SUSCEPTIBILITY OF TWO KARYOTYPIC FORMS OF Anopheles aconitus (DIPTERA: CULICIDAE) TO Plasmodium falciparum AND P. vivax

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SUMMARY

Four laboratory-raised colonies of two karyotypic forms of Anopheles aconitus, i.e., Form B (Chiang Mai and Phet Buri strains) and C (Chiang Mai and Mae Hong Son strains), were experimentally infected with Plasmodium falciparum and P. vivax using an artificial membrane feeding technique and dissected eight and 12 days after feeding for oocyst and sporozoite rates, respectively. The results revealed that An. aconitus Form B and C were susceptible to P. falciparum and P. vivax, i.e., Form B (Chiang Mai and Phet Buri strains/P. falciparum and P. vivax) and Form C (Chiang Mai and Mae Hong Son strains/P. vivax). Comparative statistical analyses of the oocyst rates, average number of oocysts per infected midgut and sporozoite rates among all strains of An. aconitus Form B and C to the ingroup control vectors, An. minimus A and C, exhibited mostly no significant differences, confirming the high potential vector of the two Plasmodium species. The sporozoite-like crystals found in the median lobe of the salivary glands, which could be a misleading factor in the identification of true sporozoites in salivary glands were found in both An. aconitus Form B and C.

KEYWORDS: Anopheles aconitus; Karyotypic form; Susceptibility; Plasmodium falciparum; P. vivax.

INTRODUCTION

Malaria remains a major health problem of the world, particularly, in the tropical41. In Thailand, four species of malaria parasites are found; the most common species are Plasmodium vivax (52.50%) and P. falciparum (45.89%), while P. malariae (0.32%) and P. ovale (one case reported from Chiang Mai province in 1996) are rare, and 1.29% are mixed infections2. The disease is generally limited to rural communities living in and near forested areas, mountains and foothills, particularly, those residing in newly opened land settlements of semi-forested areas earning their living by growing agricultural crops, and in the areas near and along the borders with the neighboring countries of Kampuchea, Laos, Myanmar and Malaysia1,2.

So far, at least six anopheline species have been incriminated as primary and secondary vectors of malaria in Thailand. The primary vectors are Anopheles dirus Peyton & Harrison, An. minimus Theobald, and An. maculatus Theobald6,12,31,39. The taxa of above three vectors are all species complexes, and the members of each complex cannot be easily distinguished from each other4,15. The secondary vectors are An. sundaiscius (Roedentwald), An. aconitus Donitz, and An. pseudovillorni (Theobald), one of the member species of An. maculatus complex8,16,17,31. For An. aconitus, it was also incriminated as a vector of malaria in other countries, i.e., Indonesia20,21, Bangladesh22, Malaysia24 and India25.

As early as 1944, An. aconitus was considered a primary vector of malaria in Thailand35. However, such implications lacked confirmation until GOULD et al.23 found one An. aconitus female positive for both oocysts and sporozoites, and another one positive for only oocysts by dissection in the rice plain just north of Bangkok in April and August, respectively. In addition, the human-baited, whom bitten during April was subsequently got infection with P. vivax. This area was known to be endemic for P. vivax essentially to the exclusion of all other Plasmodium species. Thus, the authors concluded that An. aconitus was obviously the vector. Additional positive specimens of An. aconitus have not been reported in Thailand up to this time, except the reports of positive ELISA for circumsporozoite (CS) antigens from southern Thailand23. Recently, three karyotypic forms of An. aconitus, i.e., Form A (X1, X2, Y1), B (X1, X2, Y2), and C (X1, X2, Y3) have been incriminated sympatrically from northern Thailand, while Form D (X1, X2, Y4) has been reported from only Java, Indonesia1. Apparently, little is known about the vector potential of An. aconitus in northern Thailand, particularly among the karyotypic forms, which is intensively needed to confirm its vector status. Hence the present study reports the susceptibility of An. aconitus Form B and C strains from Chiang Mai...
province (northern Thailand), Mae Hong Son province (northwest Thailand), and Phet Buri province (southwest Thailand) to *P. falciparum* and *P. vivax*.

**MATERIALS AND METHODS**

Laboratory-raised *An. aconitus* Form B and C: Three provinces, the endemic areas of malaria in Thailand, i.e., Chiang Mai (Ban Pang Ma Daeng, Maetang district), Mae Hong Son (Ban Huai Pong Kan, Muang district) and Phet Buri (Ban Tha Salao, Nong Ya Plong district), the same localities as the previous studies by JUNKUM *et al.*\(^{19}\), were provided with 5% sucrose solution until age of 4-6 days, subsequently, they were fasted for 12 hours prior to the infections. The 12-hours fasted females of *An. aconitus* Form B and C, outgroup control mosquito-vector (*An. dirus* B), and ingroup control mosquito-vectors (*An. minimus* A and C) were put in a paper cup size 8.5 cm in diameter and 11 cm in depth (50 fasted females per cup for each species), and allowed to feed on heparinized blood containing gametocytes (gametocyte density of *P. falciparum* = 21 per 1 µL; *P. vivax* = 28, 17 and 34 per 1 µL in experiment 1, 2 and 3, respectively) using artificial membrane feeding techniques as described by CHOMCHARN *et al.*\(^{10}\). The fully engorged females were separated to small paper cups (diameter 6.5 cm, depth 8 cm) with 10 mosquitoes per cup and maintained in an incubator at 27 ± 2 °C, 70-80% RH. Cotton wool pad soaked with 5% sucrose solution was provided regularly and changed every other day until the time of dissections. Eight and twelve days after feeding, the infected mosquitoes were dissected and examined for oocysts in midguts and sporozoites in salivary glands, respectively.

**RESULTS**

Oocyst rates of *An. dirus* B, *An. minimus* A and C, and *An. aconitus* Form B and C: Details of oocyst rates are shown in Table 1. Observations on dissected midguts eight days after feeding revealed that *An. aconitus* Form B were susceptible to both *P. falciparum* and *P. vivax*, and Form C was susceptible to *P. vivax*. The 100% oocyst rates and 5.22 – 126.18 average number of oocysts per infected midgut obtained from *An. dirus* B, the outgroup control mosquito-vector, indicated the all feedings were conditional experiments, which reflected on the proper density and maturity of infective gametocytes in infected blood.

In the experimental feedings of *P. falciparum*, the oocyst rates and average number of oocysts per infected midgut of *An. aconitus* Form B (Chiang Mai and Phet Buri strains) did not differ significantly (*p* > 0.05) from the ingroup control-vector, *An. minimus* A. Similar results also were obtained from statistical analysis of the oocyst rates and average number of oocysts per infected midgut between *An. aconitus* Form B strains from Chiang Mai and Phet Buri provinces.

In the experimental feedings of *P. vivax*, mostly, the oocyst rates and average number of oocysts per infected midgut of *An. aconitus* Form B (Chiang Mai and Phet Buri strains) and C (Chiang Mai and Mae Hong Son strains) did not differ significantly (*p* > 0.05) from the ingroup control-vector, *An. minimus* A, except the average number of oocysts per infected midgut of *An. aconitus* Form C (Chiang Mai strain: experiment 1) was significantly less than that in *An. minimus* A, and Form C (Mae Hong Son strain: experiment 2) was significantly greater than that in the *An. minimus* A. Similar results also were recovered from statistical analysis of the oocyst rates and average number of oocysts per infected midgut between *An. aconitus* Form B (Chiang Mai strain) and C (Chiang Mai and Mae Hong Son strains) in experiment 1 and 2, except for only the average number of oocysts per infected midgut of *An. aconitus* Form C (Mae Hong Son strain) was significantly greater than that in the Form B (Chiang Mai strain) in experiment 2.

Oocyst and sporozoite rates of *An. dirus* B, *An. minimus* A and C, and *An. aconitus* Form B and C: Details of oocyst and sporozoite rates are shown in Table 2. The dissection of midguts of *An. dirus* B, *An. minimus* A and C, and all strains of *An. aconitus* Form B and C 12
Table 1

The oocyst rates of *An. dirus* B, *An. minimus* A and C and *An. aconitus* Form B and C after feeding on blood containing gametocytes of *P. falciparum* and *P. vivax*, all dissected 8 days after feeding

<table>
<thead>
<tr>
<th>Malaria species</th>
<th>Mosquito species</th>
<th>An. dirus B</th>
<th>An. minimus</th>
<th>An. aconitus Form</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No. (%)</td>
<td>No. (%)</td>
<td>No. (%)</td>
</tr>
<tr>
<td><em>P. falciparum</em></td>
<td>Oocyst rate (No.)</td>
<td>100 (20/20)</td>
<td>91.67 (11/12)</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Average No. oocysts per Infected midgut (range)</td>
<td>84.75 ± 45.53</td>
<td>18.64 ± 22.61</td>
<td>ND</td>
</tr>
<tr>
<td><em>P. vivax</em></td>
<td>Oocyst rate (No.)</td>
<td>100 (7/7)</td>
<td>100 (5/5)</td>
<td>100 (5/5)</td>
</tr>
<tr>
<td>Experiment 1</td>
<td>Average No. oocysts per Infected midgut (range)</td>
<td>69.71 ± 26.82</td>
<td>18.00 ± 6.93</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(39-118)</td>
<td>(11-27)</td>
<td>(4-19)</td>
</tr>
<tr>
<td></td>
<td>Experiment 2</td>
<td>90.00 ( 9/10)</td>
<td>83.33 (5/6)</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Average No. oocysts per Infected midgut (range)</td>
<td>5.22 ± 3.73</td>
<td>2.00 ± 1.41</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(1-11)</td>
<td>(1-4)</td>
<td>(1)</td>
</tr>
<tr>
<td></td>
<td>Experiment 3</td>
<td>100 (11/11)</td>
<td>81.82 (9/11)</td>
<td>50.00 (5/10)</td>
</tr>
<tr>
<td></td>
<td>Average No. oocysts per Infected midgut (range)</td>
<td>126.18 ± 55.92</td>
<td>22.78 ± 15.18</td>
<td>7.80 ± 8.23</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(34-223)</td>
<td>(2-54)</td>
<td>(1-22)</td>
</tr>
</tbody>
</table>

Mosquito strain; CM: Chiang Mai, MS: Mae Hong Son, PB: Phet Buri; Oocyst rate: NS, p > 0.05, *, p < 0.05 (Fisher exact test); Average No. oocysts per infected midgut: NS, p > 0.05, *, p < 0.05 (t-test, two-sided); ND: not done.

The dissection of salivary glands 12 days after feeding on blood containing *P. falciparum* and *P. vivax* gametocytes revealed that the oocyst rates were 95% (*An. aconitus* Form B: Phet Buri strain), 96.30% (*An. aconitus* Form B: Chiang Mai strain), 100% (*An. minimus* A), and 100% (*An. dirus* B), for *P. falciparum*, and 14.28-75% (all strains of *An. aconitus* Form B and C), 39.13% (*An. minimus* C), 11.11-100% (*An. minimus* A) and 15.79-100% (*An. dirus* B) for *P. vivax*. Statistical analyses of the oocyst rates among the ingroup control mosquito-vectors, *An. minimus* A and C, and all strains of *An. aconitus* Form B and C were not done because at this period (12 days of postblood meal) the mature oocysts from the midgut of the control vectors ruptured and yielded unreliable results. Nonetheless, the satisfactory percentages of oocyst rates obtained from both outgroup and ingroup control-vectors were confirmed the conditional experiments.

Comparative statistical analyses of sporozoite rates among *An. minimus* A and C, and four strains of *An. aconitus* Form B and C of all experiments exhibited no significant differences (p > 0.05), except only *An. aconitus* Form B (Phet Buri strain) differed significantly (p < 0.05) in the experimental feeding of *P. falciparum*.

Another interesting point in the present study is the sporozoite-like crystal found in the median lobe of salivary glands of both *An. aconitus* Form B and C, i.e., Form B: Chiang Mai strain 3.70% (1/27), Phet Buri strain 20% (4/20) (experimental feeding on *P. falciparum*); Form C: Mae Hong Son strain 28.57% (4/14) (experimental feeding on *P. vivax*). The sporozoite-like crystal rather resembles a true sporozoite, particularly, when it is inside a non-squashed salivary glands. The latter has regular spindle-shaped while the former has irregular, long or short with blunt or tapered end(s) (Fig. 1). It was stable in 0.85% normal saline solution for at least half an hour and after that the dissolve of the crystal could be obviously seen, and could be easily distinguished from the true sporozoite.

**DISCUSSION**

In order to incriminate a mosquito vector in an endemic area of mosquito-borne human diseases, it is necessary to confirm the
susceptibility rate in a laboratory-bred, clean mosquito colony that
has been fed on a carrier blood containing pathogens. Thus, by using
this criterion, the susceptibility test in an experimental laboratory is
still a useful tool when suspecting the potential vector of a certain
mosquito species. Nevertheless, the susceptibility alone does not imply
an important role in the transmission of disease in nature, whereas a
refractory one can entirely rule out its significance. According to the
vectorial status of An. aconitus to P. falciparum and P. vivax as

Table 2
The oocyst and sporozoite rates of An. dirus B, An. minimus A and C and An. aconitus Form B and C after feeding on blood containing gametocytes
of P. falciparum and P. vivax, all dissected 12 days after feeding

<table>
<thead>
<tr>
<th>Malaria species</th>
<th>Mosquito species</th>
<th>An. dirus B</th>
<th>An. minimus A</th>
<th>An. minimus C</th>
<th>An. aconitus Form B</th>
<th>An. aconitus Form C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CM</td>
<td>PB</td>
<td>CM</td>
<td>MS</td>
<td></td>
</tr>
<tr>
<td>P. falciparum</td>
<td>Oocyst rate (No.)</td>
<td>100 (23/23)</td>
<td>100 (11/11)</td>
<td>ND</td>
<td>96.30 (26/27)</td>
<td>95.00 (19/20)</td>
</tr>
<tr>
<td></td>
<td>Average No. oocysts per infected midgut (range)</td>
<td>85.00 ± 26.41</td>
<td>5.45 ± 3.86</td>
<td>ND</td>
<td>13.35 ± 15.60</td>
<td>28.58 ± 28.42</td>
</tr>
<tr>
<td></td>
<td>Sporozoite rate (No.)</td>
<td>95.65 (22/23)</td>
<td>100 (11/11)</td>
<td>ND</td>
<td>70.37 (19/27)</td>
<td>45.00 (9/20)</td>
</tr>
<tr>
<td>P. vivax</td>
<td>Experiment 1 Oocyst rate (No.)</td>
<td>100 (4/4)</td>
<td>100 (2/2)</td>
<td>ND</td>
<td>ND</td>
<td>100 (4/4)</td>
</tr>
<tr>
<td></td>
<td>Average No. oocysts per infected midgut (range)</td>
<td>28.00 ± 25.07</td>
<td>5.50 ± 4.95</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Sporozoite rate (No.)</td>
<td>100 (4/4)</td>
<td>100 (2/2)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Experiment 2 Oocyst rate (No.)</td>
<td>15.79 (3/19)</td>
<td>11.11 (1/9)</td>
<td>ND</td>
<td>16.67 (2/12)</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Average No. oocysts per infected midgut (range)</td>
<td>1.33 ± 0.58</td>
<td>1.00 ± 0.00</td>
<td>ND</td>
<td>2.00 ± 1.41</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Sporozoite rate (No.)</td>
<td>80.00 (6-64)</td>
<td>33.33 (2-9)</td>
<td>ND</td>
<td>16.67 (1-3)</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Experiment 3 Oocyst rate (No.)</td>
<td>100 (17/17)</td>
<td>66.67 (12/18)</td>
<td>39.13 (9/23)</td>
<td>ND</td>
<td>75.00 (9/12)</td>
</tr>
<tr>
<td></td>
<td>Average No. oocysts per infected midgut (range)</td>
<td>33.53 ± 20.12</td>
<td>2.92 ± 1.24</td>
<td>2.89 ± 1.83</td>
<td>ND</td>
<td>2.89 ± 2.15</td>
</tr>
<tr>
<td></td>
<td>Sporozoite rate (No.)</td>
<td>100 (17/17)</td>
<td>77.78 (14/18)</td>
<td>52.17 (12/23)</td>
<td>ND</td>
<td>66.67 (8/12)</td>
</tr>
</tbody>
</table>

Mosquito strain; CM: Chiang Mai, MS: Mae Hong Son, PB: Phet Buri; Sporozoite rate: NS, p > 0.05, *p < 0.05 (Fisher exact test, χ²-test for only experiment 3); ND: not done.

Fig. 1 - Salivary glands of An. aconitus Form B. (A) Showing free flow P. vivax sporozoites from the squashed salivary glands. Note, the regular spindle-shaped sporozoites (small arrow). (B) Showing sporozoite-like crystals inside the median lobe of salivary glands (small arrow). (C) Showing free flow sporozoite-like crystals from the squashed salivary glands. Note, the irregular, long or short, crystals with blunt or tapered end(s) (small arrow).
determined by the susceptibility tests using a laboratory-bred, clean mosquito colony have never been done and/or reported before this time. The high oocyst and sporozoite rates of An. aconitus Form B strains from Chiang Mai and Phet Buri provinces to infection with P. falciparum, and Form B strains from Chiang Mai and Phet Buri provinces and Form C strains from Chiang Mai and Mae Hong Son provinces to infection with P. vivax in the present study, confirming the secondary vector status of An. aconitus as reported by GOULD et al.22. Nonetheless, further investigations on the oocyst and sporozoite rates of wild-caught female An. aconitus in an endemic area of malaria in Chiang Mai province and/or other suspected areas should be done intensively to determine its role as a naturally transmissive vector.

Many Thailand Anopheles species have been reported positive ELISA for circumsporozoite (CS) antigens of P. falciparum and P. vivax by using the whole body and/or head and thorax of mosquitoes7,8,12,14,18,25. This diagnostic tool did not fundamentally incriminate the mosquito as the natural vector, since it could be detected CS protein from the developing oocysts8, soluble CS protein shed from oocysts and sporozoites8 and CS protein in various body parts. In addition, false positive P. falciparum and P. vivax detections by ELISA were reported33. However, the mosquito species which were highly susceptible to malarial infections could not be incriminated as the potential vectors, since sporozoite did not invade salivary glands7. Judged from the above evidences, therefore, the combining of positive ELISA for CS antigens with sporozoite rate of a laboratory-bred, clean Anopheles colony should be the important evidences prior to the incrimination of potentially natural vector.

Additionally, the sporozoite-like crystal found in the median lobe of salivary glands of An. aconitus Form B and C might be one of the important, missed leading factor in the identification of true sporozoite in salivary glands of the laboratory susceptibility experiments and/or wild-caught Anopheles females. Similar results have been reported in An. sinensis Form A and B7.

RESUMO

Susceptibilidade de duas formas cariotípicas de Anopheles aconitus (Diptera: Culicidae) a Plasmodium falciparum e P. vivax

Quatro colônias desenvolvidas em laboratório, de duas formas cariotípicas de Anopheles aconitus i.e. forma B (cepa Chiang Mai e Phet Buri) e C (Cepa Chiang Mai e Mae Hong Son), foram infectadas experimentalmente com Plasmodium falciparum e P. vivax usando técnica de alimentação com membrana artificial e dissecados oito e 12 dias após alimentação da média de oocistos e esporozoitos, respectivamente. Os resultados revelaram que An. aconitus forma B e C foram suscetíveis ao P. falciparum e P. vivax. Forma C (cepa Chiang Mai e Mae Hong Son/P. vivax). Análises estatísticas comparativas das taxas de oocistos, número médio de oocistos por intestino médio infectado e taxas de esporozoitos entre todas as cepas de An. aconitus formas B e C ao grupo interno de vetores controles, An. minimus A e C, não exibiram nenhuma diferença significante, confirmando o alto potencial vetor das duas espécies de Plasmodium. Os cristais semelhantes a esporozoitos encontrados no lobo médio das glândulas salivares que poderiam ser um fator enganoso na identificação de esporozoitos verdadeiros nas glândulas salivares foram encontrados em ambos An. aconitus forma B e C.

ACKNOWLEDGEMENTS

The authors sincerely thank the Thailand Research Fund (TRF: BRG/14/2545) and the Royal Golden Jubilee Ph.D Program (Grant No. PHD/0044/2546) for financially supporting this research project, Professor Supot Wudhikarn, Dean of the Faculty of Medicine, Chiang Mai University, for his interest in this research, and the Faculty of Medicine Endowment Fund for Research Publication for its financial support in defraying publication costs.

REFERENCES

1. ANNUAL REPORT - Thailand, Division of Malaria, Department of Communicable Disease Control, Ministry of Public Health, 1998.
2. ANNUAL REPORT - Thailand, Division of Malaria, Department of Communicable Disease Control, Ministry of Public Health, 2002.


Received: 24 November 2004
Accepted: 5 August 2005