CHEMOTACTIC ATTRACTION OF *NECATOR* HOOKWORM FILARIFORM LARVAE TO SODIUM CHLORIDE

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Abstract. An investigation was carried out to elucidate the chemotactic attractive behavior of *Necator* hookworm filariform larvae to inorganic substances *in vitro*. First, an optimal concentration of these larvae against sodium chloride solutions using the agarose plate assay method was determined. The sodium chloride concentration varied from 0.1 to 1.0 molar solutions. Distilled water (DW) was used as control in all experiments. 0.5 molar was found to be an appropriate concentration to examine larval attraction. Then, chloride compounds such as NaCl, KCl, $CaCl_2$ and $MgCl_2$ were tried at 0.5 molar concentration; many larvae were attracted to NaCl and some also to KCl. Therefore, the same experiment was conducted using 0.1 molar chemical concentrations. Many larvae were attracted to NaCl; however, some larvae again moved to KCl. Next, the concentration was changed to a higher range, 1.0 molar, and as a result, NaCl only attracted the larvae. The larvae were not attracted to 1.0 molar of KCl, $CaCl_2$, and $MgCl_2$. Since the chloride anion was found not to attract larvae of this species, another experiment was conducted with 0.5 molar of the sodium compounds, Na_2CO_3, NaOH, $NaHCO_3$, NaCl, and DW. Na_2CO_3 had the strongest larval attracting ability. Other sodium compounds also attracted moderate numbers of larvae. In the inorganic substances tried, the sodium cation was found to attract *Necator* larvae, and thus the sodium cation might have an important role for finding and infecting hosts of *Necator* hookworm filariform larvae.

INTRODUCTION

The *Necator* hookworm is still prevalent in whole districts of Thailand. Eggs in stool hatch in around 7 days, and sheathed filariform larvae begin to seek their definitive host. The *Necator* species infect humans, mostly through the skin, rather than by ingesting the larvae. For the skin-penetrating parasites, host-finding behavior is very important to maintain their own generation. Before the larvae infect humans, they must search the humans' skin. Human skin must have something that cues larval attraction (Lewis *et al*, 1992). The larvae have sensory organs in their head portions, so called amphids, which are widely accepted as the main chemoreceptors in nematodes (Khlibsuwan *et al*, 1992). In the present study, an experiment was performed to determine whether or not the *Necator* hookworm has a sensitizer to inorganic substances, especially to sodium chloride.

In previous studies of hookworms, Wauters *et al* (1982), Zietse *et al* (1981), and Vetter *et al* (1985) examined the chemotactic attraction of dog serum of *Ancylostoma caninum* infective larvae. *A. caninum* larvae were attracted to canine serum. Apart from Wauters *et al* (1982), they did not use dialyzed serum. Inorganic substances were not excluded from the sera used in these experiments. Eventually, low molecular weight substances in serum attracted *A.caninum* larvae. They suggested that chemotaxis might play a very important role in enabling the larvae to find their way through the skin of the host. They used canine hookworms and canine sera for their experiments. Here, we report an attempt to characterize the chemotactic factors of inorganic substances to human-infecting *Necator* hookworm third-stage larvae. In this report, we mainly used sodium chloride, because NaCl is the most common material in human body fluids and may play a very important role for parasites finding their hosts.

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MATERIALS AND METHODS

The third-stage larvae (L_3) of *Necator americanus* used in this experiment were originally obtained from patient feces in Thailand. The seven-day copro-cultured L_3 of *N. americanus* were harvested by filter-paper culture method. The larvae were washed three times with distilled water, and then filtered through a nylon mesh 30 μ pore size (Nytrel-TI, UGB, France) to remove copro-debris, while small particle debris was removed by sucking up the supernatant after all the larvae sank to the bottom of the beaker (10 ml). In the beaker, a larvae-rich solution remained, from which only the larvae were sucked up using a small-tipped pipette. They were then transferred to a small Petri dish (2 ml). Finally, the larval number

was adjusted to 100 L_3 /5 µl. Five ml of melted 0.9% agarose (GP-42, Nakarai Tesque, Kyoto, Japan) in distilled water was poured into a polystyrene Petri dish (Nissui-P dish Co, 82mm X 15mm at bottom). After cooling, at a position 2.0 cm apart from the center dot, 4-5 dots were radially allocated at an equal distance from each other. Next, all points were marked at the outside of the bottom of each dish and each peripheral point was numbered. The center dot was prepared to place any infective larvae, while the peripheral dots were used to place a 0.5 cm round piece of filter paper (Advantec No.3. Toyo Roshi quantitative low ash). Inorganic compounds, *ie* NaCl, KCl, $MgCl_2$, $CaCl_2$, NaOH, $NaHCO_3$, and Na_2CO3 were used as attractants. All reagents were of analytical quality. Compound-impregnated pieces of filter paper (5mm in diameter) were placed on an agarose plate at the marked sites. After placing the papers, a round margin was marked along each circle at the outside of the bottom. The papers were then removed by forceps, after 45 minutes' application. As a control, distilled water (DW) was always used. Then, a 5.0 µl larval suspension containing approximately 100 viable larvae was placed at the center of the agarose plate. The water droplets were then absorbed by pilling about 2 mg of agarose powder. Next, the assay plates were covered with a lid and maintained at room temperature in darkness for 45 minutes. The number of larvae that had accumulated in the circular area (0.5 cm) was counted using a dissecting microscope (Nikon, Japan) under a dark field illuminator. Ten agarose plates with the same experimental design were simultaneously run to assay larval movement. The results were expressed as the average number of larvae ± standard deviation. Each larval number represents the mean of ten experiments. The Student's *t*-test was used in statistical analysis.

RESULTS

First, we examined whether sodium chloride had larval-attracting ability. Since human serum contains about 0.85% NaCl, which is the most of the inorganic substances in normal serum, 0.15 molar NaCl, which is a similar concentration to physiological saline solution, was examined for larval attraction on agarose plates. *Necator* hookworm larvae were found to be fond of NaCl solution. Then, we sought to determine an optimal concentration of NaCl for further studies. In the beginning, a low range of sodium chloride was used for the tests, the concentrations of which were 0.1, 0.2, and 0.5 molars. DW-dipped paper was placed adjacent to attractant ones in every experiment. As a result, 0.5 molar NaCl gathered the most larvae and the others attracted larvae depending on their concentrations (Fig 1), but there were no significant differences among them. However, there were significant differences when each was compared with the control ($p<0.01$). Next, the higher range doses of 0.5, 0.75, and 1.0 molars, were tested by the same method. The 0.5 molar concentration again attracted the most larvae. There were no significant differences in larval numbers among the three dosages (Fig 2). On the other hand, there were significant differences between those attractants and DW ($p<0.01$). As a result, we decided that the optimal concentration of sodium chloride was 0.5 molar. Further studies were carried out with this concentration.

Next, various chloride compounds were tried, NaCl, KCl, $CaCl_2$ and $MgCl_2$, for which the concentration was previously adjusted to 0.5 molar. The sodium chloride attracted the larvae most, however, potassium chloride also gathered a moderate

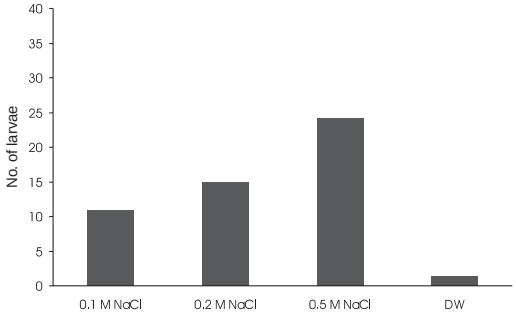

Fig 1- Chemotactic attraction of *Necator* hookworm filariform larvae to lower concentration of sodium chloride; 0.5 molar attracted the most in number of larvae. Then the larval number decreased according to the concentration. The control (DW) did not attract the larvae.

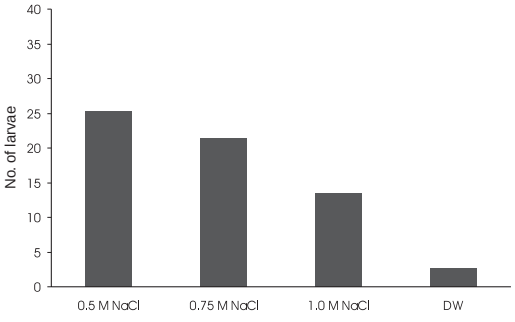

Fig 2- Chemotactic attraction of *Necator* larvae to sodium chloride, The concentrations were higher than those of Fig 1. 0.5 molar of NaCl attracted more number of larvae than 0.75 and 1.0 molars.

number of larvae(Fig 3). There was a significant difference between NaCl and KCl . It was unclear whether KCl had attracting ability or not. Therefore, we tried testing using the same chemicals in the same way, at the lower concentration of 0.1 molar (Fig 4). 0.1 molar NaCl attracted the most larvae, but KCl again gathered some larvae. There was a significant difference between NaCl and KCl ($p<0.01$). Next, we changed to much higher doses of the same chemicals, *eg* 1.0 molar. This time, only the NaCl solution could attract *Necator* larvae (Fig 5). The larvae seemed to hate the higher concentration of KCl. Since the Cl anion was found not to attract *Necator* larvae, other sodium compounds, equally adjusted to 0.5 molar, Na_2CO_3, NaOH, $NaHCO_3$, and NaCl, were examined. Na_2CO_3 showed the strongest attraction, followed by NaOH. There was a significant difference between Na_2CO_3 and NaOH ($p<0.01$). Moderate attraction was seen for $NaHCO_3$ and NaCl in the same larval numbers, and there were significant differences between these two and the former two ($p<0.01$) (Fig 6). These sodium compounds could attract the larvae, compared with the control. Eventually, sodium cation was found to attract *Necator* hookworm filariform larvae. Although Na_2CO_3 showed the strongest attraction, it is not a component of the human body. This experiment was done to show that it was not the Cl anion but the Na cation, that attracted the *Necator* infective larvae.

DISCUSSION

As for the chemotactic behavior of hookworm third-stage larvae, Zeitse *et al* (1981) examined several kinds

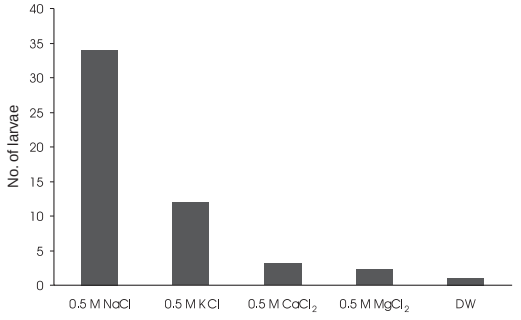

Fig 3- Chemotactic attraction of *Necator* larvae to various chloride compounds. All doses were unified as 0.5 molar and NaCl attracted the most larvae. KCl showed moderate attraction of larvae. $CaCl_2$, $MgCl_2$, and DW did not attract larvae.

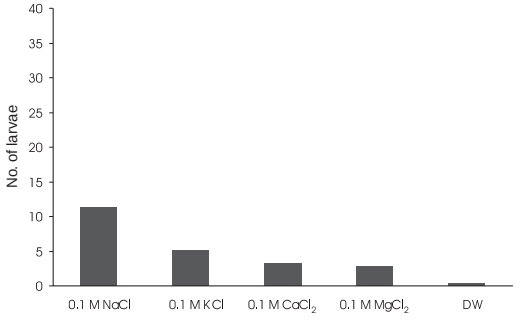

Fig 4- The same chemicals were used as Fig 3. The dosage was changed to 0.1 molar. Though 0.1 molar of NaCl was weak for attracting ability of larvae, it still attracted the most number of larvae. Some larvae were migrated to KCl, $CaCl_2$, and $MgCl_2$. There were significant differences in larval numbers between NaCl and the others.

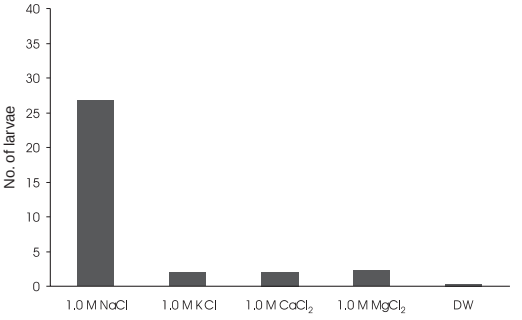

Fig 5- The same experiment was carried out changing the dosage of chemicals to 1.0 molar. NaCl attracted the most. The larvae were not attracted to other chemicals.

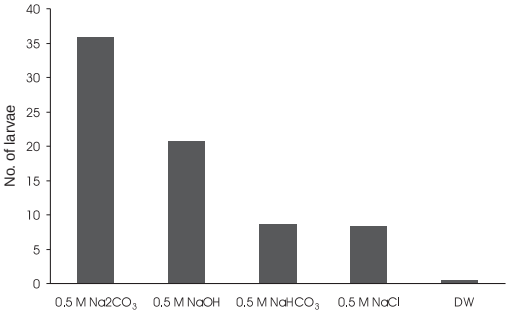

Fig 6- Chemotactic attraction of various sodium compounds to the larvae. All dosages were unified as 0.5 molars. Na_2CO_3 had the strongest attraction. Next, NaOH had moderate attraction. $NaHCO_3$ and NaCl had the same levels of attraction. The sodium compounds had significant differences when compared to control.

of mammalian and avian sera from dog, horse, cat, rat, sheep, hamster, guinea pig, rabbit, pigeon and chicken, against *Ancylostoma caninum*. The highest chemotactic activity of these sera was with dog serum, while few were attracted to rabbit and chicken sera. Next, Vetter *et al* (1985) examined the ultrafiltrated dog serum fraction by gel filtration against *A. caninum,* and concluded that chemotactic activity still remained in a molecular weight <500 Dalton. In this experiment, though the *A.caninum* were attracted to lower molecular substances (such as reduced glutathione; molecular weight 307) less than 500 Dalton, they might also be attracted to inorganic compounds included in canine serum. Wauters *et al* (1982) showed that, after ultrafiltration and dialysis, dog serum separated into several fractions; the factors causing chemotaxis against *A. caninum* again had a molecular weight of less than 500 Dalton. Their dog serum did not contain inorganic substances. This suggested that there were some cues to attract the larvae in serum, after excluding inorganic substances. Granzer and Haas (1991) demonstrated *A. caninum* larvae to be attracted by dog hydrophilic skin surface extracts, but not by skin lipids. A molecular weight of dog serum of about >10 kDa was effective for larval attraction. Their serum also did not include inorganic compounds, because they dialyzed dog serum before use. In the present study, we tried sodium chloride. *Necator* hookworm infective larvae were attracted to 0.5 molar of NaCl, which was found to be an optimal concentration for these larvae, but this was even thicker than physiological saline solution (approximately 0.15 molar). Though we could not explain why they were attracted to so thick a concentration of NaCl, their sensory organs may work in higher range concentrations. This phenomenon may also be seen in Fig 5. When no other doses of sodium chloride existed as attractants, larvae aggregated at the 1.0 molar NaCl site. Other chemicals, such as KCl, $CaCl_2$ and $MgCl_2$, as well as distilled water (DW), could not attract *Necator* larvae. These chemicals have very low volumes in serum components. The Cl anion did not attract *Necator* larvae. In Fig 6, Na_2CO_3 had the strongest attracting ability among the sodium compounds tested, because it has a double volume of sodium ion. This strongly suggests that the sodium cation attracts these larvae.

Tada *et al* (1997) reported, in a similar experiment, that *Strongyloides ratti* infective larvae prefer 0.05 molar NaCl as an optimal concentration. These larvae have more sensitive sensory organs to NaCl than those of *Necator* species. Because *S. stercoralis* does not have alternative pathways for infection, they must infect the hosts only through the skin.

As regards *Necator americanus*, Hawdon *et al* (1992) examined *in vitro* the resumption of feeding phenomenon, which was considered an indicator of the reactivation of development by arrested third-stage hookworm larvae. They concluded that *N. americanus* larvae did not resume feeding in response to glutathione, serum, glutathione plus serum, or linoleic acid. But their work was limited only to the resumption of feeding phenomenon. This was not a host-finding study for *Necator* species. We used inorganic compounds alone to attract *Necator* infective larvae and found evidence that sodium chloride was a very important substance for their chemotaxis. Though many substances exist in the human skin surface, NaCl must be one of the most important candidates for *Necator* hookworm infective larvae to find and infect their host.

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