Techniques to kill infective larvae of human hookworm *Necator americanus* in the laboratory and a new Material Safety Data Sheet

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Abstract

With increasing experimental use of the human hookworm *Necator americanus* in therapeutic trials, an evidence based approach to laboratory safety is needed to produce an accurate Material Safety Data Sheet (MSDS). Five chemical disinfectants and hot water were evaluated for their killing effect on infective larvae (iL3) of *N. americanus*. The most effective chemical was 70% ethanol which achieved 100% kill in 10 min. Dettol® was also effective after 20 min. Formalin (10% concentration) had a slow killing effect while bleach (1% concentration) and glutaraldehyde (2% concentration) were ineffective. Infective larvae were killed by hot water at 80° C or higher. In a simulated bench top spill 70% ethanol at five times the spill volume was 100% effective after 20 min. Addition of hot water was effective when the water was close to boiling, but did not kill 100% of iL3 at 80° C.

Key words: Necator americanus, hookworm, MSDS, Material Safety Data Sheet, laboratory safety

Introduction

Humans are naturally infected with three species of hookworms, *Necator americanus*, *Ancylostoma duodenale* and *Ancylostoma ceylanicum* (Nontasut *et al* 1987). Infective larvae (iL3) of human hookworms are grown in the laboratory from eggs in human faeces for diagnosis and for research. A strain of *N. americanus* from humans has also been established in hamsters and is used in research (Sen and Seth 1967). In the classical Harada-Mori technique for diagnosis of hookworms, faeces are smeared on the top half of a paper strip with the bottom immersed in water in a sealed test tube (Harada and Mori 1955). The culture is performed at 25° C under aerobic conditions and after seven days the water is examined for iL3 which have migrated from the faecal mass into the water. These iL3 can

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also be examined in greater detail to determine the species of hookworm (Pawlowski *et al* 1991). For research iL3 can be produced using a classical Harada-Mori technique or modifications including culturing faeces with charcoal and amphotericin (Kumar and Pritchard 1992). The recent hypothesis that hookworm can be used to ameliorate the severity of asthma and Crohn's disease has resulted in *N. americanus* being produced in greater numbers in laboratories for experimental infections (Falcone and Pritchard 2005; Croese *et al* 2006a). Our comments from this point will refer to *N. americanus* only, but the iL3 of *A. duodenale* are very similar (Haas *et al* 2005).

The iL3, the only stage of *N. americanus* hazardous to laboratory workers, infects humans by penetrating intact skin or mucous membranes. Penetration may be rapid, within 5 min, but can be delayed for at least 20 min owing to the inability of the iL3 to actively attach to the skin surface (Haas *et al* 2005). Penetration seems to be a two step process initially with the anterior end of the iL3 gaining mechanical entry, possibly via a skin duct or under a skin scale, and penetration being completed enzymatically (Haas *et al* 2005). Infective larvae can only move in water or a

film of water and die rapidly when dehydrated. Penetration stops even during entry if the water film containing the posterior portion of the iL3 dries (Haas *et al* 2005). Infective larvae, however, also find penetration more difficult if they are in a water droplet compared to a surface film, probably because iL3 need a mechanical purchase to start the process of entry (Goodey 1925; Benhke *et al* 1986). In experimental infections subjects usually note a pin-point intense itch commencing within 5 min of application of iL3 on a gauze pad bandaged to the skin (Croese *et al* 2006a). Presumably, this itch is a reaction to enzymes secreted by the iL3 to break down extracellular matrix during the second phase of penetration (Brown *et al* 1999).

N. americanus is classed as a level PC2 or BS2 pathogen. However, as a pathogen in the laboratory, *N. americanus* is unusual as the infective stage will seek out a human host and, having found one, will penetrate intact skin. Most other PC2 pathogens, apart from iL3 of *Strongyloides* and cercariae of schistosomes, do not have active host seeking behaviour. Activation of resting iL3 is by vibration, touch, water currents, light and warmth (Haas *et al* 2005). This important characteristic of active host seeking should guide strategies used in the laboratory situation to reduce the risks of working with iL3. In particular, since researchers have to be aware of downstream risks to other laboratory users, we propose that no hookworm iL3 should leave the laboratory alive unless allowed to do so by the researcher or technician. This should also include iL3 in laboratory waste.

Spills of liquid containing iL3 are an infection risk in the laboratory if contact is made with unprotected skin either directly or through secondary contact with iL3 on laboratory benches or other items.

We decided it was timely to review the MSDS for *N. americanus* to ensure that laboratory scientists dealing with human hookworms had accurate information on the hazard of *N. americanus* and strategies to minimise this. We also wished to use techniques based on best evidence to manage spills of iL3 onto inanimate surfaces and skin.

We searched Medline, Current Contents, Google Scholar and finally Google using the search terms "Necator MSDS" and "Necator Material Safety Data Sheet". There were no hits on the academic search engines and only one primary source was found on Google. The only MSDS apparently available was at Health Canada at the Office of Laboratory Security - http://www.phac-aspc. gc.ca/msds-ftss/msds107e.html, and the same page appeared at several other sites. This website is now under review.

The MSDS for *N. americanus* suggested that spills be managed in the following manner: *"Allow aerosols to settle; wearing protective clothing, gently cover the spill with absorbent paper towel and apply 1% sodium hypochlorite, starting at the perimeter and working towards the centre; allow sufficient contact time (30 min) before clean up."* (Health Canada 2001). Since we were not confident that 1% bleach was the ideal killing agent for iL3, we decided to evaluate techniques that could be used to kill hookworm iL3 in the laboratory situation.

Materials and methods

Infective larvae grown from the faeces of an experimentally infected human volunteer (Croese *et al* 2006b) using a Harada-Mori technique were examined in deionised water under a dissecting microscope. Actively swimming larvae were collected using a pipette and placed in a small volume of water (0.1 mL) in a glass cavity block. Larvae were tested in groups from 10 to 30, until a total of at least 30 iL3 had been tested for each intervention. Two mL of the liquid to be tested was added to the cavity block by disposable plastic transfer pipette. The liquid current caused iL3 to commence moving. Activity of iL3 was assessed at 5, 10, 15 and 20 min after addition of the liquid using the dissecting microscope and observing iL3 for 2 min. Twenty minutes was chosen as an end point as the maximum time contaminated liquid should be left in the laboratory environment. If no movement of iL3 was seen, stimulation was given in the form of vibration of the liquid. Activity was classified as: i) swimming with a sinusoidal motion (normal), ii) occasional slight movement but no progression (inactive), and iii) no movement (dead). An iL3 was classed as "dead" if no movement was observed for 2 min with stimulation provided by mechanical vibration (tapping cavity block) sufficient to move the larvae. To evaluate the efficacy of the best techniques in a simulated spill situation, approximately 200 iL3 in 1 mL of water were placed on a piece of hard plastic sheet, five times the volume of test solution added and the solution was subsequently transferred to a glass Petri dish and examined under a dissecting microscope to detect viability.

Chemicals tested for a killing effect on iL3 were 70% ethanol, 10% buffered neutral formalin, 2% glutaraldehyde, 1% bleach and Dettol® (full strength and 0.5%) (Reckitt Benckiser Plc, Berkshire,

UK). Although glutaralydehyde and formalin should not be used in an open laboratory environment, they were chosen for possible use in closed containers such as culture vials. In addition the killing effect of hot water at 50°C, 60°C, 70°C and 80°C was tested. Control groups of iL3 were held in deionised water.

Results

Of the five chemicals tested, the most effective killing agent was 70% ethanol, followed by Dettol® (Table 1). Formalin (10%) killed iL3 slowly and bleach (1%) and glutaraldehyde (2%) were ineffective with iL3 remaining fully active in bleach. Although all iL3 in glutaraldehyde at 20 min were alive, 100% were not swimming normally. Heat was 100% effective only at 80°C (Table 1). At 70°C 97% of iL3 were killed immediately and the survivors failed to move normally indicating a high level of efficacy. Some iL3 exposed to heat <70°C and glutaraldehyde showed a "stunned" response initially and moved from the "no movement - dead" category to slight or normal movement categories with time. Glutaraldehyde, for example, had 5/34 larvae appearing dead at 5 min, but at 10 and 15 min this had reduced to 3 and then zero larvae appearing dead. At 15 min all 34 larvae were showing signs of toxicity, but were not completely immobile; hence the "dead" category reduced over time.

For the simulated spill we tested 70% ethanol and hot water at 80°C and 98°C (near boiling). At 20 min all iL3 were dead after treatment with five times the volume of 70% ethanol. For hot water, all iL3 were dead after addition of near boiling water (98°C), but for water at 80°C two iL3 of approximately 200 were still alive.

Discussion

The only strategy that killed iL3 immediately was the application of heat through water at 80°C (Table 1). Hot water could be used on heat resistant surfaces in the laboratory environment. However, in the simulated spill although water at 80°C killed most iL3, a small percentage survived indicating that the temperature of water needed to be above 80°C to be fully effective. Attaining a killing temperature is critical since if iL3 are not killed initially, there is no residual killing effect to increase the cumulative mortality as with chemicals. A practical recommendation is to use water that is close to boiling point. The usual care would also be required in using hot water at temperatures greater than scalding temperature.

Ethanol (70%) was the most effective chemical tested and achieved 100% kill of iL3 in the simulated spill when five times the volume of ethanol was added. Formalin (10%) had a slow killing effect which had not reached a maximum at 20 min. Although formalin should not be used in an open laboratory environment as it creates an additional hazard of inhalation of a dangerous vapour, it could be used to kill iL3 in closed containers such as Harada-Mori cultures.

Dettol® at the dilution (0.5%) recommended by the manufacturer quickly damaged larvae although death lagged behind the less severe effects. Death of iL3 did not occur more rapidly with full strength Dettol® (Table 1). Application of Dettol® or ethanol to areas of potential contact of iL3 onto skin could be a useful strategy to stop penetration. This should be done rapidly as penetration can take less than 5 min, particularly if iL3 are in a surface film and not in droplets of

Table 1

Cumulative mortality (no movement of iL3 of Necator americanus after 2 min of observation) over time of iL3 exposed to test liquids and hot water.

	Number iL3	5 min	10 min	15 min	20 min
Dettol® (full strength)	30	20.0%	96.7%	96.7%	100.0%
Dettol® 0.5%	30	50.0%	90.0%	100.0%	100.0%
Ethanol 70% (cavity block)	45	95.6%	100.0%	100.0%	100.0%
Bleach 1%	47	0.0%	0.0%	0.0%	2.1%
Formalin 10%	30	3.3%	6.7%	86.7%	90.0%
Glutaraldehyde 2%	34	14.7%	8.8%	0.0%	0.0%
Water 50°C	35	8.6%	2.9%	5.7%	5.7%
Water 60°C	30	60.0%	60.0%	23.3%	40.0%
Water 70°C	30	96.7%	100.0%	96.7%	100.0%
Water 80°C	30	100.0%	100.0%	100.0%	100.0%
Control	60	0.0%	0.0%	0.0%	0.0%

water. Obviously, if Dettol® or ethanol is not rapidly available, the site of contamination should be quickly flooded with water under pressure to wash the iL3 off the skin and then dried rapidly. If available, use of dry heat via a hair dryer would, in theory, increase the likelihood of killing larvae and preventing penetration. However, if a hair dryer is not immediately available in the laboratory, time should not be spent on obtaining one. Albendazole (400 mg orally) reduced the success of experimental infections of *N. americanus* in human volunteers and could be used if topical treatment fails to prevent penetration of iL3 (Cline *et al* 1984).

Glutaraldehyde (2%) and bleach (1%) had no killing effect on iL3. Hence, the recommendation to use bleach for spills of iL3 in the current MSDS for *N. americanus* is dangerous as it implies that the iL3 are killed by bleach when our results indicate they are unaffected.

Based on this study at least two protocols for handling spills of iL3 onto inanimate surfaces can be recommended: 1) flood with five times the volume of 70% ethanol, wait 20 min, wipe up liquid and dispose into a sealed container; 2) on heat resistant surfaces only: flood with five times the volume of water close to boiling, wait 5 min, wipe up liquid and dispose into a sealed container.

This study has confirmed the value of using heat and ethanol to kill iL3 on items used in research and hookworm culture. Ethanol can be added to sealed containers such as old Harada-Mori cultures. Ethanol or water close to boiling can be used to flood containers containing slides, disposable test tubes and pipettes used to manipulate iL3 prior to disposal.

Finally it should be emphasised that no live iL3 should accidentally leave a laboratory since iL3 can migrate (if a water film is available) and actively seek out human hosts unlike most other pathogens.

Conclusion

The MSDS (Health Canada 2001) fails to highlight that the only infective stage of *N. americanus*, and hence the only stage associated with any hazard, is the iL3. The recommendation to use bleach or glutaraldehyde to kill iL3 is also not supported by our studies. We present an MSDS for *N. americanus* iL3 that incorporates the new data and more clearly sets out the hazards and their management.

Material Safety Data Sheet for *Necator americanus*

Section 1 — Description of the Product

Live infective larvae (iL3) of *Necator americanus* (human hookworm, New World hookworm); class Nematoda, family Ancylostomatidae. Adults are parasitic nematodes living in the small intestine of humans.

Dimensions of infective larvae: Length 630-670 μm, width 20-30 μm at mid-body. Not visible to naked eye.

Section 2 - Information on the Parasite

Infective larvae are grown from eggs of *N. americanus* passed in faeces of a host and cultured in faecal culture in an aerobic environment at 23-28° C for 7-10 days. The infective larva is the third stage larvae, the previous two rhabditiform stages feeding on faecal bacteria. Infective larvae do not feed, have a limited life span (maximum 6 weeks) and die from dehydration if not kept in a moist environment. Infective larvae are produced from eggs passed by a human host or from a laboratory strain of *N. americanus* that is adapted to hamsters.

Section 3 — Hazards Identification

Primary Hazard: Infective larvae in water drops or films coming into contact with skin or mucous membranes.

Special Hazard: Infective larvae are motile and given a water film to move in they can actively seek human hosts, rather than passively infect like most other laboratory pathogens.

Parasite Behaviour: Infective larvae will actively seek to penetrate intact skin. Adults, eggs and rhabditiform larvae of *N. americanus* are not infectious. Only third stage larvae (iL3) are infectious. Infective larvae can penetrate intact skin and mucous membranes and can swim in water including a water film. They will actively seek out a mammalian host, moving up a heat gradient. Once iL3 make contact with the skin, they invade using mechanical and enzymatic mechanisms, penetrating initially

under skin scales and down hair follicles. When iL3 penetrate, a pin-point itching sensation is felt. The penetration site is marked by a small red spot within several hours that progresses to a red papule and fades within a week. Larvae reach the small intestine via blood-lung migration which involves entry into dermal capillaries, travel through the right side of the heart to the pulmonary capillaries, escape into the alveoli, passage up the airways, travel down the oesophagus, and through the stomach to establish in the small intestine where maturity occurs.

Infectious dose: One iL3 is capable of penetrating intact skin and establishing in the small intestine, but at least one male and one female are required for mating and to produce eggs in faeces.

Transmission: Transmission is by penetration of iL3, typically through intact skin, but also through mucous membranes. Penetration is rapid; the human host notes an itch at the penetration site usually within 5 min.

Monitoring: Hookworms suck blood and initiate an allergic response in the small intestine which commences 3 wk after penetration. Clinical signs occur at the penetration site (red itchy papules) and during the intestinal establishment phase at 3-8 wk (abdominal pain and episodic diarrhoea). Since each papule marks the site of entry of an individual iL3, the number of iL3 penetrating the skin can be enumerated. Signs of pulmonary passage are typically minor, at most a slight cough for several days. Establishment in the small intestine is typically asymptomatic until 3 wk at which time abdominal pain with intermittent diarrhoea may commence and last for 4 wk. Heavy infections may cause anaemia. Humans with no prior anaemia and with adequate iron intake can tolerate populations of at least 30 worms with no development of anaemia. If adult worms of both sexes are present, eggs may appear in faeces at 5 wk or more after penetration. If only one sex is present, eggs will not be detected in faeces. The number of worms in the small intestine and their location can be monitored by capsule endoscopy and other endoscopic techniques.

Person-to-Person Communicability: Nil.

Laboratory-Acquired Infections: None reported.

Section 4 - First-aid Measures and Medical Management

First-Aid: Wash site of contact immediately with 70% ethanol or Dettol®. If these chemicals are not quickly available, wash the site immediately with running water and dry the skin to remove all water film. If immediately available in the laboratory, use a hair dryer to dry the site using a hot air stream.

However, if a hair dryer is not immediately available, time should not be wasted on obtaining one and the site can be dried by paper towels.

Medical Management: Adult and juvenile hookworms in the small intestine can be treated with oral pyrantel, mebendazole, albendazole, levamisole, or nitazoxanide. Albendazole 400 mg orally can kill migrating juveniles of *N. americanus* and could be used in laboratory accidents to prevent establishment in small intestine. Seek medical advice prior to treatment.

Immunisation: None commercially available.

Section 5 - Accidental Release Measures

Chemicals to Kill iL3: Infective larvae can be killed within 10 min by 70% ethanol and within 15 min by 0.5% Dettol®. Bleach is ineffective.

Physical Inactivation: Infective larvae can be killed immediately by addition of hot water at near boiling point. Infective larvae also die when the water film they are in completely evaporates.

Spills: Avoid any contact between liquid containing iL3 and skin or clothing. Wear gloves; carefully apply five times the volume of 70% ethanol. Allow sufficient contact time (15 min) before wiping up liquid to leave dry surface. On heat resistant surfaces, another option is to flood with water at near boiling point, leave 5 min before wiping up liquid to leave dry surface. Dispose into a sealed container.

Section 6 – Handling and Storage

Sources in Laboratory: High risk specimens are faecal cultures aged 5 days or more which contain iL3. This would rarely occur in routine aerobic faecal bacterial plates since they are usually disposed of prior to 7 days. High risk items are faecal cultures of 5 days or more, particularly agar plate and Harada-Mori cultures. Human faeces inside containers may also become infectious if left for 5 days or more.

Other Precautions: Cultures of iL3 should be in a sealed container whenever they are not being accessed by scientific staff and should be labelled as infectious.

Other Storage Requirements: Store at 25° C; storage in dark will prolong the life of iL3.

Section 7 –Personal Protection

Containment Requirements: Physical containment level 2 (PC2) or biosafety level 2 (BS2)

practices and containment equipment are needed for all activities involving iL3.

Protective Clothing: Laboratory coat with long sleeves and gloves should be worm at all times when working with live iL3.

Section 8 - Physical and Chemical Properties

Physical Properties: Not visible to naked eye. Infective larvae can only survive in a water film and will die rapidly when outside a water film. Killed within 5 min by heat >70° C.

Section 9 - Ecological Information

Infective larvae naturally survive in the external environment, living in water films for up to 6 wk and seeking a human host. No live iL3 should be unintentionally released from the laboratory.

Section 10 - Disposal Considerations

All iL3 should be killed by 70% ethanol prior to routine disposal. Ethanol 70% should be added to all fresh faeces containing *N. americanus* eggs prior to routine disposal. No live iL3 should be unintentionally allowed to leave the laboratory.

Section 11 - Transport Information

Infective larvae can be shipped in water in a sealed container with air.

Section 13 – Other Information

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References

Behnke JM, Wells C, Brown J. 1986. An improved technique for experimental infections with skin penetrating nematode larvae (*Necator americanus*). *Int J Parasitol* 16: 461-464.

Brown A, Girod N, Billett EE, Pritchard DI. 1999. *Necator americanus* (human hookworm) aspartyl proteinases and digestion of skin macromolecules during skin penetration. *Am J Trop Med Hyg* 60: 840-847.

Cline BL, Little MD, Bartholomew RK, Halsey NA. 1984. Larvicidal activity of albendazole against *Necator americanus* in human volunteers. *Am J Trop Med Hyg* 33: 387-394.

Croese J, O'Neil J, Masson J, Cooke S, Melrose W, Pritchard D, Speare R. 2006. A proof of concept study establishing *Necator americanus* in Crohn's patients and reservoir donors. *Gut* 55: 136-137.

Croese J, Wood MJ, Melrose W, Speare R. 2006. Allergy controls the population density of *Necator americanus* in the small intestine. *Gastroenterology* 131: 402-409.

Goodey T. 1925. Observations on certain conditions requisite for skin penetration by the infective larvae of *Strongyloides* and ankylostomes. *J Helminthol* 3: 51-62.

Haas W, Haberl B, Idris SI, Kallert D, Kersten S, Stiegeler P. 2005. Behavioural strategies used by the hookworms *Necator americanus* and *Ancylostoma duodenale* to find, recognise and invade the human host. *Parasitol Res* 95: 30-39.

Harada Y, Mori O. 1955. A new method of culturing hookworms. *Yonago Acta Med* 1: 177-179.

Health Canada. 2001. Material Safety Data Sheet – Infectious Substances. *Necator americanus*. Online: http://www.phac-aspc.gc.ca/msds-ftss/msds107e.html. Accessed 11 April 2007.

Falcone FH, Pritchard DI. 2005. Parasite role reversal: worms on trial. *Trends Parasitol* 21: 157-160.

Kumar S, Pritchard DI. 1992. Secretion of metalloproteases by living infective larvae of *Necator americanus*. *J Parasitol* 78: 917-919.

Nontasut P, Singhasivanon V, Maipanich W, Yamput S, Visiassuk K. 1987. Comparative study of different doses of mebendazole in hookworm infection. *Southeast Asian J Trop Med Public Health* 18: 211-214.

Pawlowski ZS, Schad GA, Stott GJ. 1991. *Hookworm infection and anemia. Approaches to prevention and control*. Geneva: World Health Organization.

Sen HG, Seth D. 1970. Development of *Necator americanus* in golden hamsters *Mesocricetus auratus*. *Indian J Med Res* 58: 1356-1360.