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Neem Extract as a Control Tool for Vector-borne Diseases: An Example of Visceral Leishmaniasis in Bangladesh

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ABSTRACT Neem (*Azadirachta indica* A, Juss) oil has been suggested to be effective against the carriers of some important vector-borne diseases. In a laboratory setting, azadirachtin was effective against *Phlebotomus argentipes* (sandfly), a vector of visceral leishmaniasis (VL). The effectiveness of neem oil indoor-spray to control VL in an endemic area of Bangladesh was done in sub-district of Mymensingh where the highest incidence of VL occurs in Bangladesh. Households were assigned to intervention and control groups by cluster randomization. Each group had approximately 750 households. Although the sero-conversion rate detected by rK39 dipstick and total number of sandflies were not statistically different between the intervention and control groups, the rate of blood-fed flies was significantly lower among intervention houses two years after the intervention. The present studies demonstrate that neem oil and azadirachtin were effective for sandflies and thus may provide a new capacity for other important vector-borne disease control.

KEY WORDS : Neem, azadirachtin, vector-borne diseases, leishmaniasis, sandfly, *Phlebotomus argentipes*

INTRODUCTION

Leishmaniasis affects more than 12 million people in the world (WHO, 1990). Bangladesh, Brazil, India and Sudan account for approximately 90% of the estimated global burden of leishmaniasis. Visceral leishmaniasis (VL) or kala-azar is caused by the protozoan parasite *Leishmania donovani* and transmitted by the sandfly *Phlebotomus argentipes*. After DDT for indoor spraying was withdrawn from most countries, the incidence of kala-azar is on the rise in some countries including Bangladesh. Although the recent invention of rapid diagnosis, such as the rK39 dipstick, has facilitated easy diagnosis, effective prevention methods have not been developed for VL control. VL is a fatal disease if it is not treated. With the consideration of the severity of illness, an effective prevention tool is long waited and has a high priority.

Leishmaniasis can be prevented by improving housing conditions, e.g., by building concrete houses (Lane, 1991; Alexander and Maroli, 2003) but this method is far beyond the reach of the poor living in endemic communities. Although spraying DDT had been used for vector-borne disease control, DDT

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has been banned from most countries (Hertig and Fairchild, 1948; Joshi and Rai, 1994; Kaul *et al.*, 1994; Pandya, 1983; Kumar *et al.*, 1995; Mukhopadhyay *et al.*, 1996; Kishore *et al.*, 2004). Alternative chemicals for residual spray are too expensive for routine public health use.

Neem oil, a natural pesticide, extracted from neem seeds contains "azadirachtin" that has recently gained recognition as an agrichemical by the Food and Drug Administration (FDA) in the U.S., Ministry of Health, Labor and Welfare in Japan and the Australian Pesticides and Veterinary Medicines Authority (APVMA) in Australia. Azadirachtin has been reported to effectively prevent larvae from feeding (Mordue and Blackwell, 1993). Many insects including malaria transmitting mosquitoes are sensitive to neem oil (Dhar *et al.*, 1998; Nathan *et al.*, 2005). Some reports suggest a role for neem oil against *Phlebotomus* sp. (Sharma and Dhiman, 1993; Srivansan and Kalyanasundaram, 2001). Our preliminary study conducted in a VL endemic area of Bangladesh showed that neem oil reduces vector density seven days after spraying (Wagatsuma *et al.*, 2006). In this study we evaluated the effectiveness of neem oil indoor-spraying to control VL in an endemic area of Bangladesh.

MATERIALS AND METHODS

Study Area

Mymensingh is the most endemic district for VL in Bangladesh and 50% of the country's total VL cases are reported from this district. In Mymensingh, Fulbaria and Trishal upazilas (subdistricts) report the most cases with more than 30 cases per 10,000 people per year. However, the actual incidence rate is expected to be much higher than reported. Trishal consists of 12 unions with an area of 339 km^2. This study was conducted in two unions of Trishal upazila, Mymensingh District.

Study Design

At the beginning of the study we enumerated the total number of households in the study area and designated an individual household as a cluster in this study. We randomly selected 3,400 households and then selected a single individual (indicator person) from each household for a sero-prevalence survey before neem oil spraying intervention. Children less than three years of age were excluded from the study due to difficulties in collecting blood samples. After receiving informed consent, field research assistants completed a household roster and recorded persons treated for VL in the household over the past three years and persons considered to have current VL based on a simplified history. Untreated VL suspected patients were referred to the governmental hospital for appropriate case-management. Selected individuals provided a few drops of finger prick blood before the intervention and a rapid rK39 dipstick test was performed on the collected blood samples. Sero-negative subjects were randomly assigned to the intervention and control groups.

For the intervention households, neem oil solution (neem oil mixed with soap water) was sprayed. For control households no intervention was carried out. Neem oil was applied bi-weekly during the summer months (April to September) and monthly in other months. Sero-negative subjects were repeatedly tested with rK39 dipstick one year later (a follow-up survey was also conducted two years after the intervention, but data are currently under analysis so the results are not shown here). The sero-positivity rates among sero-negative subjects in the intervention group were compared with the control group. Sandflies were collected from 108 houses randomly selected prior to intervention. Mosquito collections were repeated for the same set of houses during September-November one year after neem intervention. Two years after the intervention, a different set of 85 houses per group were randomly selected for sandfly collection in May-July and sandfly species were identified for 51 houses per group (51/85 = 60%).

Blood Test for Sero-conversion Survey

Blood specimens were tested by rK39 dipstick tests provided by Kalazar Detect™ from InBios International Inc., Seattle, USA. This test is currently recommended by WHO. We followed the standard procedures for the rK39 dipstick test (Sarker *et al.*, 2003; Bern *et al.*, 2000; Chappuls *et al.*, 2003).

Neem Oil Spraying

Commercially available neem oil (Neem Oil®, Neem Foundation, Dhaka, Bangladesh) was used. The azadirachtin content was 300 ppm in the neem oil as confirmed by the International Institute of Biotechnology and Toxicology (IIBAT). Neem oil was diluted to a 0.5% solution with a detergent (Jet®, P&G, Bangladesh) as an emulsifier (Wagatsuma *et al.*, 2006). Knapsack sprayers were used to coat the mud walls with the neem oil solution up to a height of 3 m from the floor. Each house was sprayed bi-weekly in April to September and monthly during the remaining months (October to March).

Sandfly Collection and Identification

Our previous study in Bangladesh indicated the number of sandflies collected by light traps correlated well with the risk of leishmaniasis (Wagatsuma *et al.*, 2006; Alam *et al.*, 2009), so the same traps were used for the collection of sandflies in this study. CDC Miniature Light Traps Model-512 (John W. Hock Company, Florida, USA.) were set at 16:00–19:00 and recovered at 6:00–9:00. The trap was set in the bedroom where the indicator person slept (blood samples were also collected from the indicator person). Traps were set twice (two nights) per house for sandfly collection and the average number of sandflies per house per trap was calculated.

Our previous studies in Bangladesh suggested that sandfly numbers fluctuate by month of collection with a peak in April to July (Bern *et al.*, 2005; Wagatsuma *et al.*, 2006). As trapped sandfly numbers were lower than previous studies and one year after intervention, we collected sandflies during the peak months (May to July) two years after intervention. A different set of 85 houses per group (intervention and control groups) were selected for these sandfly collections.

Collected sandflies were preserved in 100% ethanol and dissected under a stereomicroscope. PVA mounting medium (BioQuip Products, Inc, USA) was used for slide preparations, and the slides were examined under a compound microscope for species identification with the keys developed by Lewis (1978) to identify the leishmaniasis vector. We specifically identified *Phlebotomus argentipes* to distiguish from non-vector species (non-vector sandflies were not identified to species).

RESULTS

Sero-prevalence and Sero-conversion

A total of 1,550 persons were identified as sero-negatives by the rK39 dipstick kit before intervention with the neem oil spray. Subjects were allocated into two groups, 770 in the intervention group and 780 in the control group. There was no significant difference in sero-prevalence between the intervention and control groups (7.0 and 6.8% respectively; χ^2-test, $P > 0.05$). One year after intervention, 33 of 487 (6.8%) subjects from the intervention group and 22 of 459 (4.8%) from the control group became sero-positive, but the difference was not statistically significant.

Sandfly Population

As shown in Table 1, the average numbers of sandflies per house per trap before and one year after intervention were not significantly different.

Table 1. Trap catches of sandflies before and after intervention with neem oil treatment

Treatment	Before intervention		One year after intervention	
	No. of houses	No. of sandflies (house/night)[a,c]	No. of houses	No. of sandflies (house/night)[a,c]
Neem oil	59	4.1 (0 – 11)[b]	51	4.5 (0 – 46)
Control	49	4.0 (0.5 – 9.5)	49	4.9 (0 – 27)

[a]Sandflies were collected for two nights per house in the same houses before and one year after neem intervention.
[b]Number in parentheses indicates the range.
[c]There was no significant difference in number of flies between treatments before and one year after intervention by t-test at $P < 0.05$.

Table 2. Number of engorged and unengorged female *P. argentipes* trapped in houses with and without spraying neem oil

Treatment	No. of sandflies (%)		
	Engorged	Unengorged	Total
Neem oil	13 (14.9)*	74 (85.1)*	87 (100)
Control	22 (33.8)	43 (66.2)	65 (100)

* Significantly different within the same column by chi-square test at $P < 0.05$.

The average number of sandflies per house per trap was 4.1 in the intervention houses and 4.0 in the control houses. There was also no significant difference between the groups one year after intervention (Table 1).

Two years after intervention, sandflies were collected in May to July 2008 (suspected peak months for sandfly density in this area) in a different set of 169 houses (84 from the intervention group and 85 from the control group). Trapping was not conducted in one house in the intervention area because no one was living in the house.

A total of 6,123 sandflies were collected, with 3,376 from the neem oil intervention area and 2,747 from the control area. The average number of sandflies per trap was 20.1 in the intervention area and 16.1 in the control area. There was no significant difference in sandfly numbers between neem oil and control groups by t-test. These houses showed higher numbers of sandflies than the numbers trapped one year after intervention (t-test; $P < 0.05$). Chi-square test showed that the proportion of female sandflies to male sandflies in the intervention area was significantly lower than the control area ($P < 0.001$).

Number of *P. argentipes* in the Neem Intervention Group

For identification, 1,712 sandflies were dissected and 1,693 sandflies identified, 19 sandflies (17 females and 2 males) were impossible to identify because the spermatheca and other body parts were not clearly visible. From the intervention area 959 sandflies were identified (317 females and 542 males) and 485 sandflies (155 females and 319 males) were *P. argentipes*. From the control area, 744 sandflies (447 female and 297 male) were identified and 265 sandflies were *P. argentipes* (103 females and 162 males). The proportion of *P. argentipes* was significantly higher in the intervention area compared to the control area ($P < 0.001$).

Trapping of Engorged and Unengorged *P. argentipes* Populations

Table 2 shows the number of engorged and unengorged *P. argentipes* trapped in houses treated with neem oil. There was a significant reduction in the number of engorged *P. argentipes* in the intervention group; 13/87(14.9%) in the intervention group vs 22/65(33.8%) in the control group ($P < 0.05$, Chi-square test). More unengorged *P. argentipes* were found in the neem intervention group.

Trapping of Engorged *P. argentipes* and other Species

Number of engorged *P. argentipes* and other species (non-vectors not identified into species level) was 13 and 15 respectively in the intervention area and 22 and 3 respectively in the control area

Table 3. The number of engorged sandfly species trapped in houses with and without spraying neem oil

Treatment	No. of female sandflies (%)		
	P. argentipes	Others	Total
Neem oil	13 (46.4)*	15 (53.6)*	28 (100)
Control	22 (88.0)	3 (12.0)	25 (100)

* Significantly different within the same column by chi-square test at $P < 0.05$.

(Table 3). In the control area, the number of engorged *P. argentipes* was seven times more than other species. Chi-square test showed that the proportion of engorged *P. argentipes* in the control area was significantly higher than the intervention area ($P = 0.002$). The results also showed other species (non-vectors) were more in the neem intervention houses.

Mosquito Trapping

We recovered 3,691 mosquitoes from the traps (1,422 from the intervention area and 2269 from the control area). The average number of mosquitoes per trap was 8.5 (SD = 7.4) in the intervention area and 13.3 (SD = 12.2) in the control area. These numbers were significantly higher in the control area than the intervention area ($P < 0.01$). More than 20 mosquitoes per trap were collected from 7 (8.3%) houses in the intervention area and 18 (21%) houses in the control area.

DISCUSSION

This study showed a significant reduction in engorged *P. argentipes*, a vector for visceral leishmaniasis, in houses sprayed with neem oil extracts. This is the first evidence that regular spraying of neem oil extracts may be effective in a field setting for control of *P. argentipes* vectors. There was no direct evidence in reduction of the number of vector sandflies, but a reduction the number of engorged *P. argentipes*, indicates the impact of azadirachtin is rather anti-blood feeding or repellant rather than direct killing of sandflies. We found unengorged vector and non-vector mosquitoes were more in the neem sprayed houses. Neem may attract sandflies, but reduce blood-feeding. Regular neem application showed no affect on the total number of sandflies even two years after treatment, indicating the effect of azadirachtin or other chemical compounds in the neem oil may not be harmful to sandfly populations.

A previous study conducted in the same area reported *P. argentipes* were 59.2% of the total collection (Alam *et al.*, 2009). The other sandfly species were *Sergentomyia* sp. (*S. shortti*, *S. barraude*, *S. babu babu*, *S. indica*, *S. pertubans* were the most common). Among the *Sergentomyia* species, *S. shorttii* contributed 14.4% of the total collection. Density of sandflies, both vector and non-vector species, were significantly higher in endemic areas than non-endemic areas. Compared to the sandfly density (number of sandflies per house per trap) of 13.5 in this previous study, our study showed a relatively low number of sandflies. This indicates that our neem study area may be categorized as a low endemic area. However, we found much higher vector density when we collected sandflies in May-July. This confirms that sandfly density fluctuates by season with a peak during the early monsoon months.

Sergentomyia spp. are the major non-vector species co-existing in the VL endemic areas of Bangladesh. Vector species appear to prefer human blood more than non-vector sandflies (Katakura, personal communication). To clarify this characteristic, further studies are required to identify the sources of blood in engorged sandflies.

Adult *P. argentipes* are usually detected in human dwellings and cow-sheds, and larvae are recovered from domestic and peri-domestic areas such as basements and cellars of houses, cracks in mud floors and walls, soil in human dwellings, animal borrows and animal shelters (Modi and Tesh, 1983; Pandya and Niyogi, 1980; Dhiman *et al.*, 1983; Ghosh and Bhattacharya, 1991). Neem oil spraying on mud walls may effect the immature stages as well as the adults (Feliciangeli, 2004). Although further studies are required to clarify the effect of azadirachtin on *P. argentipes*, the finding in this study indicate that neem oil extracts may be a good alternative for effective vector control of leishmaniasis. In addition to high environmental safety, other benefits include the complex structure of azadirachtin makes it difficult for the development of resistance in insects (Medina *et al.*, 2004).

More neem oil products have appeared in the market since we conducted this study. If good quality neem extracts with high concentrations of azadirachtin can be used, further reduction of engorged vectors may be achieved without hampering

barriers of community acceptance, e.g., smell of neem oil solution after spraying. In Thailand, community produced neem oil is promoted. If the quality of products can be maintained by internationally standardizing the production procedures, an effective tool for vector control may be developed. It is important that international biopesticide societies promote and support the correct use of neem oil for vector control in the future.

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Socio-economic Report

Acceptance for Neem Oil as a Visceral Leishmaniasis Vector Control Tool in Bangladesh

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ABSTRACT In Bangladesh, 20 million people are considered at risk of developing visceral leishmaniasis (VL). In this study, neem oil was found to be effective against the vector *Phlebotomus argentipes*. To promote neem oil as a public health tool for VL prevention, information about the human perception of use is required. This study was conducted to explore the willingness of people to accept neem oil products for VL control. We conducted a questionnaire study in three VL endemic villages in the Mymensingh district during the months of June-July 2008. In this area, intervention studies using neem oil have been conducted since 2006. Neem oil was monthly sprayed on approximately 750 randomly selected mud-walled houses. Our study population was 120 randomly selected women aged 25–50 years. Ninety-three questionnaires were successfully collected. The majority of respondents (47; 51%) showed interest in purchasing neem oil at the current market price (30 taka for a 70 ml container). In addition, 16 (17%) respondents replied they would purchase it if the price was lower. In conclusion, neem oil showed acceptance as a method for VL prevention in endemic communities of Bangladesh. Further studies are required to identify what information is appropriate to widely promote neem oil.

KEY WORDS : *Azadirachta indica*, neem oil, leishmaniasis, vector control.

INTRODUCTION

Visceral leishmaniasis (VL) is one of the most neglected diseases in the world. Presently, there are no well-defined models for cost-effective control of VL vectors (WHO, 2007). In the world, each year approximately 500,000 cases of VL are reported (90% in Bangladesh, Brazil, India, Nepal and Sudan) with an estimated more than 50,000 deaths. Actual global mortality from VL may be higher because in many countries the disease is not recognized or is frequently undiagnosed, especially where there is no access to medication (WHO 2007).

In Bangladesh, VL is transmitted by the sandfly *Phlebotomus argentipes*. Because of the biological characteristics of sandflies, the risk of disease is higher for people living in mud-walled houses. Many methods have been applied for vector control. The use of indoor residual insecticides, e.g. DDT spraying, is a simple method for control. However many countries have stopped the use of DDT because of it's toxicity to mammals including humans. In addition, development of resistance against DDT

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in *P. argentipes* is reported (Mukhopadhyay *et al.*, 1996). Still DDT has been used continuously in some developing countries.

Insecticide-treated bednet (ITN) is an effective method for reducing man-vector contact. This method is useful because less insecticide is used and people can purchase bednet as a domestic item. However, ITN does not reduce the number of female *P. argentipes* (Dinesh *et al.*, 2008a,b). Currently, no well-defined method for cost-effective control exists (WHO, 2007). Development of a successful vector control method for VL is needed.

Azadirachta indica is a tree commonly found in Southeast Asian countries and is called the "neem" tree. Neem oil (extract from its seeds) has been used in various ways since ancient times. Neem products show a wide range of effects against vector insects that are useful for VL control as antifeedants (Jeniffer and Alasdair, 2000), insect growth regulators and repellents (Sharma and Dhiman, 1993). Azadirachtin, a complex tetranortriterpenoid limonoid from the neem seeds is the main component responsible for both antifeedant and toxic effects against insects (Isman, *et al.*, 1990). Neem oil contains other active substances that work with azadirachtin (Govindachari *et al.*, 2000) and it is difficult for resistance to develop against the many active substances at once.

In an earlier study, 2 and 5% neem oil formulations with mustard oil showed 100% protection from bites of female *P. argentipes* for 12 hrs when applied on volunteers' skin (Sharma and Dhiman, 1993). In addition, neem oil was also effective against *Anopheles* and *Gambiae* mosquitoes. It was highly toxic to mosquito larvae and inhibited the development of pupae (Okumu *et al.*, 2007). However, effectiveness of neem oil in controlling numbers of *P. argentipes* in field conditions has not been evaluated. Because of low migratory activity in *P. argentipes* adults, the distance of susceptible persons from patients highly correlates with the risk for contracting VL. Living in the same house with a VL patient is associated with a 26-fold increased risk, and living within 50 m a 3-fold risk compared with people living more than 50 m away (Bern *et al.*, 2005). To effectively reduce VL cases, community-based methods are required for VL control in endemic countries.

This study is part of a main umbrella neem oil intervention study being conducted to estimate the effects of neem oil against the sandfly vector. In the local villages of Bangladesh, neem oil is not available and rural people do not know about neem oil (oil can be purchased in selected shops in big cities). There is no previous study reporting the perceptions of local residents on the use of neem oil and willingness to pay for neem oil products for VL control. The objective of this study was to explore the willingness of rural people to pay for neem oil products for VL control. Information about the peoples' attitudes toward available neem products can be helpful for improving perceptions and familiarizing rural residents with neem oil products.

MATERIALS AND METHODS

Population Study

We conducted a questionnaire study in three VL endemic villages in Kanihali Union of Mymensingh, Bangladesh. More than 50% of VL cases in Bangladesh are reported from the Mymensingh district. In this area, a neem oil intervention study for VL prevention has been operating since 2006. Neem oil was sprayed monthly at 770 randomly selected mud-walled houses in three villages, and 780 houses were randomly selected as controls from five other villages apart from the intervention villages.

In July 2007, we conducted an open-ended questionnaire study by in-person interviews. Interviews were conducted by a researcher (M.F.) assisted by a local interpreter. The interpreter was trained for assistance with general knowledge of the disease and neem oil use. This study aimed to pilot a small number of people (about 30 people including both males and females, all more than 18 years old and living in the intervention or control villages). Results of the pilot study (data not shown) showed that female respondents were more interested in family health and neem oil use. Especially women between 25 to 50 years of age expressed their opinions more clearly than others. We also found it

was difficult to ask people in the control area about neem oil because they lacked previous exposure to neem oil use. Neem oil was not available in these villages, so people could not clearly respond to the questions. Based on the pilot study in 2007, we randomly selected 120 women between the ages of 25 to 50 years that lived in the intervention houses for more than three years.

Data Collection

An interviewer-administered questionnaire study of 120 women was carried out during June and July 2008. The interviewers, trained in both class sessions and in the field, conducted all interviews. During the study period, the researcher (M.F.) supervised the interviewer by reviewing the forms and conducting spot checks in the field. A structured interviewer-administered questionnaire was used to collect information on bednet use, attitude toward neem and cosmetic use. We asked questions about cosmetics to obtain specific information about the prices of products they used daily. This information can help us to explore the niche for neem oil. The questionnaire consisted of fifteen questions written in English. The questionnaire was pretested with women 25 and 50 years old who lived in the study area that were not selected for the actual study. Based on the pretest, necessary modifications were made.

For the study in 2008, two trained bilingual interviewers (bilingual in Bengali and English, one man and one woman) administered the questionnaires in face-to-face interviews. The interviewers asked questions orally in Bengali and recorded the answers. Interviewers had finished their training before the study started. They were requested to finish the questionnaires within ten to fifteen minutes but not in less than ten minutes. To confirm respondent's answers interviewers repeated the answers orally.

Each interview was conducted in the respondent's house or sometimes another place convenient for the respondent (e.g., in the farm). When respondents could not be contacted, we revisited them at least two times on different days to achieve a better coverage. In addition to the written consent obtained for the perception and behavioral studies at the beginning of the main study in 2006, the purpose of the interview was explained again to the participants in 2008, and verbal consent was obtained before beginning each interview. Respondents were also told they did not have to answer any questions if they did not want to. Background information on socio-economic status was extracted from the main intervention study (length of schooling, land ownership, etc.).

Data Analysis

Data from the questionnaires were entered by the double data-entry method and analyzed with SPSS Statistics 17.0.

Table 1. Selected socio-economic information about respondents

Questions	Answers	No. of respondents (%)
Age of respondents	25–30	34 (37)
(years)	31–35	16 (17)
	36–40	19 (28)
	41–45	12 (13)
	46–50	12 (13)
	Total	93 (100)
Land ownership	Do not have own land	30 (33)
	Have own land	62 (67)
	Total	92 (100)*
School Education	Never attended	64 (69)
	Attended a school	29 (31)
	Total	93 (100)

* Information about one respondent's land ownership was missing.

Table 2. Perception of neem oil smell after application

Questions	Answers	No. of respondents (%)
Does the smell of neem oil disturb you ?	Substantial	56 (60)
	High	7 (8)
	Moderate	10 (11)
	Slight	10 (11)
	Not at all	10 (11)
	Total	93 (100)
How many days can you Detect the smell ? (days)	≤5	1 (1)
	4	3 (3)
	3	16 (17)
	2	33 (36)
	1	35 (38)
	≤1	5 (5)
	Total	93 (100)

RESULTS

Coverage

Out of 120 selected persons, 93 interviews (78%) were successfully completed. The reasons for non-participation were: migrated (n = 18), absent (n = 6), and not found in the house member list (n = 3).

Socio-economic and Demographic Characteristics

Table 1 shows the socio-economic information obtained from respondents. Respondent's average age was 35 years (range: 26 to 50 years, SD = 7.2). Majority of the respondents (69%) never attended school and were illiterate; 62 (67%) respondents had their own land and only 5 (5%) respondents had connection to owned electricity. Women respondents who always used a bed net when they slept accounted for 60% of respondents; 38% said they sometimes use bed net and only 2% women said they never used a bed net.

Neem Oil Odour

Table 2 shows respondent's perception toward the smell of neem oil after application. Eighty-three (89%) respondents replied the smell of neem oil disturbed them of which 56 (60%) respondents said the smell of neem oil was extremely disturbing. Some respondents who extremely disliked the neem smell said they felt dizzy after neem oil application. Only 10 (11%) of the respondents replied the smell did not disturb them at all. However, many respondents said the smell would not be a problem if neem oil worked to prevent VL. Eighty-nine (96%) respondents said the smell was gone within three days after neem oil application.

Table 3. Perception of change in disturbance from insects after neem oil application

Questions	Answers	No. of respondents (%)
Change in the number of insects perceived	Substantially decreased	11 (12)
	Moderately decreased	12 (13)
	Slightly decreased	29 (31)
	No change	40 (30)
	Increased	1 (1)
	Total	93 (100)
Change in the frequency of insect bites	Substantially decreased	11 (12)
	Moderately decreased	7 (8)
	Slightly decreased	28 (30)
	No change	46 (50)
	Increased	1 (1)
	Total	93 (100)

Reduction of Insects and Insect Bites

Table 3 shows respondent's perception about changes in insect population after neem oil intervention. According to 52 (56%) respondents there was decrease in the number of insects inside their houses and 25% respondents said the number decreased substantially (combined for the responses of "substantially decreased" and "moderately decreased"). According to 46 (50%) of the respondents the frequency of insect bites inside houses decreased after neem oil spray intervention and out of these 11 (12%) women were of the opinion that the frequency of insect bites was substantially low. However, 46 (50%) respondents had observed no change in number.

Acceptance of Control Strategy

Among 93 respondents, 47 (51%) persons were interested in buying existing neem products (30 taka for a 70 ml container). In addition, 16 persons were ready to purchase neem if the price were lower. They were interested in using neem oil if they could afford the product. The willingness to pay for neem oil based on personal perceptions of changes in the number of insect bites is given in Fig. 1. Among respondents interested in buying neem oil (respondents who replied they could spend more than 1 taka for neem oil), 34 (54%) had observed the decrease in insect bites after neem oil application. This proportion was higher than among respondents that did not want to buy neem oil (12; 40%). However, there was no statistical difference in the perceptions of the two groups.

Of the respondents that wanted to buy neem oil 41 (65%) owned land. This proportion was similar to to the respondents that did not want to buy neem oil (21; 70%). Information about one respondent's land ownership was missing.

DISCUSSION

This study showed that the majority of people in the Mymensingh District of Bangladesh were interested in buying neem oil. Although the current price is not a small amount of money for the respondents, more than half replied that they were interested in buying the existing neem products. In addition, 16 (17%) of the respondents said they would purchase neem oil if the price was lower. In Bangladesh, diagnosis and treatment of VL are extremely expensive compared to the income of affected families (Anoopa, *et al.*, 2006). Furthermore, during the interview we observed that most respondents knew who was affected by VL in their neighborhood. Under these circumstances, respondents appear to think seriously about VL and will spend money for prevention. Therefore, neem oil has a possibility of becoming a popular method for VL prevention in endemic communities.

More than half of the respondents also mentioned that neem oil application reduced the total number of insects in the bed rooms at night. The proportion of respondents who replied insect bites had decreased after neem oil application was higher among respondents that wanted to buy than those who did not want to buy neem oil. The malaria study

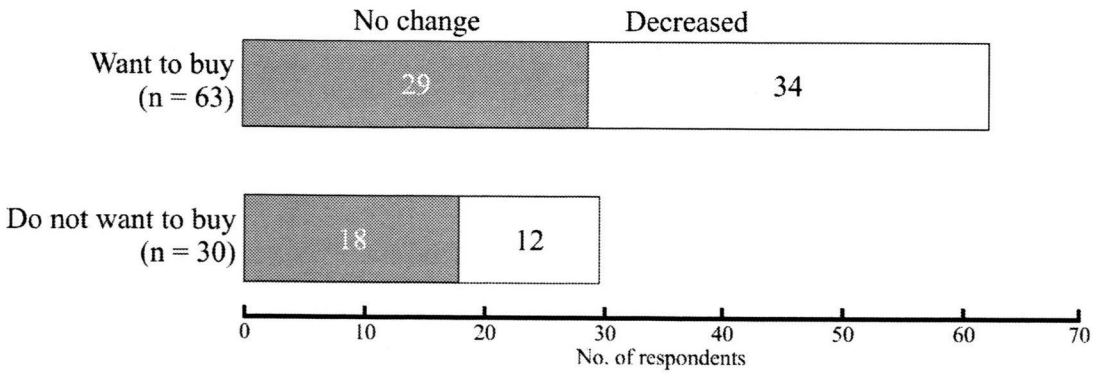

Fig.1. Willingness to pay based on personal perception of insect bite reduction.

in Kenya shows that the reason respondents valued bed net use was mainly to avoid mosquito bites. Malarial control was the second reason for the use of bed nets (Ng'ang'a *et al.*, 2009). A positive attitude about the attractive benefits of vector control methods can help to widely promote its use.

The proportion of landowners who were for or against the purchase of neem oil was similar. This suggests that the willingness to pay for neem oil is not affected by socio-economic status which is in contrast to the support for common prevention tools such as bed nets significantly affected by socio-economic status (Chuma *et al.*, 2009). Neem oil may become a common prevention tool even among poor people despite the lack of a statistical difference in this study. Further studies are needed to examine the relationship between socio-economic status and a willingness to pay for neem oil among the people living below poverty line.

Those respondents who did not like to buy neem oil were averse due to bad smell of neem oil due to sulfur compounds that have a strong garlic-like odour (Koul, 2004). However these sulfur compounds also potentially contribute to, at least in part, the insect repellency of neem oil (Balandrin *et al.*, 1988; Koul, 2004). If the odor is removed from neem oil, important active constituents may also be lost.

Although the current price of neem oil is a great expense for respondents, this study showed the majority of people were interested in buying neem oil. Still there were many people that did not want to purchase neem oil and we could not show a significant relationship between a willingness to pay and other response variables. Further studies are required to clarify other factors related to acceptance in order to pay for neem oil in vector control intervention.

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Rhoptry neck protein RON2 forms a complex with microneme protein AMA1 in *Plasmodium falciparum* merozoites ☆

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ABSTRACT

Erythrocyte invasion is an essential step in the establishment of host infection by malaria parasites, and is a major target of intervention strategies that attempt to control the disease. Recent proteome analysis of the closely-related apicomplexan parasite, *Toxoplasma gondii*, revealed a panel of novel proteins (RONs) located at the neck portion of the rhoptries. Three of these proteins, RON2, RON4, and RON5 have been shown to form a complex with the microneme protein Apical Membrane Protein 1 (AMA1). This complex, termed the Moving Junction complex, localizes at the interface of the parasite and the host cell during the invasion process. Here we characterized a RON2 ortholog in *Plasmodium falciparum*. *Pf*RON2 transcription peaked at the mature schizont stage and was expressed at the neck portion of the rhoptry in the merozoite. Co-immunoprecipitation of *Pf*RON2, *Pf*RON4 and *Pf*AMA1 indicated that the complex formation is conserved between *T. gondii* and *P. falciparum*, suggesting that co-operative function of the rhoptry and microneme proteins is a common mechanism in apicomplexan parasites during host cell invasion. *Pf*RON2 possesses a region displaying homology with the rhoptry body protein *Pf*RhopH1/Clag, a component of the RhopH complex. However, here we present co-immunoprecipitation studies which suggest that *Pf*RON2 is not a component of the RhopH complex and has an independent role. Nucleotide polymorphism analysis suggested that *Pf*RON2 was under diversifying selective pressure. This evidence suggests that RON2 appears to have a fundamental role in host cell invasion by apicomplexan parasites, and is a potential target for malaria intervention strategies.

1. Introduction

Malaria is one of the most prevalent and deadly global infectious diseases, more than half of the world's population is at the risk of infection, and over 300 million people develop clinical disease each year of which 2 million are fatal [1]. Clinical malaria results from the replication of protozoan parasites of the genus *Plasmodium* in the circulating erythrocytes of the host. During the time between release from a rupturing mature schizont-infected erythrocyte and invasion of new erythrocytes, merozoites are transiently exposed in the circulation, and are thus potentially vulnerable to attack by preventive measures based upon immunological or biochemical methods. To design such tools, it is important to understand the molecular composition of the merozoite and the structure-function makeup of the molecular interactions that occur as the merozoite recognizes and gains entry into a host cell.

Like most apicomplexan parasites, the malaria merozoite invades host cells via a multistep process initiated by reversible binding to the erythrocyte surface. Subsequently, a high affinity attachment occurs between the apical end of the merozoite and the host cell, followed by the movement of the junctional adhesion zone (moving junction) around the merozoite toward its posterior pole. Finally the merozoite invaginates into the erythrocyte by forming a nascent parasitophorous vacuole [2]. The moving junction is one of the most distinctive features of apicomplexan invasion and was first observed in

Abbreviations: aa, amino acid(s); Ab, antibody; AMA1, apical membrane antigen 1; GST, Glutathione S transferase; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; RON, rhoptry neck protein.

☆ Sequence data from this article have been deposited with the GenBank™/EMBL/DDBJ databases under accession numbers AB444588–AB444592.

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Plasmodium species in the late 1970s [3], but the molecular nature of its structure remains unresolved.

Recent studies in *Toxoplasma gondii* suggest that host cell invasion involves protein discharge from at least two apical secretory organelles, the micronemes and rhoptries, based on the observation that a microneme protein, Apical Membrane Protein 1 (AMA1), forms a complex with three rhoptry neck (RON) proteins: RON2, RON4 and Ts4705 (RON5) [4–6]. These proteins have predicted orthologs in *P. falciparum*, and the RON4 ortholog has been reported to associate with *Pf*AMA1 [7] and to be localized at the moving junction [8], suggesting that the complex (and likely its function) is conserved between *T. gondii* and *P. falciparum* [7]. Attempts to knock-out the AMA1 gene locus were unsuccessful in both *Plasmodium* [9] and *T. gondii* [10], and the conditional reduction of *Tg*AMA1 expression severely impaired the cell invasion ability of *T. gondii* [11], indicating AMA1 has an essential function. The conservation of the RON proteins among apicomplexan parasites suggest that their functions and protein interactions are also conserved in the biology of host cell invasion. However, in *Plasmodium*, the details of this complex have yet to be fully characterized. In this study, to better understand the moving junction complex formation in *Plasmodium*, we sought to characterize *Pf*RON2 and determine the nature of its interaction with *Pf*RON4 and *Pf*AMA1.

2. Materials and methods

2.1. Malaria parasites

P. falciparum cloned lines 3D7, HB3, Dd2, 7G8, FVO, and D10 were maintained *in vitro*, essentially as previously described [12].

2.2. DNA and RNA isolation

Genomic DNA (gDNA) was isolated from *P. falciparum* using IsoQuick™ (Orca Research Inc., Bothell, WA). To determine transcription levels throughout the asexual stages, schizonts were purified by differential centrifugation on a 70%/40% Percoll-sorbitol gradient, after which released merozoites were allowed to invade uninfected erythrocytes for 4 h before the clearance of all remaining schizonts using 5% D-sorbitol. Fractions of the culture were harvested immediately and 24 h later, and then at 6 h intervals thereafter. Total RNA was isolated from parasite-infected erythrocytes stored at −20 °C in RNA*later*™ (Qiagen, Valencia, CA), using the RNeasy Mini Kit (Qiagen). Following DNase treatment, complementary DNA (cDNA) was generated with random hexamers using an Omniscript Reverse Transcription Kit (Qiagen).

2.3. Polymerase chain reaction (PCR) amplification and sequencing

A TBLASTN search was performed against the *P. falciparum* genome database (3D7 parasite line) via PlasmoDB website (http://www.plasmodb.org/) [13] using the *Tg*RON2 amino acid sequence as a query. To evaluate the polymorphism of *Pf*RON2, five pairs of overlapping primers were used for PCR amplification from HB3, FVO, Dd2, D10, and 7G8 parasite lines, and sequences were determined by direct sequencing of the PCR-amplified DNA fragments using an ABI PRISM® 3100-*Avant* Genetic Analyzer (Applied Biosystems, Foster City, CA). Oligonucleotides used were as follows: fRON2.F2 (5′-GATTCCAATAATTATAATTCTGATAATG-3′) and fRON2.R2 (5′-CGTAAAATATTCATTATATGAAAGATATGC-3′), fRON2.F3 (5′-GCATTAGGAGAACTTGTTGAACCA-3′) and fRON2.R3 (5′-CATAATATCTAAATAGGTTTTTGCTGAC-3′), fRON2.F4 (5′-GGATTAGTATTTTTATATGCAATGATTG-3′) and fRON2.R4 (5′-GTTATTTTCTAATAAATGTTTACTATCTTC-3′), fRON2.F5 (5′-GATAAATGGGATCAATTTATAAATAAGG-3′) and fRON2.R5 (5′-GCTAGCTACTGGTCCTGCACCT-3′), and fRON2.F6 (5′-ATGCAATTACCTTACTTAAGTCAAATG-3′) and fRON2.R6 (5′-ATATAAAATGAAAATAACAGAAAAGGTTATG-3′).

2.4. Quantification of pfron2 transcripts

Transcription of *ron2* was evaluated in the HB3 parasite line by real-time reverse transcription (RT)-PCR using a QuantiTect SYBR Green PCR Kit (Qiagen) and a LightCycler System (Roche, Basel, Switzerland). As a control, transcription of *ama1* and *rhoph2* was also evaluated. Oligonucleotides used were as follows: fRON2.qF (5′-CAGAACTAAGCAAACATGTAAAACATG-3′) and fRON2.qR (5′-GTATAACGCCTTGCTCATTTCCTG-3′) for *pfron2* (product size is 133 bp); fAMA1.qF (5′-GGAAGAGGACAGAATTATTGGGAAC-3′) and fAMA1.qR (5′-CCTGAATCTTCTTGTTGGTATGTATG-3′) for *pfama1* (product size is 137 bp); fRhopH2.qF (5′-GTAACAACACTTACTAAGGCAGACT-3′) and fRhopH2.qR (5′-GTACAAAGCTACAATATTGTTAGATCT-3′) for *pfrhoph2* (product size is 210 bp). The same oligonucleotides were used to PCR-amplify DNA fragments to be ligated into the pGEM-T Easy® plasmid (Promega, Madison, WI) which was used to make a standard curve to evaluate the copy number of each transcript.

2.5. Antibodies

A DNA fragment encoding amino acid positions (aa) 21–98 of *Pf*RON2 was PCR-amplified from *P. falciparum* 3D7 gDNA and ligated into pEU-E01GST-N2, an expression plasmid with N-terminal glutathione S transferase (GST)-tag followed by a PreScission Protease cleavage site, designed specifically for the wheat germ cell-free protein expression system (CellFree Sciences Co., Ltd., Matsuyama, Japan) [14], to produce recombinant GST-fused fRON2N protein (GST-fRON2N). Oligonucleotides used in the PCR amplification were fRON2.SalF1 (5′-*GTCGAC*TCAGAACTAAGCAAACATGTAAAACATG-3′) and fRON2.SalR1 (5′-*GTCGAC*CCCATTATTCATTTCACTACCAGGA-3′) (SalI restriction sites are underlined). Produced GST-fRON2N was captured using a glutathione-Sepharose 4B column and eluted with 10 mM reduced gluthathione, pH 8.0. To generate anti-*Pf*RON2 sera, BALB/c mice were immunized subcutaneously with 20 μg of purified GST-fRON2N emulsified with Freund's adjuvant. A Japanese white rabbit was immunized subcutaneously with 500 μg of purified GST-fRON2N with Freund's adjuvant for the first time, followed by 250 μg thereafter. All immunizations were done 4 times at 3 week intervals, prior to collection of antisera. Rabbit anti-*Pf*RhopH2 serum was obtained from I. Ling (National Institute for Medical Research, UK) [15], Rabbit anti-*Pf*AMA1 serum was obtained from C. Long (National Institute of Health, USA), and mouse monoclonal anti-*Pf*RON4 antibody (Ab; 26C64F12) was obtained from J.-F. Dubremetz (Université de Montpellier 2, France) [7]. Rabbit anti-Clag3.1 serum was as previously described [16].

2.6. SDS-PAGE and Western blot analysis

The recombinant protein, GST-fRON2N, was digested with a PreScission Protease at 4 °C overnight before analysis. Triton X-100 extracts of *P. falciparum* or recombinant proteins were dissolved in SDS-PAGE loading buffer, incubated at 100 °C for 3 min, and subjected to electrophoresis under reducing conditions on a 5–20% polyacrylamide gel (ATTO, Japan). Proteins were then transferred to a 0.22 μm PVDF membrane (BioRad, Hercules, CA). The proteins were immunostained with antisera followed by horseradish peroxidase-conjugated secondary Ab (Biosource Int., Camarillo, CA) and visualized with Immobilon™ Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA) on RX-U film (Fuji, Japan). The relative molecular sizes of the parasite-encoded proteins were calculated by reference to molecular size standards (BioRad).

2.7. Immunoprecipitation

Immunoprecipitation was carried out as previously described [17]. Briefly, proteins were extracted from late schizont parasite pellets by

1% Triton X-100 treatment in phosphate-buffered saline (PBS) containing cOmplete Proteinase Inhibitor Cocktail Tablets (Roche). Supernatants (50 μl) were pre-incubated at 4 °C for 1 h with 20 μl of 50% protein G-conjugated beads (GammaBind Plus Sepharose; GE Healthcare) in NETT buffer (50 mM Tris–HCl, 0.15 M NaCl, 1 mM EDTA, and 0.5% Triton X-100) supplemented with 0.5% BSA (fraction V; Sigma-Aldrich). Recovered supernatants were incubated with rabbit antisera (anti-*Pf*RON2, anti-*Pf*AMA1, or anti-*Pf*RhopH2) or mouse anti-*Pf*RON4 Ab with gentle rotation at 4 °C for 2 h and then 20 μl of 50% protein G-conjugated beads were added. After 1 h incubation at 4 °C, the beads were washed once with NETT-0.5% BSA, once with NETT, once with high-salt NETT (0.5 M NaCl), once with NETT, and once with low-salt NETT (0.05 M NaCl and 0.17% Triton X-100). Finally, proteins were extracted from the protein G-conjugated beads by incubation with SDS-PAGE reducing loading buffer at 100 °C for 3 min. Supernatants were collected for Western blot analysis.

2.8. Indirect immunofluorescence assay

Thin smears of schizont-enriched *P. falciparum*-infected erythrocytes (Dd2 parasite line) were prepared on glass slides and stored at −80 °C. The smears were thawed, formaldehyde-fixed, and preincubated with PBS containing 5% non-fat milk at 37 °C for 30 min. They were then incubated with antisera at 37 °C for 1 h, followed by fluorescein isothiocyanate (FITC)-conjugated goat anti-(IgG and IgM) secondary Ab (Jackson ImmunoResearch Laboratories, West Grove, PA) and Alexa546-conjugated goat anti-(IgG and IgM) secondary Ab (Invitrogen, Carlsbad, CA) at 37 °C for 30 min. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). Slides were mounted in ProLong Gold antifade reagent (Invitrogen) and viewed under oil-immersion. High resolution image-capture and processing were performed using a confocal scanning laser microscope (LSM5 PASCAL; Carl Zeiss MicroImaging, Thornwood, NY). Images were processed in Adobe Photoshop (Adobe Systems Inc., San José, CA).

2.9. Immunoelectron microscopy

Parasites were fixed for 15 min on ice in a mixture of 1% paraformaldehyde–0.1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). Fixed specimens were washed, dehydrated, and embedded in LR White resin (Polysciences, Inc., Warrington, PA) as previously described [18,19]. Thin sections were blocked at 37 °C for 30 min in PBS containing 5% non-fat milk and 0.01% Tween 20 (PBS-MT). Grids were then incubated at 4 °C overnight with mouse anti-*Pf*RON2 or control sera in PBS-MT. After washing with PBS containing 10% BlockAce (Yukijirushi, Sapporo, Japan) and 0.01% Tween 20 (PBS-BT), the grids were incubated at 37 °C for 1 h with goat anti-mouse IgG conjugated to 10 nm gold particles (Amersham Life Science, Arlington, IL) diluted 1:20 in PBS-MT, rinsed with PBS-BT, and fixed on ice for 10 min in 2.5% glutaraldehyde to stabilize the gold. Then the grids were rinsed with distilled water, dried, and stained with uranyl acetate and lead citrate. Samples were examined with a transmission electron microscope (JEM-1230; JEOL Ltd., Tokyo, Japan).

2.10. Primary structure analysis of the protein

Signal peptide sequence was evaluated by SignalP3.0 [20]. Transmembrane region was evaluated by TMpred [21] and TMHMM2.0 [22]. Low complexity region was evaluated by Globplot 2.3 [23]. Amino acid sequence alignment was generated by MUSCLE [24].

2.11. Statistical analysis

Number of nonsynonymous substitutions over numbers of nonsynonymous sites (d_N), number of synonymous substitutions over numbers of synonymous sites (d_S), and their standard errors were computed using the Nei-Gojobori method with Jukes-Cantor correction implemented in MEGA 4.0.1 [25]. Standard errors were estimated using the bootstrap method with 500 replications. The statistical difference between d_N and d_S was tested using a one-tail Z-test with 500 bootstrap pseudosamples.

3. Results

3.1. RON2 orthologs of apicomplexan parasites

Using *Tg*RON2 as a query in BLAST analyses [26], and similar analyses using the predicted orthologs thus identified, we found RON2 orthologs in *P. falciparum* (*Pf*RON2; PF14_0495, PlasmoDB), *P. yoelii* 17XNL strain (*Py*RON2; PY06813, TIGR), *P. knowlesi* H strain (*Pk*RON2; PKH_125430 or PK14_2335w, Sanger Centre), and *P. vivax* Sal-I strain (*Pv*RON2; Pv117880, TIGR), *P. berghei* (*Pb*RON2; Contig5108), *P. chabaudi* (*Pch*RON2; Contig882.0), *Theileria annulata* (*Ta*RON2; Fig. S1A, TA19445 and TA19390, Sanger Centre [27]), *Theileria parva* (*Tp*RON2; Fig. S1B, TP01_0014, TIGR [28]), and *Babesia bigemina* (*Bbig*RON2; Fig. S1C, Contig3449, Sanger Centre). The RON2 were fragmented in the *P. berghei*, *P. chabaudi*, *T. annulata*, and *T. parva* genome nucleotide sequence databases, and full-length versions were constructed (supplementary Table S1).

3.2. PfRON2 protein structure and similarity to RhopH1/Clag proteins

The full-length *Pf*RON2 protein consists of 2189 residues with a putative signal peptide sequence at its N-terminus from amino acid positions (aa) 1 to 20. An interspecies variable region (aa 55–878), exhibiting low complexity and many repeats [23], was identified by comparing 6 *Plasmodium* RON2 amino acid sequences (Figs. 1 and S2). A BLASTP search using the conserved region of *Pf*RON2 (aa 879–2189) as a query identified *P. vivax* RhopH1/Clag homolog (XP_001616939.1, aa 251–394; E=0.001) as possessing homology with *Pf*RON2 aa 1105–1259. A Position-Specific Iterated BLAST search using *Pf*RON2 aa 1105–1259 as a query converged at iteration 3 and indentified most of the RhopH1/Clag genes in *Plasmodium* species. Alignment of RhopH1/Clag with RON2 from multiple genera identifies a predicted globular domain that is likely stabilized by disulfide bonds between 4 conserved Cys residues (Fig. 2). Three transmembrane regions were predicted by TMpred, however TMHMM2.0 predicted only a single transmembrane region for all *Plasmodium* RON2 orthologs assessed.. Interestingly, TMpred predicted a putative transmembrane region in the region conserved between RhopH1/Clag and RON2 (Fig. 2). Because RhopH1/Clag is a component of a soluble protein complex, we considered that these predicted transmembrane regions in RhopH1/Clag and RON2 constitute a likely hydrophobic region buried within a globular domain. Another predicted transmembrane region at aa 1114–1133 in *Pf*RON2 is also possibly hydrophobic region buried within a globular domain. TMpred considers the observation that there is an overrepresentation of positively charged amino acid

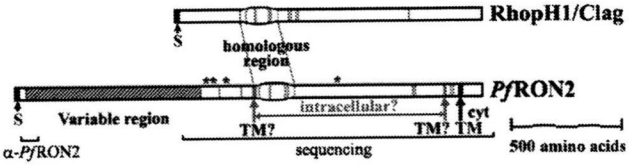

Fig. 1. Schematic representation of *Pf*RON2. S and TM indicate putative signal peptide (aa 1–20) and transmembrane sequences, respectively. The shaded box indicates an interspecies variable region. Vertical red bars indicate conserved Cys residues among orthologous sequences. Homologous region between RhopH1/Clag and RON2 is indicated by a yellow box. The region used to generate anti-*Pf*RON2 sera (α-*Pf*RON2) and the region sequenced in the laboratory lines (sequencing) are indicated. Asterisks indicate polymorphic sites.