AN IMPROVED TECHNIQUE FOR EXPERIMENTAL INFECTIONS WITH SKIN PENETRATING NEMATODE LARVAE (*NECATOR AMERICANUS*)

JERZY M. BEHNKE, CATHERINE WELLS and JILL BROWN

MRC Experimental Parasitology Research Group, Zoology Department, University of Nottingham, University Park,
Nottingham NG7 2RD, U.K.

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Abstract—BEHNKE J. M., WELLS C. and BROWN J. 1986. An improved technique for experimental infections with skin penetrating nematode larvae (*Necator americanus*). *International Journal for Parasitology* 16: 461-464. Conventional techniques for infecting experimental animals with skin penetrating nematode larvae create inconsistencies and variation in parasite establishment. A method is described which has been used to improve considerably the degree of uniformity of infection of laboratory rodents with the larvae of *N. americanus*. Infective larvae were placed onto the gauze of a surgical plaster and attached to the shaved backs of adult hamsters and mice. These were secured with a further layer of tape encircling the animal and removed 24 h later.

INDEX KEY WORDS: Nematoda; *Necator americanus*; skin penetration; mouse; hamster.

INTRODUCTION

EXPERIMENTAL infections of laboratory animals by skin penetrating nematode larvae (e.g. *Nippostrongylus brasiliensis*, *Strongyloides ratti* and *Necator americanus*) often result in highly variable and unpredictable establishment of the parasite concerned. Experiments utilizing such species necessitate large experimental group sizes in order to permit evaluation of the statistical significance. In some parasites the percutaneous route can be bypassed (e.g. *N. brasiliensis*) and the larvae can be injected subcutaneously (still ensheathed) without loss of infectivity. In other species (e.g. *N. americanus*) subcutaneously injected larvae do not establish as well as when presented via the skin surface. The larvae of *N. americanus* are believed to exsheath on the skin surface prior to penetration (Augustine, 1923; Goodey, 1925) and are known to stay at the skin site for at least 48 h following entry through the epidermis (Behnke, Paul & Rajasekariah, 1986). It is possible that exsheathment prior to penetration and/or passage through the skin represent components of the infection process which are obligatory to the successful continuation of development (Matthews, 1977).

The variation in establishment of *N. americanus* in laboratory rodents, using conventional percutaneous infection techniques, presented a continuing hindrance to our investigations of the host/parasite relationship. Experiments were therefore carried out to evaluate alternative means of infection and the results are presented in this paper. Furthermore, we describe here an improved technique which in our

laboratory has eliminated many of the original problems and has resulted in significantly higher and more uniform establishment of *N. americanus* in our experimental animals.

MATERIALS AND METHODS

Animals. Male, 5-10-week-old C57BL₁₀ and BALB/c mice were used in this study and were treated with piperazine citrate (100 mg kg⁻¹ body wt) 14 and 7 days prior to infection. Syngeneic DSN hamsters were purchased from Intersimian Ltd, and were bred and maintained under conventional animal house conditions in this department. All animals were given food and water *ad libitum*.

The parasite. Infective larvae of *N. americanus* were obtained in 1983 from Dr. Rajasekariah of CIBA-GEIGY Hindustan Ltd., Bombay, India. Passage in neonatally infected hamsters and maintenance techniques have been described (Sen & Seth, 1970; Sen, 1972; Behnke *et al.*, 1986).

Conventional techniques for infecting laboratory rodents. Female hamsters were mated concurrently to provide litters aged 2-4 days at infection. Neonates were removed and strapped to the surface of a warm bench top incubator using strips of adhesive tape. The required dose of larvae in 10-25 µl distilled water was placed onto the abdominal surface. When the water had evaporated the infection site was kept moist by covering with gauze onto which drops of water were placed at regular intervals. After 90 min exposure the neonates were replaced with their mothers. Mother aggression on return of the neonates resulted in considerable loss of animals. This was partially reduced by providing valium (0.2 mg l⁻¹) in the mothers' drinking water from 24 h prior to until 48 h post infection. Adult hamsters and mice were anaesthetised with Sagatal (M & B Veterinary Products), shaved on the abdomen and immobilized on a bench surface

with adhesive tape for 90 min and infected as above. This prolonged restraint combined with the necessity to re-anaesthetise animals inevitably resulted in losses, particularly as some strains (e.g. C57BL₁₀) are more susceptible to anaesthesia than others. This method is both labour intensive and time consuming.

Modified technique. The adult animals were immobilised using Sagatal and a band of skin immediately behind the forelimbs was shaved encircling the animal. The required dose of infective larvae was applied to the gauze of a plaster (Johnson & Johnson Ltd.) of the sort used for minor cuts and abrasions. The plaster was placed onto the shaved belt such that the gauze made contact with the back of the animal and the adhesive ends joined across the abdomen. A further layer of surgical tape, passing over the plaster, was used to secure it in place for 24 h after which time it was removed. Although the animals were somewhat encumbered by the plasters, they appeared to feed and drink normally after regaining consciousness. Not entailing additional doses of anaesthetic nor prolonged restraint this technique is considered to be far less stressful than that employed previously. The method was adapted for use with neonatal hamsters. Fine plasters were cut and applied to the neonates as described above, but without the necessity of anaesthesia or shaving. Complete litters could be infected in less than 10 min, and produced no signs of stress or aggression by the mothers on return of their litters. The plasters were again removed 24 h after infection.

Recovery and enumeration of larvae in lungs. The methods used to recover lung stage *N. americanus* have been described previously (Behnke *et al.*, 1986).

Statistical analysis. Where appropriate the mean worm burden (MWB) $\pm$ standard error of mean (s.e.m.) have been analysed by the non-parametric Mann-Whitney *U* test (Sokal & Rohlf, 1969). A value of $P \leq 0.05$ was considered significant.

RESULTS

Infection of mice and neonatal hamsters by the conventional technique

Table 1 presents examples of the variation in the number of parasites recovered from individual animals given a uniform dose of infective larvae by the conventional techniques. All the animals used were syngeneic and were treated identically. Despite this, the results show that a considerable degree of variation was encountered with recoveries ranging from 0 to 60% of the infection dose. Such variation would preclude the use of this infection technique in experiments seeking to identify subtle differences in worm survival.

In some experiments the gauzes were examined after the exposure period, for the presence of larvae which had failed to penetrate. Most gauzes contained at least a few (<10) active larvae although occasionally considerably more larvae were observed (>50% of infection dose). However, we were unable to establish a negative correlation between the number of larvae remaining on the gauzes and lung worm recoveries. Additional factors must therefore contribute to variation in worm burden.

Comparison of different routes of infection

Although it was unlikely that neonatal hamsters might be infected by means other than the percutaneous route, the possibility that improved establishment might be achieved in mice by employing alternative routes of infection was investigated. The results are summarized in Table 2.

Despite the variation in worm recovery, it is clear from both experiments that mice infected percutaneously harboured significantly more worms than animals infected by the other routes. It would appear therefore that the percutaneous route of infection for the larvae of *N. americanus* cannot be bypassed without considerable loss of infectivity.

Evaluation of the modified technique for percutaneous infection with N. americanus

Experiments were carried out to determine whether the method for percutaneous infection could be improved. The modified technique finally adopted was described earlier, but is here evaluated alongside the conventional methods used in previous studies. The results are summarised in Table 3.

There can be little doubt that the modified method, employing plasters, was found to be far more efficient than the conventional technique. Not only did significantly more larvae establish in mice and adult hamsters but in these groups and in neonatal hamsters establishment was considerably more uniform.

DISCUSSION

Conventional methods for infecting laboratory animals with skin penetrating larvae, in addition to creating variable and inconsistent worm burdens, are time consuming and costly because the animals have to be immobilised under anaesthetic for prolonged

TABLE 1—EXAMPLES OF VARIATION IN WORM BURDEN FOLLOWING CONVENTIONAL PERCUTANEOUS EXPOSURE OF MICE AND NEONATAL HAMSTERS TO INFECTION WITH *N. americanus*

Expt.	Species	No. of larvae given	Individual worm recovery*	MWR $\pm$ s.e.m.
1	C57BL ₁₀ (♂) mice	300	16, 23, 26, 32, 47, 112, 199	65.0 $\pm$ 25.5
2	C57BL ₁₀ (♂) mice	300	0, 5, 6, 13, 35, 50,	18.2 $\pm$ 7.4
3	BALB/C (♂) mice	400	3, 43, 58, 82, 94, 113	65.5 $\pm$ 16.1
4	Hamsters (2-day-old, ♂ & ♀)	120	5, 6, 10, 10, 43, 51	20.8 $\pm$ 8.5
5	Hamsters (2-day-old, ♂ & ♀)	200	2, 4, 53, 119	44.5 $\pm$ 27.5

* All the animals were killed 6 days following infection and larvae were recovered from the lungs.

TABLE 2—COMPARISON OF THE ESTABLISHMENT OF *N. americanus* IN MICE INFECTED VIA DIFFERENT ROUTES. THE MICE IN BOTH EXPERIMENTS WERE MALE BALB/c AND WERE GIVEN 320 L3 (EXPT. 6) OR 250 L3 (EXPT. 7) ON DAY 0. THE ANIMALS WERE KILLED ON DAY 5 AND THE WORMS WERE RECOVERED FROM THE LUNGS

Route of infection*	Expt. 6		Expt. 7	
	No. mice	MWR $\pm$ S.E.M.	No. mice	MWR $\pm$ S.E.M.
Oral		N.d.	9	1.7 $\pm$ 0.4
I.P.	5	1.0 $\pm$ 0.5	10	5.0 $\pm$ 2.0
S.C.	5	17.0 $\pm$ 8.5	10	23.3 $\pm$ 3.5
P.C.	3	85.7 $\pm$ 17.5†	7	98.9 $\pm$ 24.5‡

* I.P. = Intraperitoneal, S.C. = subcutaneous, P.C. = percutaneous.

N.d. = Not done.

Statistical analysis:

† Significantly different from S.C. at $P \leq 0.025$.

‡ Significantly different from S.C. at $P \leq 0.005$.

TABLE 3—COMPARISON OF THE MODIFIED AND CONVENTIONAL TECHNIQUES FOR PERCUTANEOUS INFECTION WITH THE INFECTIVE LARVAE OF *N. americanus*. THE MICE WERE AUTOPSED 5 DAYS FOLLOWING INFECTION WHEREAS ALL HAMSTERS WERE KILLED ON DAY 6. WORMS WERE RECOVERED FROM THE LUNGS ONLY

Expt.	Animals (No. larvae administered)	Treatment	Individual worm recovery	MWR $\pm$ S.E.M.
8	BALB/c mice (250 L3)	C/PI†	17, 23, 65, 114, 135, 160, 178	98.9 $\pm$ 24.5*
		M/PI**	169, 176, 183, 197, 229	190.8 $\pm$ 10.6*
9	21-day hamsters (150 L3)	C/PI	2, 16, 17, 20, 21, 25, 28, 30, 31, 34, 42	25.2 $\pm$ 3.2†
		M/PI	55, 58, 94, 103, 124, 126, 150	101.4 $\pm$ 13.4†
10	28-day hamsters (150 L3)	C/PI	2, 2, 5, 5, 10, 10, 18	7.4 $\pm$ 2.2‡
		M/PI	14, 28, 37, 53, 58, 61	41.8 $\pm$ 7.6§
		M/PI (plasters removed after 90 min)	12, 13, 14, 15, 28, 31	18.8 $\pm$ 3.4§
11	2-day hamsters (170 L3)	C/PI	21, 23, 41, 50, 55, 63, 80, 96	53.6 $\pm$ 9.2†
		M/PI	47, 66, 70, 73, 75, 76, 85, 94	73.3 $\pm$ 4.9†

Statistical analysis of results. Groups with the same superscript were compared and found to have the following levels of significance: * $P \leq 0.01$; † $P \leq 0.001$; ‡ $P \leq 0.005$; § $P = 0.025$; † $P > 0.05$.

† C/PI = Conventional technique; ** M/PI = Modified technique.

periods of time. The results presented in this paper have clearly demonstrated that the modified technique utilizing plasters has a number of advantages over the conventional methods, summarized as follows: (1) An increase in the number of larvae established in exposed animals; (2) Greater consistency of worm burdens within identically treated groups; (3) A reduction in deaths under anaesthetic (less anaesthetic is required and animals are not restrained);

(4) Fewer animals are thus required per group; (5) Fewer larvae are needed because of improved establishment; (6) The method is less time consuming and is less labour intensive; (7) The animals are subjected to less stress and trauma during infection.

The most obvious variables between individual animals exposed to infection by the conventional techniques were the rate of drop evaporation, variation in the interval between infection and the application of a

moist gauze, the temperature of animals under anaesthesia and the varying response to anaesthetic. The modified technique standardised some of these variables. The infective larvae of *N. americanus* cannot penetrate when surrounded by a deep layer of liquid, but require a shallow film for penetration (Goodey, 1925). Application of the larvae in a drop of water to plaster gauzes would have resulted in uniform spreading in the gauze material and a shallow film containing the larvae would have been created over the skin surface when the plasters were attached to the animals. Presentation of larvae via gauzes on plasters therefore created uniform conditions from the outset and this would have contributed to improving the consistency of subsequent worm burdens.

We have evaluated the modified technique only in relation to the infection of laboratory rodents by *N. americanus*. It is, however, possible that the same method could be used with equivalent success for other skin penetrating nematodes. Finally, it is interesting to note that although to our knowledge the method described in this paper has not been used previously to infect laboratory rodents, Augustine (1923) applied the larvae of *N. americanus* on moist gauze bandages, fastened with adhesive tape to the forearms of human volunteers.

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