness was as follows

and in a decreasing order: T. nelsoni and T. spiralis; T. nelsoni and T. nativa; and T. nelsoni and T. pseudospiralis. The remotest relationship was between T. spiralis and T. nativa. By contrast, our results indicate the remotest relationship between T. spiralis and T. spiralis var. pseudospiralis (raccoon isolate).

All hybrids established in this study had low RCI-values initially but increased with subsequent passages with consistent and relatively intermediate RCI-values from those of parental stock. The one exception was from wolverine-polar bear hybrids where the RCI-value from generation one was similiar to one of the parents i.e. 24.40 for hybrid and 22.23 for wolverine isolate parent. One could argue that an error was made in sexing as multiple-breeding-pairs were used but when RCI-values of subsequent generations of this hybrid are compared they were much higher than the wolverine isolate parent stock and the difference between generation one and three is greater than normal variations found between any three continuous generations in the wolverine isolate (see Chapter II, Table I). In fact, for one of the wolverine-polar bear crosses we are probably observing hybrid vigour based on the high RCI-value of the hybrid in generation three. This is the first time that we have observed a RCI-value of a hybrid greater than either parent. The RCI-values of the hybrid of polar bear-raccoon isolate increased when passaged through mice-quail-hamster for several generations.

All hybrids of raccoon isolate lacked cysts in muscle stage of the life cycle and showed distinct features: (a) RCI was low initially, usually lower than either of the parental stock, (b) RCI increased in subsequent generations, (c) RCI-values were influenced by host and (d) there was no hybrid breakdown.

Interbreeding between raccoon and other Trichinella isolates represents the first observation of reproductive compatibility between these isolates. Britov (1977) reported successful fertilization by the presence of zygotes but was unable to recover live embryos or larvae when T. pseudospiralis was crossed with T. spiralis, T. nelsoni and T. nativa. The level of compatibility between sperm and ova of the raccoon isolate and the wolverine and/or polar bear isolate must be extremely low since 110 females crossed with wolverine and polar bear males did not produce larvae in vitro at Day 7. Nevertheless, the low recovery of muscle larvae suggest some compatibility between sperm and ova of the parasite. What is even more remarkable is upon establishment of a hybrid, even though numbers of muscle larvae were low initially, the species of experimental host had a dramatic influence on infectivity by raising the RCI-value. This relationship between host and infectivity of Trichinella needs further study.

There is no doubt that we have differences in interbreeding experiments that are pronounced such as the inability of pig

and raccoon isolates to interbreed but the pig isolate interbreeds with the arctic isolates (wolverine and polar bear) which in turn breed with the raccoon isolate. It is interesting that the raccoon isolate recovered in Russia interbreeds with arctic isolates from North America. All these observations suggest a latitudinal cline of variants or isolates with distinct characteristics, but still allowing some gene flow between the isolates. If Trichinella does not have several separate species how are these differences explained? It is possible that we are dealing with a series of semispecies or incipient species in various stages of speciation. Most will probably disappear or become part of the normal variation of T. spiralis while others such as the raccoon isolate may progress to a full species designation particularly if it stays relatively isolated geographically and utilizes a bird host.

CHAPTER IV

SENSITIVITY OF *TRICHINELLA* SP. ISOLATES TO THIABENDAZOLE

INTRODUCTION

Thiabendazole, 2-(4-thiazolyl)-benzimidazole is a broad-spectrum anthelmintic (Brown et al. 1961; Cuckler 1961) which has marked activity against experimental infections of Trichinella spiralis (Campbell 1961; Campbell and Cuckler 1962, 1964a; Spaldonova et al. 1965, 1978; Kozar and Kozar 1967; Kociecka 1971; Ozeretskovskya et al. 1974; Ruitenberg and Steerenberg 1974; Campbell and Blair 1978). Kociecka (1971) emphasized the importance of T. spiralis strains and suggested that differences in strain sensitivity to thiabendazole may contribute to differences in experimental results. Ozeretskovskaya et al. (1970) found that an Arctic strain of T. spiralis in mice was more susceptible to thiabendazole and the effect of thiabendazole on T. pseudospiralis (Garkavi 1972) has been studied by Spaldonova et al. (1978). These studies used one dosage only and time of treatment was either during the intestinal or early muscle stage. As thiabendazole is still used in the treatment of human trichinellosis, and is well tolerated by humans, its activity against enteral and parenteral phases of Trichinella isolates at low sustained dosages warranted further investigation.

The objective of the present study was to investigate the comparative action of thiabendazole on three geographical isolates of Trichinella, and T. spiralis var. pseudospiralis

in mice during the intestinal, dissemination and muscle phases at sustained low and high dosages.

MATERIALS AND METHODS

Parasites, animals and inoculation procedures

Four Trichinella isolates designated as Trichinella (pig; 43°00'N, 81°00'W; 1952); Trichinella (polar bear; 58°00'N, 95°00'W; 1976) Trichinella spiralis var. pseudospiralis (raccoon; 1972) and Trichinella (wolverine; 55°00'N, 100°00'W; 1979) were used for the experiments. The reasons for considering T. spiralis var. pseudospiralis are outlined by Belosevic and Dick (1980b). For simplicity throughout this Chapter I will refer to these Trichinella as pig, polar bear, raccoon or wolverine isolates. We consider both the polar bear and wolverine isolates to be northern or Arctic strains of T. spiralis (Dick and Belosevic 1978; Dick and Chadee 1980). All four Trichinella isolates were maintained in outbred Swiss Webster mice [Crl:COBS CFW (SW)], but experimental infections used 50-60 day old outbred male white mice [Crl:COBS CD-1 (ICR), Charles River Breeding Laboratories, Wilmington, Mass.] as previous workers (Blair and Campbell 1971) used this strain of mice. Isolation procedures, preparation of inocula, and infection of hosts were determined by standardized techniques (see Chapter I, p. 6). Mice were routinely inoculated with 400 larvae/mouse by gastric intubation and were given commercial lab chow (Wayne Lab-Blox) and water ad libitum. Reconstituted thiabendazole-treated food and water was given ad libitum during drug treatment experiments.

Drug preparation

Thiabendazole (Merck and Co. kindly donated through the courtesy of W. C. Campbell) was prepared in the diet at a $^{w}/w$ mixture of finely ground commercial feed. The drug-food mixture was thoroughly mixed with a household blender and placed in enamel trays. Water was added to the mixture and kneaded, then this mixture was molded into pellets and allowed to dry at 60C in an air-stream oven for 12 hr. In all experiments drug-food mixtures were prepared two days prior to treatment. Although drug-food mixtures were consistent throughout these experiments I took the additional precaution of feeding several experimental groups with the same batch of medicated feed. Each experimental group was infected with a different Trichinella isolate.

In vitro release of newborn larvae

Outbred male white mice [Crl:COBS CD-1 (lCR)] were infected with 400 larvae/mouse by gastric intubation for each of the four Trichinella isolates and were given reconstituted thiabendazole-food (0.03 - 0.06%) on Day 2 to 7 postinfection. Day 2 postinfection was chosen as it was post copulation and Day 7 postinfection was the day of peak larval production. Isolation and procedures used for in vitro inoculation and counting of newborn larvae was determined by standardized techniques (see Chapter II, p. 17).

Larval counts from female worms within and between mice were analyzed for variation in treated and control groups.

Longevity and reversibility effect of thiabendazole induced sterilization in T. spiralis during the intestinal phase

This experiment investigated if chemosterilization was permanent and if the isolates responded in a similiar manner. Groups of outbred white mice [Crl:COBS CD-1 (1CR)] infected with 400 larvae/mouse were given reconstituted thiabendazole-food at sterility dosages (0.03 or 0.05%) for the four Trichinella isolates. Mice were fed medicated food from Day 2 to 7 postinfection and placed on commercial lab chow thereafter. Female worms isolated from the intestine were examined for in vitro larval release on a daily basis from Day 7 postinfection until the worms left the small intestine. In vitro larval release by females from untreated mice was monitored from Day 4 onwards. Isolation, in vitro inoculation of female worms and counting of newborn larvae were determined by standardized techniques (see Chapter II, p.17).

Muscle invasion of larvae from drug-treated worms

To determine if larvae from drug-treated worms were capable of muscle invasion, and to test if in vitro drug analysis could be compared to the muscle phase, 15 mice/

Trichinella isolate were infected with 400 larvae/mouse and given sterility drug dosages (0.03 or 0.05% thiabendazole) from 2 to 7 days postinfection and then placed on commercial lab chow until 40 days postinfection. The procedures for isolation of muscle larvae and determination of reproductive capacity index (RCI^{I}) were determined by standardized techniques. (see Chapter I, p. 6).

Effect of thiabendazole on disseminating and muscle larvae of Trichinella

The RCI-value (as a measure of drug efficacy) was estimated 40 days postinfection unless otherwise stated. To determine the effect of thiabendazole on disseminating larvae, 10 mice [Crl:COBS CD-1 (1CR)]/group/Trichinella isolate were infected with 400 larvae/mouse and placed on medicated feed (0.03 - 0.1% thiabendazole) from 4 to 20 days postinfection. All mice after treatment were fed normal lab chow and necropsied at 40 days postinfection. Although the length of the intestinal phase and presumably the disseminating phase differed among the isolates a strict drug regime was used for comparative purposes. The early muscle phase was considered to be 20 to 40 days postinfection and the effect of thiabendazole at dosages from 0.03 to 0.5% was determined for the isolates. During the late muscle phase infected mice were placed on

medicated food (0.03 - 0.1% thiabendazole) 40 to 60 days postinfection and necropsied Day 60. In both the early and late muscle phase 10 mice/drug dosage/*Trichinella* isolate was used. The number of larvae in the musculature of treated and untreated mice was determined in groups of 2, and compared within and between groups. At high drug dosages many loosely coiled, c-shaped and motionless larvae (dead larvae) were observed but were not included in the determination of RCI-value. Worms that survived sub-lethal drug dosages were tested for infectivity.

Statistics

Mean larval production during the intestinal phase was subjected to a variety of statistical tests; Bartlett's test for homogeneity of variance, analysis of variance [one-way (1V), two-way (2V)] using Biomedical Computer Programs (BMDP), and Student's *t*-test for differences among means. The RCI-value (expressed as percentage reduction of muscle larvae) for the dissemination and muscle phases of *T. spiralis* was analysed using simple linear regression (sub-program regression, SPSS), and a comparison of slopes of the regression line (Brownlee 1968). A value of < 0.05 probability was considered significant.

RESULTS

In vitro larval release from Day 7 female *Trichinella* treated with thiabendazole at various concentrations from 2 to 7 days postinfection

The results of 24-hr *in vitro* larval release from Day 7 females for four *Trichinella* isolates are summarized in Table I and represent data from three replications. One-way analysis of variance showed a significant decrease ($p < .001$) in *in vitro* larval production with increasing drug dosages for the *Trichinella* isolates. A dosage of 0.05% thiabendazole caused total sterility in the pig and raccoon isolate, while a dosage of 0.03% caused chemosterilization in two Arctic *Trichinella* isolates (polar bear and wolverine) (Table I). There was a significant difference ($p < .001$) in the overall sensitivity among the isolates to thiabendazole treatment when all isolates were compared using two-way analysis of variance. The polar bear and wolverine isolates were more sensitive to the thiabendazole dosage of 0.03% ($p < .05$) than either the pig or raccoon isolate. At a level of 0.01% thiabendazole in the diet a varied response was noted with the polar bear being the least sensitive (42% efficacy). Untreated worms began larval production *in vitro* 4 or 5 days postinfection (Figs. 1 and 2), while female worms for all isolates 4 to 6 days postinfection treated with 0.03 or 0.05% thiabendazole were sterile and the uterus void of developed larvae. Recovery for adult worms was not significantly reduced at Day 7 postinfection for any drug dosage.

Table I. The effect of various dosages of thiabendazole on the intestinal phase of Trichinella; in vitro larval release/female/24-hr at Day 7.

Isolate	Untreated	Drug Dosages[a]							
		0.01%	0.02%	0.025%	0.03%	0.04%	0.045%	0.05%	0.06%
	$\bar{x}$ ±SD	$\bar{x}$±SD	$\bar{x}$±SD	$\bar{x}$±SD	$\bar{x}$±SD	$\bar{x}$±SD	$\bar{x}$±SD	$\bar{x}$±SD	$\bar{x}$±SD
T. spiralis	70.08±20.12	19.54±3.31	5.65±7.33	-	1.62±2.40	0.40±0.64	0.004±0.01	0	0
(pig)	(n=100)[b]	(n=180)	(n=200)		(n=190)	(n=220)	(n=180)	(n=270)	(n=20)
% efficacy		72	92		98	99	99	100	100
T. spiralis	25.60±8.87	10.84±4.56	2.64±1.55	1.67±1.03	0	0	-	0	0
(polar bear)	(n=91)	(n=170)	(n=170)	(n=150)	(n=240)	(n=40)		(n=20)	(n=20)
% efficacy		42	90	93	100	100		100	100
T. spiralis var. pseudospiralis	21.75±9.07	6.17±1.42	4.02±1.82	2.90±1.93	0.42±0.31	0.12±0.23	0.09±0.15	0	0
	(n=100)	(n=150)	(n=200)	(n=150)	(n=200)	(n=200)	(n=150)	(n=250)	(n=20)
% efficacy		72	82	87	98	99	99	100	100
T. spiralis	29.57±9.51	4.29±2.18	0.56±1.14	0.06±0.14	0	0	0	-	-
(wolverine)	(n=100)	(n=150)	(n=210)	(n=200)	(n=260)	(n=210)	(n=150)		
% efficacy		85	98	99	100	100	100		

[a] Drug treatment from Day 2 to 7 postinfection

[b] Number of female worms

- Drug dosage was not examined

Fig. 1. Reversibility effect of thiabendazole induced sterilization in T. spiralis during the intestinal phase. Sample size (n = 50) unless indicated by numbers in brackets. Arrow = day fecund female worms were observed following drug treatment 2 to 7 days postinfection.

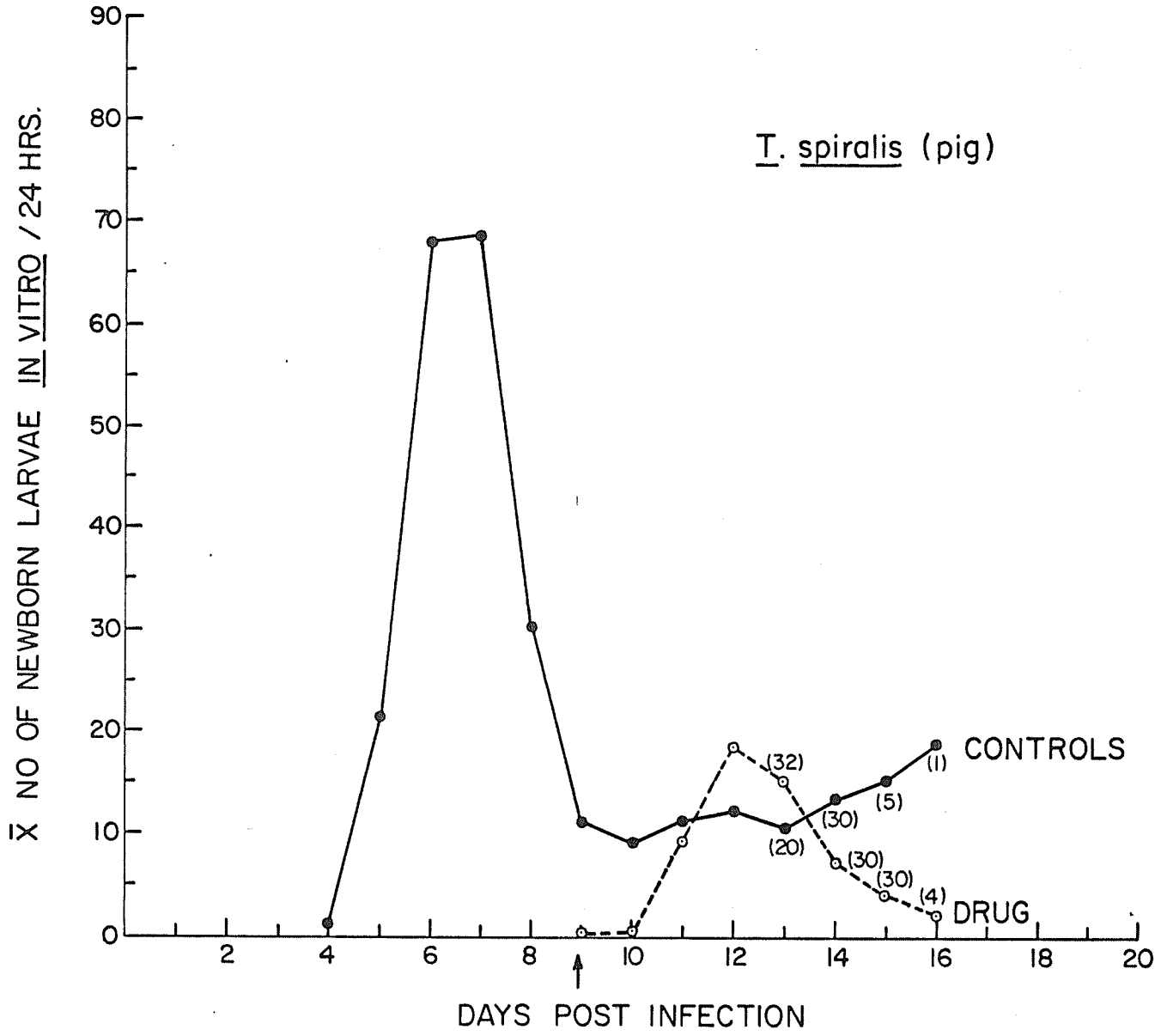
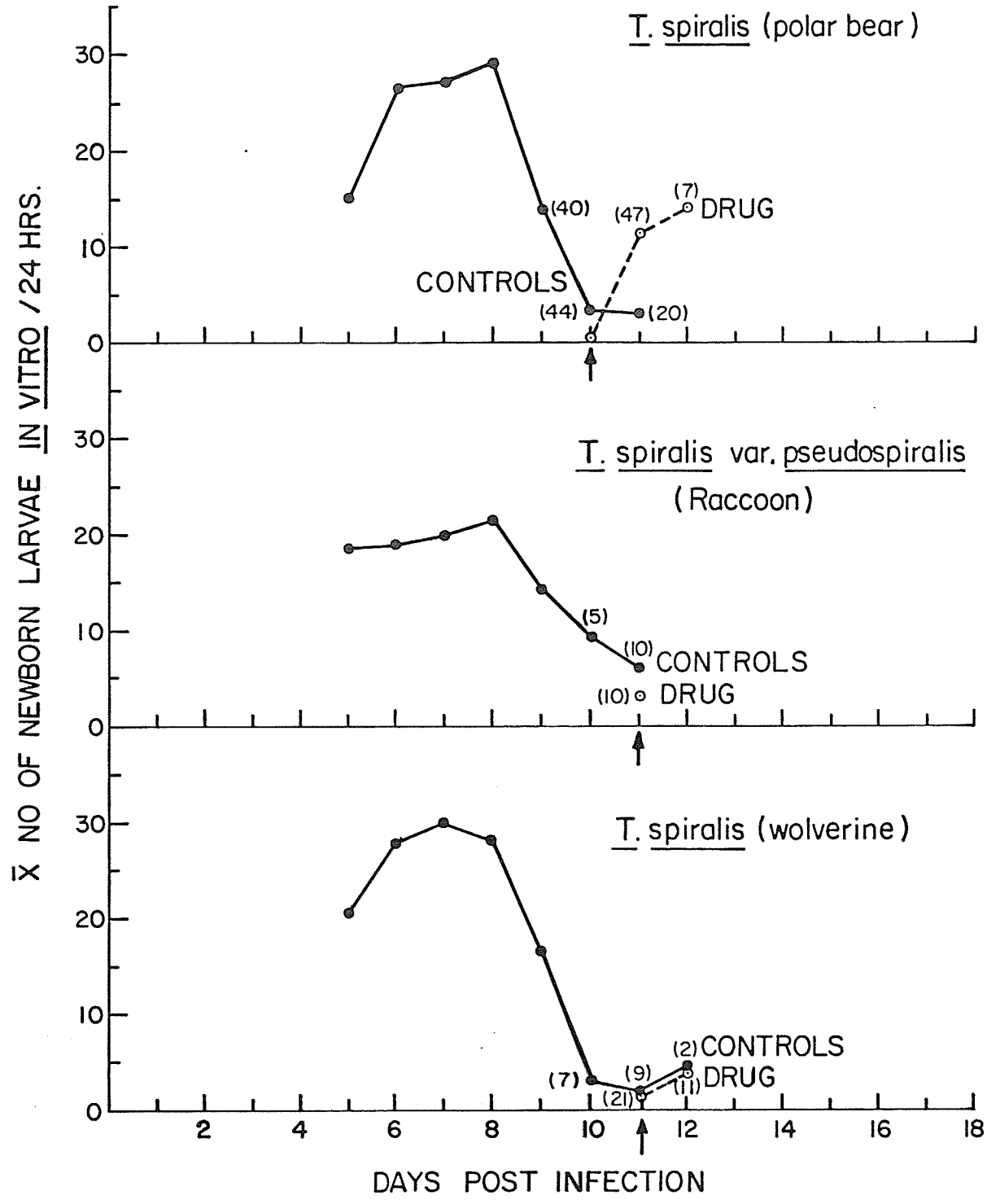

T. spiralis (pig)
X̄ NO OF NEWBORN LARVAE IN VITRO / 24 HRS.
90
80
70
60
50
40
30
20
10
0
2
4
6
8
10
12
14
16
18
20
DAYS POST INFECTION
CONTROLS
DRUG
(1)
(5)
(30)
(20)
(32)
(30)
(30)
(4)

Fig. 2. Reversibility effect of thiabendazole induced sterilization in T. spiralis during the intestinal phase. Sample size (n = 50) unless indicated by numbers in brackets. Arrow = day fecund female worms were observed following drug treatment 2 to 7-days postinfection.


T. spiralis (polar bear)
30
20
10
0
(40)
(47)
(7)
DRUG
CONTROLS
(44)
(20)
T. spiralis var. pseudospiralis
(Raccoon)
(5)
(10)
CONTROLS
(10)
DRUG
T. spiralis (wolverine)
(2) CONTROLS
(7)
(9)
(11)
DRUG
(21)
2
4
6
8
10
12
14
16
18
X̄ NO OF NEWBORN LARVAE IN VITRO /24 HRS.
DAYS POST INFECTION

Longevity and reversibility effect of thiabendazole induced sterilization following drug treatment from 2 to 7 days postinfection during the intestinal phase

Longevity and release of newborn larvae during the intestinal phase for untreated and drug-treated worms are outlined in Figs. 1 and 2. Female worms of the pig isolate following treatment with 0.05% thiabendazole produced larvae *in vitro* 2 days postinfection and continued until the females left the intestine on Day 16. Drug-treated worms peaked in larval production by Day 12 and produced significantly ($p < .05$) more newborn larvae than the controls (Fig. 1). Females of the polar bear isolate were fecund 3 days post-drug treatment at a dosage of 0.03% thiabendazole and by Day 11 produced significantly ($p < .05$) more larvae than the controls and the intestinal phase was only 12 days (Fig. 2). Drug treatment of 0.05% thiabendazole for females of the raccoon isolate began larval production 4 days post-treatment and the intestinal phase was only 11 days giving little time for larva deposition (Fig. 2). Similarly, for the wolverine isolate after drug treatment of 0.03% thiabendazole, fecund female worms were producing larvae from Day 4 post-treatment until they leave the intestine by Day 12 (Fig. 2). *In vitro* larval production by individual females for all isolates (Figs. 1 and 2) for untreated and drug-treated groups was

continuous during the intestinal phase. The majority of larvae (80%) were produced in the untreated group, regardless of the isolate, between Day 5 and 8 postinfection. The length of the intestinal phase, though different among the isolates, was the same in the control and drug-treated group (Figs. 1 and 2).

Reversibility effect of thiabendazole induced sterilization: Comparison with intestinal and muscle phase

Treatment with thiabendazole from 2 to 7 days postinfection and recovery of larvae at Day 40 postinfection was done to determine if there were differences in drug efficacy among the Trichinella isolates during the intestinal and muscle phase. Results are summarized in Table II. Larvae produced by females following drug treatment from 2 to 7 days during the intestinal phase were capable of muscle invasion. Furthermore, percent efficacy between in vitro and in vivo experiments showed no significant differences among the isolates. For example, percent efficacy of pig isolate females was 79% for in vitro studies and 83% for in vivo studies (Table II). The number of newborn larvae produced following drug treatment (Figs. 1 and 2; Table II) and the RCI-value were directly related to the length of the intestinal phase. Muscle larvae following drug treatment were infective to mice when transferred and examined 40 days postinfection.

Table II. Total production of *Trichinella* larvae by female worms *in vitro* throughout the intestinal stage and in muscle following treatment with thiabendazole from Day 2 to 7 postinfection

		UNTREATED		TREATED			
Isolate	Drug dosage for sterility	$\bar{x}$ larval prod. *in vitro* of intestinal phase	$\bar{x}$ index reproductive capacity/animal $\pm SD^c$	$\bar{x}$ larval prod *in vitro* of intestinal phase	%efficacy *in vitro*	$\bar{x}$ index reproductive capacity/animal $\pm SD^{c,d}$	%efficacy muscle
Pig	0.05%	274 (n=506)[a]	94.18±7.74 (n=30)[b]	58 (n=142)[a]	79	16.00±2.23 (n=15)[b]	83
Polar bear	0.03%	117 (n=314)	43.73±6.40 (n=30)	26 (n=54)	78	2.00±0.11 (n=15)	95
Raccoon	0.05%	105 (n=265)	19.49±4.35 (n=30)	3 (n=10)	97	0.10±0.01 (n=15)	99
Wolverine	0.03%	134 (n=268)	42.51±4.51 (n=30)	6 (n=32)	96	0.40±0.02 (n=15)	99

[a] Number of female worms

[b] Number of animals

[c] Day 40 postinfection

[d] Muscle larvae were infective to mice

Therapy of the dissemination phase

Thiabendazole-treated food at concentration of 0.03 to 0.1% from 4 to 20 days postinfection was highly effective against the Trichinella isolates (Table III). Thiabendazole at 0.03% dosage was 67% effective for the pig isolate and 85% effective or higher for the other isolates while at dosages of 0.05 to 0.1% efficacy was 95% or higher. Only in the raccoon isolate was 100% efficacy observed at 0.1% thiabendazole, but regardless of the Trichinella isolate, overall drug sensitivity was similar and not significantly different among the isolates (Table III). Female worms examined in vitro from Day 5 to 12 treated with 0.05% thiabendazole from 4 to 20 days postinfection were sterile at Days 9 to 10 for the polar bear, raccoon and pig and wolverine isolate, respectively. Regardless of the isolate, 50% live and 50% motionless larvae were observed by Days 6 and 7 postinfection.

Therapy of the muscle phase

Mice treated at dosages of 0.05 to 0.5% from 20 to 40 days postinfection elicited a varied response among Trichinella isolates (Table IV). There was an increase in drug efficacy for all isolates with increasing drug concentrations. At 0.5%, thiabendazole was only 74% effective for the pig isolate

Table III. The effect of thiabendazole on the dissemination phase (4 to 20 days postinfection) of Trichinella.

	Untreated	Drug Dosages			
		0.03%	0.05%	0.75%	0.1%
Isolate	$\bar{x} \pm SD$	$\bar{x} \pm SD$	$\bar{x} \pm SD$	$\bar{x} \pm SD$	$\bar{x} \pm SD$
Pig	94.18±7.74[a]	31.04±1.84	2.24±0.44	0.23±0.03	0.005±0.003
	(n=30)[b]	(n=10)	(n=10)	(n=10)	(n=10)
% Efficacy		67	98	99	99
Polar bear	43.73±6.40	2.57±0.39	1.51±0.33	0.54±0.01	0.035±0.006
	(n=30)	(n=10)	(n=10)	(n=10)	(n=10)
% Efficacy		94	97	99	99
Raccoon	19.49±4.35	2.59±0.35	0.29±0.12	0.016±0.004	0
	(n=30)	(n=10)	(n=10)	(n=10)	(n=10)
% Efficacy		87	99	99	100
Wolverine	42.51±4.51	4.38±0.86	0.18±0.03	0.075±0.021	0.025±0.011
	(n=30)	(n=10)	(n=10)	(n=10)	(n=10)
% Efficacy		90	99	99	99

[a] RCI Day 40 postinfection

[b] Number of animals

Table IV. The effect of thiabendazole on the early muscle phase (20 to 40 days postinfection) of Trichinella.

Isolate	Untreated	Drug Dosages							
		0.03%	0.05%	0.075%	0.1%	0.15%	0.25%	0.35%	0.05%
	$\bar{x} \pm SD$	$\bar{x} \pm SD$	$\bar{x} \pm SD$	$\bar{x} \pm SD$	$\bar{x} \pm SD$	$\bar{x} \pm SD$	$\bar{x} \pm SD$	$\bar{x} \pm SD$	$\bar{x} \pm SD$
Pig	94.18±7.47[a]	90.32±4.78	59.44±4.72	54.62±3.90	62.20±3.44	61.12±7.66	54.48±10.23	26.25±11.33	24.30±5.17[c]
	(n=30)[b]	(n=10)	(n=10)	(n=10)	(n=10)	(n=10)	(n=10)	(n=10)	(n=10)
% Efficacy		4	37	42	34	35	42	72	74
Polar bear	43.73±6.40	43.96±3.43	43.20±6.19	30.38±2.29	29.58±5.49	33.92±8.13	18.48±2.60[c]	0.75±0.33	0.001±0.001
	(n=30)	(n=10)	(n=10)	(n=10)	(n=10)	(n=10)	(n=10)	(n=10)	(n=10)
% Efficacy		o	o	31	32	22	58	98	99
Raccoon	19.49±4.35	17.37±3.52	18.28±4.80	17.73±3.90	18.85±3.35	17.54±2.75[c]	3.31±1.21[c]	0.001±0.001	0
	(n=30)	(n=10)	(n=10)	(n=10)	(n=10)	(n=10)	(n=10)	(n=10)	(n=10)
% Efficacy		11	6	9	3	10	83	99	100
Wolverine	42.51±4.51	37.54±5.11	35.12±4.52	33.58±3.64	31.18±3.19	30.50±4.45	15.00±7.14	11.91±2.68[c]	0.07±0.05
	(n=30)	(n=10)	(n=10)	(n=10)	(n=10)	(n=10)	(n=10)	(n=10)	(n=10)
% Efficacy		12	17	21	27	28	65	72	99

[a] RCI Day 40 postinfection

[b] Number of animals

[c] Muscle larvae were not infective to mice

compared to 99% or higher for the other isolates (Table IV). Except for the pig isolate, all other isolates at dosages of 0.35 or 0.5% had few live muscle larvae. A fifty percent reduction of muscle larvae (effective dosage) occurred between dosages of 0.15 to 0.25% thiabendazole for the polar bear, raccoon and wolverine isolates but at higher drug levels (0.25 - 0.35%) for the pig isolate. When overall drug sensitivities were compared the raccoon, polar bear and wolverine isolates were significantly ($p < .05$) more sensitive to thiabendazole treatment than the pig isolate (Fig. 3). While the pig isolate was initially more sensitive to drug treatment at low dosages, it is the least sensitive of all the isolates at higher dosages (Fig. 3). Worms that survived sublethal dosages, and in one instance well below the effective dosage, 0.15% for raccoon, were not infective to mice.

Treatment with thiabendazole at dosages of 0.03 to 0.1% from 40 to 60 days postinfection had little or no effect on muscle larvae (Table V). Only the polar bear and wolverine isolates had a significant reduction of 26 and 37%, respectively, of muscle larvae at 0.1% thiabendazole. Overall drug sensitivity was not significantly different among the isolates (Table V).

Fig. 3. Regression between drug dosage and percent efficacy muscle larvae from mice treated with thiabendazole 20 to 40 days postinfection. Equation of regression lines and R^2 coefficients are: pig $y = 20.60 + 116.37 x$, $R^2 = 0.747$; polar bear $y = 0.35 + 224.63 x$, $R^2 = 0.896$; raccoon $y = -6.65 + 249.40 x$, $R^2 = 0.844$ and wolverine $y = 6.97 + 190.10 x$, $R^2 = 0.975$.

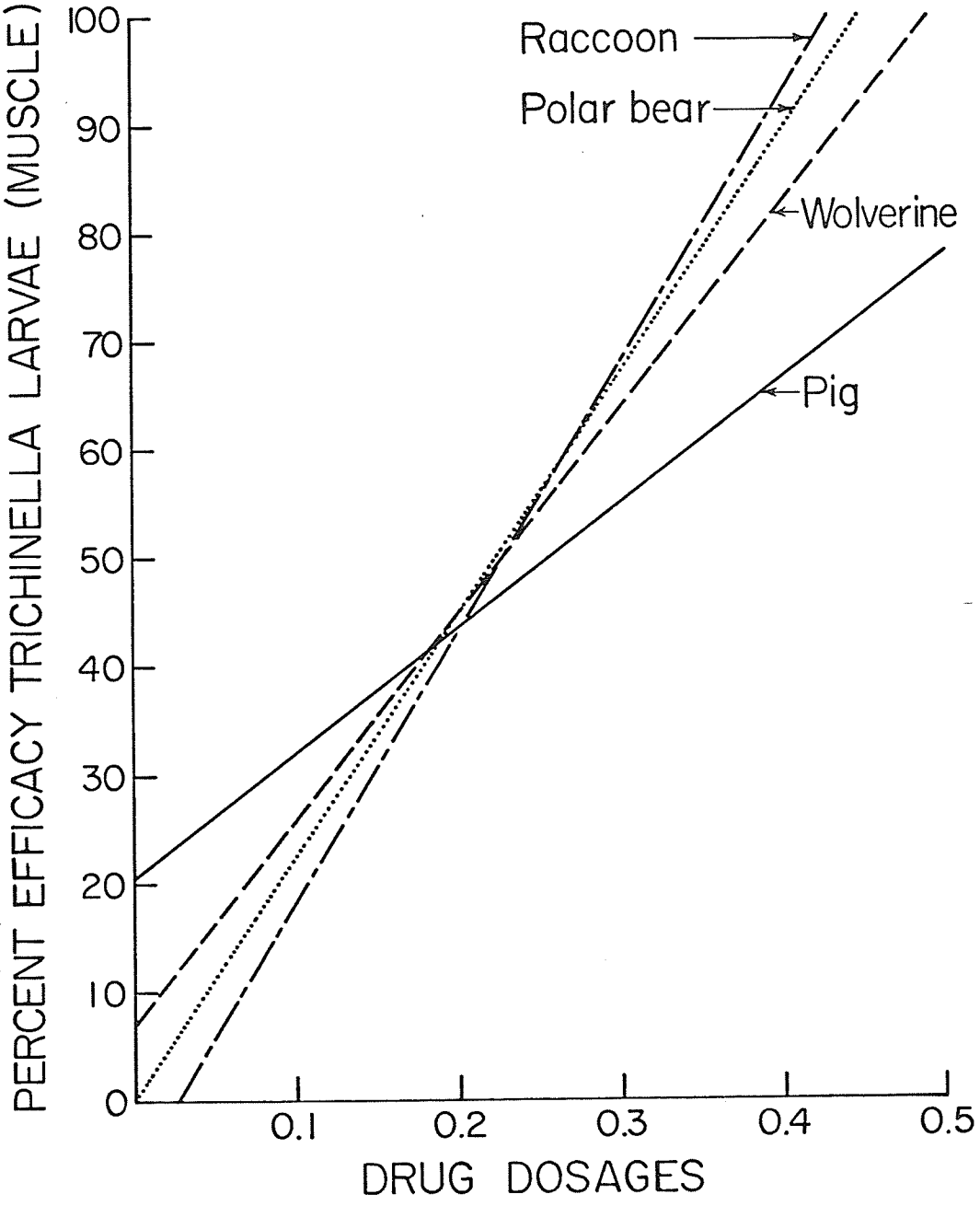

PERCENT EFFICACY TRICHINELLA LARVAE (MUSCLE)
100
90
80
70
60
50
40
30
20
10
0
0.1
0.2
0.3
0.4
0.5
DRUG DOSAGES
Raccoon
Polar bear
Wolverine
Pig

Table V. The effect of thiabendazole on the late muscle phase (Days 40 to 60 postinfection) of Trichinella.

Isolate	Untreated $\bar{x} \pm SD$	Drug Dosages 0.03% $\bar{x} \pm SD$	0.05% $\bar{x} \pm SD$	0.075% $\bar{x} \pm SD$	0.1% $\bar{x} \pm SD$
Pig	98.80±8.23[a]	98.80±9.33	86.38±6.44	89.48±10.35	92.52±8.69
	(n=10)[b]	(n=10)	(n=10)	(n=10)	(n=10)
% Efficacy		0	13	9	6
Polar bear	40.65±3.17	40.00±7.14	38.57±5.39	41.14±3.60	30.14±4.89
	(n=10)	(n=10)	(n=10)	(n=10)	(n=10)
% Efficacy		0	0	0	26
Raccoon	20.75±2.72	18.81±3.22	18.45±4.48	19.30±3.57	18.41±6.69
	(n=10)	(n=10)	(n=10)	(n=10)	(n=10)
% Efficacy		9	11	7	11
Wolverine	44.62±4.56	36.45±6.50	38.77±7.55	38.20±5.06	28.13±4.89
	(n=10)	(n=10)	(n=10)	(n=10)	(n=10)
% Efficacy		18	13	14	37

[a] RCI Day 60 postinfection

[b] Number of animals

DISCUSSION

Low dosages of thiabendazole during the intestinal phase of trichinosis decreased or inhibited larval production in mice. Changes in the susceptibility of Trichinella to thiabendazole during the first few days of infection has been reported (Campbell and Cuckler 1964a; Campbell and Hartman 1968; Spaldonova et al. 1978), mebandazole (Fernando and Denham 1976; McCracken 1978), parbendazole (Theordorides and Landerman 1969) and for cambendazole (Campbell and Yakstis 1970; Duckett and Denham 1970; Spaldonova and Corba 1977). In this study low sustained dosages two days after infection were effective in suppressing larval production. Differences in the susceptibility of these isolates to drug treatment is of considerable interest. For example, both the pig and raccoon isolate were completely sterile at 0.05% thiabendazole, however, at the lower dosage of 0.03% chemosterilization in the polar bear and wolverine isolates were noted. It is evident that the polar bear and wolverine isolates are more susceptible to drug treatment in the intestine. Thiabendazole at 0.05% is known to cause chemosterilization in T. spiralis (Campbell and Cuckler 1964a) and at 100 mg/kg by Timonov and Bryntseva (1978). Chemosterilization occurring at 0.03%, to our knowledge, represents the lowest dosage causing complete

sterility. Chemosterilization of female worms was not restricted to Day 7 but worms were also sterile from Day 4 to 6 for treatment initiated by Day 2 with thiabendazole dosages of 0.03 or 0.05%. Chemosterilization was not permanent and has also been shown to be reversible by Blair and Campbell (1971) and by Timonov and Bryntseva (1978).

The number of larvae released by females post-drug treatment for any isolate was found to be directly related to the length of the intestinal phase, that is, those isolates with a short intestinal phase (polar bear, raccoon and wolverine) had lower numbers of larvae released in vitro and therefore a lower RCI-value following treatment. In this study newborn larvae were recovered from in vitro experiments 2, 3 and 4 days post-treatment. Although Blair and Campbell (1971) did not find any newborn larvae 3 days post-drug treatment differences between this study and their results may be related to drug regime as they used 3 to 7 days versus 2 to 7 in this study. Nevertheless, results confirm those of Blair and Campbell (1971) in that larvae produced following drug treatment are capable of muscle invasion. Timonov and Bryntseva (1978) noted that larvae did not penetrate into muscular fibres and thus were incapable of muscle invasion following thiabendazole treatment. In this study a significantly greater number of larvae were produced

by females following drug treatment than untreated worms on the same day. This suggests that thiabendazole suppresses embryogenesis and differentiation, but when treatment was terminated larvae developed rapidly and are produced continuously during the intestinal phase. For all the isolates at least 90% of the female worm population produced larvae *in vitro* following drug treatment. The effect of thiabendazole on spermatogenesis or oogenesis at low sustained dosages during the first two days of infection is not known. Timonov and Bryntseva (1978) showed that thiabendazole treatment of 100 mg/kg 4 to 5 days postinfection caused considerable damage in spermatogenesis, oogenesis and embryogenesis, and a great number of eggs were observed in the 2 to 4 stage of blastomere. In this study female worms from both treated and untreated experiments were expelled from the intestine and the same rate and larvae produced at the onset of worm expulsion were successful in establishing themselves in the muscles. Thiabendazole does not influence the host immune response during rejection of adults in the primary gut phase of *Trichinella*. When percentage drug efficacy was compared *in vitro* (24-hr larval release) and *in vivo* (RCI-value), remarkably similar results were obtained by the biology of the *Trichinella* isolate must be known for this comparison. In addition, muscle larvae

following drug treatment were infective to mice when transferred and examined 40 days postinfection.

Treatment with low concentrations of thiabendazole during the dissemination phase, from 4 to 20 days, was highly effective in reducing the numbers of muscle larvae at 40 days postinfection for all Trichinella isolates. Although other workers chose different times of treatment (Campbell and Cuckler 1964a; Spaldonova et al. 1978) we chose 4 to 20 days for reasons outlined in materials and methods. Since 80% of disseminating larvae were produced between 6 and 8 days postinfection the high efficacy obtained in this study indicated the drug's lethal effect on these larvae and may explain differences between this study and Spaldonova et al. (1978). Spaldonova et al. (1978) obtained a 59.5% reduction in muscle larvae for T. pseudospiralis when mice were treated 8 to 11 days postinfection. It is possible that the drug had a direct effect on larvae produced from Day 8 to 11 or there may have been a decrease in drug sensitivity by the larvae produced on Day 5 to 7 postinfection. Similarity, Campbell and Cuckler (1964a) observed low efficacies for T. spiralis at a low dosage (0.025%) but obtained an 85 to 100% reduction in muscle larvae when mice were fed 0.05% thiabendazole 7 to 14 or 14 to 21 days postinfection. In this study, regardless of the drug dosage high efficacies were obtained for three isolates while larvae of the raccoon isolate were absent from muscles.

This high efficacy cannot be attributed to the drug's lethal action on disseminating larvae only but must also be due to chemosterilization of adult female worms in the intestine. Support for this comes from an experiment with a drug treatment of 0.05% thiabendazole where continued larval production by females *in vitro* was found starting with Day 4 or 5 and continuing until Day 9 or 10 postinfection. This continuous larval production by females was true for all isolates prior to chemosterilization at Day 9 or 10 postinfection. Also, 2 to 3 days after initiating drug treatment and then evaluating *in vitro* larval production by females 50% live and 50% motionless larvae were observed. This was found for all *Trichinella* isolates and suggested that unborn larvae may be affected by thiabendazole in the uterus of the female worms. The high efficacy obtained during the disseminating phase at low sustained dosages could be attributed to several factors which include the drug's lethal action on disseminating larvae, direct drug action on unborn larvae and chemosterilization of female worms.

Drug treatment of *Trichinella* during the early muscle phase (20 to 40 days postinfection) gave variable results between isolates. A decline in sensitivity to thiabendazole is evident as larvae invaded muscles and those isolates most susceptible to drug treatment were polar bear, wolverine, and raccoon. Similarly, Ozeretskovskaya *et al.* (1970) noted

that thiabendazole was less active on muscle stages of synanthropic (pig) strain of Trichinella than on Arctic strains and suggested this was related to difficulty of the drug penetrating the fibrous capsule. Work by Campbell and Cuckler (1964a) found that a 2 week treatment with 0.5% thiabendazole and examination 3 weeks postinfection produced a 99% reduction of muscle larvae. In this study, a dosage of 0.5% produced an efficacy of 74% for the pig isolate but the same dosage was 99 to 100% effective against polar bear, wolverine or raccoon isolates. Differences in the sensitivity to thiabendazole treatment during the early muscle phase clearly exists among the Trichinella isolates. Muscle larvae of the raccoon isolate presumably are in direct contact with thiabendazole since they lack a cyst, however, this isolate was the least susceptible at low dosages but was the most sensitive at higher dosages. The fact that Spaldonova et al. (1978) found no differences in susceptibility between T. spiralis and T. pseudospiralis at 100 mg/kg may be related to the duration of their drug treatment which was 28 to 31 days postinfection. Treatment with 0.1% thiabendazole in this study from 20 to 40 days postinfection was only 3% effective and differed from Spaldonova et al. (1978) who achieved 30% efficacy.

In all isolates examined there was a critical effective dosage which caused a threefold-or-more increase in drug

efficacy and once that dosage was achieved drug efficacy increased substantially with increasing dosage. For example, the raccoon isolate at a dosage of 0.15% was only 10% effective, whereas, a dosage of 0.25% was 83% effective, and at 0.5% 100% effective. Muscle larvae that survived sub-lethal dosages and in one instance well below the sub-lethal level, 10% efficacy at 0.15% thiabendazole for the raccoon isolate, were not infective to mice examined 40 days postinfection. Campbell and Cuckler (1964a,b) also showed that larvae recovered from thiabendazole-treated animals were non-infective to another host. Loss of infectivity after treatment has also been reported with metrifonate (Lamina 1970) and parbendazole (Finefield 1972). It is not known how long larvae could remain alive in the muscle or if the damage caused is irreversible following thiabendazole treatment but conceivably larvae are damaged sufficiently allowing cellular infiltration and eventual resorbtion of Trichinella from the host muscle. As muscle larvae grow older, and presumably with the progress of encapsulation, they are more resistant to thiabendazole treatment. When drug treatment began 40 days postinfection for 20 days at sustained low dosages thiabendazole was virtually ineffective in reducing numbers of muscle larvae. In fact only the Arctic isolates, polar bear and wolverine, responded at 0.1% and a significant reduction in muscle larvae was observed. Despite the decline in drug sensitivity during the late muscle phase differences in the susceptibility to thiabendazole treatment among the Trichinella isolates were detected.

Although the exact mechanism of thiabendazole action on T. spiralis is not known a number of factors acting independently or in concert may be responsible. During the enteral phase, developmental changes such as molting patterns, changes in the location of the worms relative to the mucosa, or basic biochemical differences in energy metabolism between larval and adult stages (Campbell 1967) and between strains of T. spiralis may account for changes in drug sensitivity. Also, muscle larva cyst morphology and biochemical and physiological requirements may differ among the isolates. Immature and adult worms meet their energy requirements by oxidative and fermentative pathways (Ferguson and Castro 1973), while in larvae endogenous carbohydrates are metabolized to phosphoenolpyruvate (Castro and Fairbairn 1969; Ward et al. 1969). As such, those anthelmintics that inhibit the fumarate reductase system, like thiabendazole and cambendazole, would be effective. This is known to occur with Haemonchus contortus (Malkin and Camacho 1972; Prichard 1973). Other proposed mechanisms have been reviewed by Campbell and Blair (1974). Whatever the mode of action, thiabendazole is highly effective against the Trichinella isolates during the intestinal, disseminating and early muscle phase at various treatments. In addition, differences in the susceptibility to thiabendazole

treatment is related not only to the strain of Trichinella but also to the stage in the parasite's life cycle. Distinct biological characteristics between these isolates clearly account for differences in the results obtained. Furthermore, the raccoon isolate which lacks a cyst stage in the muscle reacts similarly to the Arctic forms (polar bear and wolverine), indicating that thiabendazole activity may be related more to the metabolic activity of an isolate rather than to a physical barrier like the cyst wall.

GENERAL CONCLUSIONS

From this study the following conclusions are made:

1 (a) Trichinella spiralis larvae recovered from frozen muscles from wild carnivores were infective to experimental mice.

(b) A system to designate isolates is outlined and defined. A typical designation should include host species, latitude, longitude and year parasite recovered e.g. (wolverine, 55°00N, 100°00'W; 1979).

2 (a) Biological characteristics of two geographically closely situated Trichinella isolates (wolverine and polar bear) were compared and found to share some features (resistance to freezing, opaqueness of cuticle, and intestinal position). Other characteristics such as virulence, 24-hr in vitro larval release, RCI-values, rate of calcification and longevity in the small intestine were significantly different. These differences are probably part of the normal biological variability for T. spiralis.

(b) Comparison of biological characteristics for the wolverine isolate in different hosts species revealed differences in intestinal position, 24-hr in vitro larval release and RCI-values.

3 (a) Single-pair-breeding experiments between wolverine and raccoon or between polar bear and pig isolates showed reproductive isolation, but multiple-pair-breeding

trials showed reproductive compatibility except between pig and raccoon isolate combinations which did not breed.

(b) High breeding success in single and multiple-pair-breeding trials between wolverine and polar bear suggested a high degree of reproductive compatibility between these two isolates.

(c) Absence of newborn larvae *in vitro* for the raccoon isolate when crossed with either wolverine or polar bear males indicated a low level mating success, initially.

(d) Hybrids from all multiple-pair crosses had low RCI-values initially but increased with subsequent passages and differed from those of parental stock suggesting no sexing error was made.

(e) There was no hybrid breakdown and host influenced RCI-values of the hybrids.

(f) Mating success suggested isolates are semispecies or incipient species.

4 (a) Thiabendazole treatment at various dosages was effective in chemosterilizing adult females of pig, wolverine, polar bear and raccoon isolates in the intestine; highly effective against disseminating larvae and effective in reducing numbers of muscle larvae during the early muscle phase of infection.

(b) Trichinella isolates sensitivity to thiabendazole treatment varied with the most susceptible in the intestinal phase being wolverine and polar bear isolates. Disseminating larvae were highly susceptible to drug regardless of Trichinella isolate or drug dosages. Treatment during the early muscle phase at high dosages was effective against polar bear, wolverine and raccoon isolates but less so against the pig isolate. Low dosages were ineffective in reducing numbers of muscle larvae during the late muscle phase for all Trichinella isolates.

(c) Susceptibility to thiabendazole treatment is related not only to the strain of Trichinella but also to the stage in the parasite's life cycle.

(d) Similarities in susceptibility to thiabendazole treatment for wolverine, polar bear and raccoon isolates suggested that thiabendazole activity is related to the metabolic acitivity of an isolate rather than to a physical barrier such as a cyst wall.

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(Ru) in Russian

APPENDIX I

Detailed taxonomic history of *Trichinella*[1]

Phylum	Nematoda	
Class	Adenophora (Aphasmidea)	
Order	Trichurata	
Family	Trichinellidae	Ward, 1907
Syn.	Trichinidae	Cobbold, 1879
Genus	*Trichinella*	Railliet, 1895
Syn.	*Trichina*	Owen, 1835
Type of genus:	*T. spiralis*	(Owen, 1835)
Syns.	*T. canis*	Kraemer, 1853
	T. circumflexa	Polonio, 1860
	T. pseudalius	Dengler, 1863
	Pseudalius trichina	Davaine, 1863
Other species:	*T. nativa*	Britov and Boev, 1972
	T. nelsoni	Britov and Boev, 1972
	T. pseudospiralis	Garkavi, 1972

[1] YAMAGUTI, S. 1961. Systema Helminthum. *The nematodes of vertebrates*. Volume III. Part 1. Interscience Publishers INC., New York. Interscience Publishers Ltd., London.

APPENDIX II

Definition of terms as used in this thesis

The words 'variety' and 'strain' are used throughout and their meaning is that of a geographical race. Britov 1969, 1971, Britov et al. 1971 used the term 'variety' to denote geographical 'races' of Trichinella. Similarly, the term 'isolate' has been used by Read and Schiller 1969 and Arakawa and Todd 1971, to denote geographical 'races' of the parasite. Consequently, I felt justified to use these terms throughout the thesis and their meaning is that of a 'geographical isolate'.

Definition of terms as used in context of this thesis are:

1. Geographical isolate. A population or group of populations prevented by an extrinsic barrier from free gene exchange with other populations of the species (Mayr 1975).
2. Synonymy. A chronological list of the scientific names which have been applied to a given taxon, including the dates of publication and the authors of the names (Mayr 1969).
3. Biological species concept. A concept of the species category stressing reproductive isolation, and the possession of a genetic program effecting such isolation (Mayr 1969).
4. Deme. A local population of a species; the community of potentially interbreeding individuals at a given locality (Mayr 1969).

5. Species. Groups of interbreeding natural populations that are reproductively isolated from other such groups (Mayr 1940).
6. Subspecies. A geographically defined aggregate of local populations which differs taxonomically from other subdivisions of the species (Mayr 1969).
7. Semispecies = incipient species. Populations that have acquired some, but not yet all, attributes of species rank; borderline cases between species and subspecies (Mayr 1969).
8. Sibling species. Pairs or groups of closely related species which are reproductively isolated but morphologically identical or nearly so (Mayr 1969).

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