



Research Article

## Prevalence of *An. culicifacies* sibling species and vector incrimination of *Anopheles* mosquitoes in Magway and Bago Region of Myanmar

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### ARTICLE INFO:

#### Article History:

Received: 01/07/2016  
Revised: 02/08/2016  
Accepted: 16/08/2016  
Available Online: 01/09/2016

#### Keywords:

Malaria,  
*Anopheles culicifacies*,  
monoclonal antibodies,  
Bowbin village,  
Christopher stage ovary,

**Abstract:** The present study was conducted in Thayetchaung village (Bowbin dam area) of Ghobinkauk Township, Bago Region and Maybinthar village, Myothit Township Magway Region to determine the present of individual vector species and the prevalence of sibling species *Anopheles culicifacies* from December 2013 to November 2014. Adult Anopheline mosquitoes were collected by different collection methods according to WHO guideline. Christopher stage ovaries were collected from *An. culicifacies* for ovarian nurse cell chromosome study and head and thorax were dissected for vector incrimination study. During the study periods, a total of 1727 and 507 female *Anopheles* comprising 11 and 10 species were collected from Thayetchaung and Maybinthar villages respectively. *An. vagus*(576) was the predominant species followed by *An. annularis*(430) > *An. philippnensis*(238) > *An. maculatus* (164) > and *An. culicifacies*(150) in Thayetchaung. In Maybinthar, highest density of *An. culicifacies*(237) were collected, followed by *An. annularis*(60) > *An. maculatus*(42). The main vector *An. minimus* was observed 31 in Bowbin and 24 in Maybinthar. Man biting rate of *An. minimus* was found 1.25, 1.25 indoor and 1.5, 1.00 outdoor bite/man/night and for *An. culicifacies* was found 1.25, 1.25 indoor and 3.00, 5.25 outdoor bite/man/night in Thayetchaung and Maybinthar respectively. *An. culicifacies* B was present in both study villages and species A, C, D and E were absent. Species B was predominantly higher in the study village of Myothit Township (61.24%) than Thayetchaung village Ghobinkauk Township (38.76%). Vector incrimination study found that only 1 well of pool samples of *An. minimus* was found 3.226% (1/31) of *Pv247* sporozoite positive in Thayetchaung village. *An. minimus* was a potential vector of malaria. None of the specimens belonging to *An. culicifacies* B was found with circumsporozoite antigen of any of the *Plasmodium* species in both areas. Therefore *An. culicifacies* is not important vector of malaria, *An. dirus* and *An. minimus* are the major vectors of malaria in Myanmar.

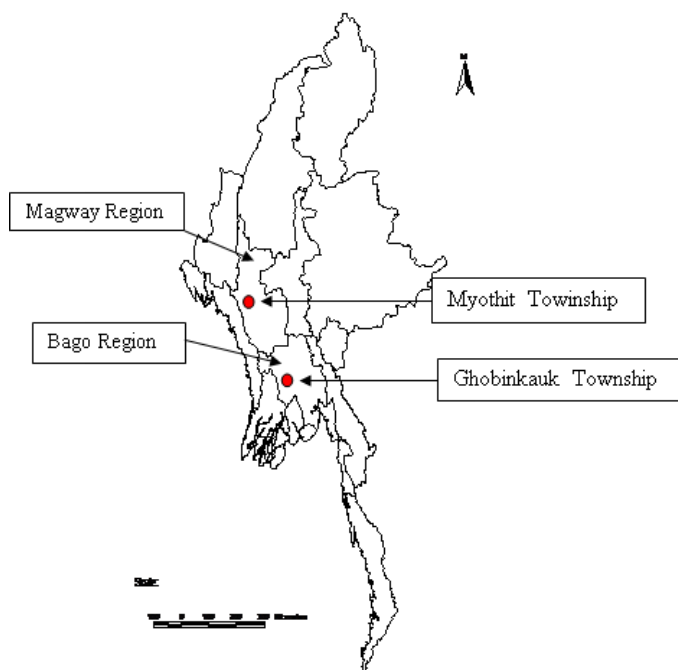
### INTRODUCTION

Malaria remains a major health problem in Myanmar.

According to Ministry of Health (MOH) malaria has been a priority public health problem in Myanmar. According to Health in Myanmar 2014, morbidity and mortality rate were 24.35/1000 population and 12.62/100000 population respectively in 1990 and 6.44/1000 population and 0.48/1000,000 populations respectively in 2013 [1]. *Plasmodium falciparum* is the predominant species but now *P. vivax* is gradually rising in Myanmar [2]. These parasites are transmitted by *Anopheles* mosquitoes. Although more than 37 *Anopheles* mosquitos' species are exist in Myanmar, only few species are involved in the transmission of malaria. *An. dirus* and *An. minimus* are the main vectors of malaria but *An. maculatus*, *An. vagus*, *An. annularis*, *An. philippnensis*,

*An. stiphensi* and *An. culicifacies* are secondary or suspected vectors of malaria in Myanmar [3]. The *Anopheles* mosquitoes breeding sites are very greatly depending on the climatic conditions and availability of water. In Myanmar *An. minimus* and *An. dirus* are considered to be one of the most efficient malaria vectors due to their ability to adapt so rapidly. Previously *An. minimus* was found foothill and forest fringe but now it is found in rice field in plain areas. *Anopheles dirus* species are also bred in deep forest areas but now their larvae were found in men made wells in Mon State and Thanintheryi Region [4, 5] because certain environmental changes like deforestation and vegetation clearance for crop plantations and also rapid growth of population and installation of new rural areas may lead to an increase abundance of mosquito larval habitats [6, 7]. Well breeding *An. dirus* from Mon State was cyto-taxonomically identified as sibling species D [8]. A recent study of cyto-taxonomic identification in Anopheline

mosquitoes in Kamamaung Township Kayin State revealed that *An.minimus* A, *An.dirus* D and *An.culicifacies* B sibling species were present in this area [9].



**Figure 1: Thayetchaung village, Bowbin dam areas**

*Anopheles maculatus* are main vector of malaria in Malaysia [10] but it is a secondary vector of malaria in Myanmar and widely distributed in hilly and plain areas of Southeast Asia including Myanmar. About 30 *Anopheles* taxa have been identified so far as species complexes they are important vectors of malaria in different parts of the World. Members of a species complex, commonly known as sibling species/isomorphic or cryptic species, are reproductively isolated evolutionary units with distinct gene pools and hence, differ in biological characteristics which determine their potential in the transmission of diseases. *Anopheles culicifacies* species complex are widespread distribution in Asia, from Iran, Afghanistan and Pakistan in west and India to Bangladesh, Myanmar and Thailand in the east. It is also found in Nepal and Southern China in the North and Sri Lanka in South [11]. *Anopheles culicifacies* is a fresh water breeder and found three sibling species A, B, and D were recorded in Myanmar [12, 8] and India [13] but in Sri Lanka *An.culicifacies* sibling species E is breeding in brackish waters [14]. *Anopheles culicifacies* is a complex of 5 sibling species designated as species A, B, C, D and E. Species B is not the vector but all other species transmit malaria, although their vectorial capacity varies greatly [15]. It is the vector of rural and peri-urban malaria in the peninsular India [13]. Species A and D are efficient malaria vectors, whereas species B is regarded as a poor vector of malaria in India [16]. In Sri Lanka, however, species B has long been considered to be an important malaria vector. *Anopheles culicifacies* of A-E have been detected in Sri Lanka with species E being incriminated as the major malaria vector of *P. falciparum* and *P. vivax* [17, 18]. *Anopheles culicifacies* is the secondary vector of malaria and widely distribution in Myanmar [12, 19]. Each sibling

species may have its own distribution and biology. Large number of sibling species B of *An.culicifacies* adult and larvae were found in hyper-endemic foothill areas of Paukkaung Township, Bago Region in Myanmar [20, 21]. Main vectors of *An.dirus* and *An.minimus* are lurking in some malaria endemic areas in Myanmar where the suspected vectors as *An. culicifacies*, *An. maculatus* and *An.annularis* are presented abundantly [12]. Vector incrimination is a prerequisite for understanding the role of Anopheline in malaria transmission and has been used to determine which species is the most important vector [22-24]. It also is used to compare the contribution of individual species to overall malaria transmission [25].

Very few literatures were available about sibling species of *Anopheles* in Myanmar. Therefore the present study was undertaken in Bawbin dam area of BagoYoma forest fringe area and Maypinthar village of plain area in Magway Regions to fine out the *An.culicifacies* sibling species prevalence and potential vector of malaria.

## MATERIALS AND METHODS

### Study areas and study period:

(1) Entomological survey was conducted in Thayetchaung village (Bawbin dam area), Ghobinkauk Township Bago Division (2) Maypinthar village Myothit Township Magway Region seasonally from December 2013 to November 2014 (Fig. 1).

### Thayetchaung village (Bowbin dam area) Ghobinkauk Township Bago Division

Thayetchaung village (Bowbin dam area) is situated beside the Bowbin dam. It is 15 kilometer away from Puiy - Paukkaung road. The population is about 1000. Almost all of the households have one to two mosquito nets which are used for the prevention of mosquitoes bite.

### Maypinthar village Myothit Township Magway Region

Maypinthar village, Myothit Township Magway Region was selected for the collection of *Anopheles* mosquitoes and larvae. Maypinthar village is situated beside the Magway Naypyidaw road. The village is 10 kilometer away from Son dam. Maypinthar Myothit car road is across the village. The population is about 2000. Ninety percent of the households have one to two mosquito nets.

### Adult mosquito's collection

Adult mosquitoes were collected by WHO sucking tube with several mosquitoes catching methods using cattle baited big net trap (330x330x180cm) collection, indoor and outdoor human bait mosquito collection and indoor morning resting mosquito in both areas seasonally at 18:00 to 01:00 hours for 5 days.

### Larvae collection

*Anopheles* Larvae were collected in and around 3-kilometer radius from the study villages. Water pools, sand pools, footprints, rock pools and slowly running water in river band were detected.

## Identification of Mosquitoes

Wild caught adult mosquitoes and adult mosquitoes emerged from pupae collection during larva surveys were identified by morphologically identification method using adult *Anopheles* mosquito identification keys of different authors. [19, 26-29].

## Ovary collection and processing

Blood fed *An.culicifacies* were separate out by the help of sucking tube to paper cup with glucose for ovary development. Humidity and temperature were maintained by covering with water soak towel (27°C, 90%). When ovary development was reached to semi-gravid stage (Christophe stage) ovary specimens were dissected and preserved in Cornoy's fixative in screw type bottle. Samples were store in 4°C in refrigerator till ovarian nurse cell polytene chromosome study.

## Identification of sibling species

Preserved ovaries of *An.culicifacies* were processed in 50% propionic acid and stained with 2% lacto-acetoorcein stain according to the method Green and Hunt [30] for making polytene chromosome preparations. The chromosome complement of individual mosquitoes was examined under compound light Olympus microscope for species diagnostic inversions used for identification of the members of *An.culicifacies* complex [11].

## Salivary gland collection

An average of 575.6 female mosquitoes collected per seasonal from study areas was tested for species-specific circumsporozoite protein (CSP) using standard procedure for the enzyme-linked immunoabsorbent assay (ELISA) [31,32]. The head and the thorax of individual mosquitoes were separated from the body using a clean blade (cleaned with alcohol after each dissection) and 10 each head and thorax of same species was put in individual microfuge tubes containing silica gel as preservative for detection of

sporozoite antigen in salivary glands. All collected samples were store in a freezer at - 4°C. This procedure reduced the probability of detection of CS antigen from other parts of the body [33]. Head and thorax of individual vectors were coded identically for correlating the results,

## Vector incrimination

Head and thorax of individual *Anopheles* species and *An.culicifacies* specimens identified at sibling species level by cyto-taxonomic technique were processed for sporozoite detection in the salivary glands. Homogenates of 10 pools head and thorax of individual species in grinding buffer was put into each wall and tested for the present of Circumsporozoite proteins using *P.falciparum*, *P.vivax*<sub>210</sub> and *P.vivax*<sub>247</sub> specific monoclonal antibodies by ELISA [31,32].

## Statistical analysis

Data entry and analysis were carried out using Microsoft excel software. Sibling species identification and sporozoite positivity were determined for the abundance of sibling species and *Plasmodium* parasite species.

## RESULTS

Table 1 shows that a total of 1727 female *Anopheles* comprising 11 species from Thayetchaung village (Bowbin dam areas) and 507 female *Anopheles* comprising 10 species from Maypinthar village were collected during the study periods. The highest number (576) of *An.vagus* was collected, followed by *An.annularis* (430), the lowest number (4) of *An.kochi* was collected in Bowbin dam areas. In Maypinthar, highest number of *An.culicifacies* (237) was collected, followed by *An.annularis* (60), the lowest number (2) of *An.tessellatus* was observed. The main vector *An.minimus* was observed (31) in Thayetchaung village, Bowbin area and (24) in Maypinthar village.

**Table 1:** Distribution of *Anopheles* mosquitoes species in two different areas

| Species                      | Thayetchaung village Ghobinkauk Township<br>Bago Division (Bowbin dam areas) |                           |                            |                | Maypinther village, Myothit Township Magway<br>Region |                           |                            |                |
|------------------------------|--|---------------------------|----------------------------|----------------|---|---------------------------|----------------------------|----------------|
|                              | Total<br>collected<br>mosquitoes   | Indoor<br>biting<br>(MBR) | Outdoor<br>biting<br>(MBR) | Animal<br>bait | Total<br>collected<br>mosquitoes                      | Indoor<br>biting<br>(MBR) | Outdoor<br>biting<br>(MBR) | Animal<br>bait |
| <i>An. minimus</i> (MV)      | 31   | 5<br>(1.25)               | 6<br>(1.5)                 | 20             | 24  | 5<br>(1.25)               | 4<br>(1.0)                 | 15             |
| <i>An. annularis</i> (SV)    | 430  | 2<br>(0.5)                | 8<br>(2.0)                 | 420            | 60  | 0                         | 1<br>(0.25)                | 59             |
| <i>An. aconitus</i> (SV)     | 20   | 0                         | 0                          | 20             | 34  | 0                         | 0                          | 34             |
| <i>An. barbirostris</i>      | 73   | 2<br>(0.5)                | 5<br>(1.25)                | 66             | 37  | 0                         | 2<br>(0.5)                 | 35             |
| <i>An. culicifacies</i> (SV) | 150  | 5<br>(1.25)               | 12<br>(3)                  | 133            | 237   | 5<br>(1.25)               | 21<br>(5.25)               | 211            |
| <i>An kochi</i>              | 27   | 0                         | 0                          | 27             | 0   | 0                         | 0                          | 0              |
| <i>An. karwari</i>           | 4  | 0                         | 0                          | 4              | 0   | 0                         | 0                          | 0              |
| <i>An. maculatus</i> (SV)    | 164  | 3<br>(0.75)               | 6<br>(1.5)                 | 155            | 42  | 0                         | 1<br>(0.25)                | 41             |

**Table 1** (continued)

| Species                      | Thayetchaung village Ghobinkauk Township Bago Division (Bowbin dam areas) |                     |                      |             | Maypinther village, Myothit Township Magway Region |                     |                      |             |
|------------------------------|---|---------------------|----------------------|-------------|--|---------------------|----------------------|-------------|
|                              | Total collected mosquitoes  | Indoor biting (MBR) | Outdoor biting (MBR) | Animal bait | Total collected mosquitoes                         | Indoor biting (MBR) | Outdoor biting (MBR) | Animal bait |
| <i>An.philippnensis</i> (SV) | 238   | 0                   | 5<br>(1.25)          | 233         | 25   | 0                   | 0                    | 25          |
| <i>An. stephensi</i> (SV)    | 14  | 0                   | 0                    | 14          | 21   | 0                   | 0                    | 21          |
| <i>An. tessellates</i> (SV)  | 0   | 0                   | 0                    | 0           | 2  | 0                   | 0                    | 2           |
| <i>An. vagus</i> (SV)        | 576   | 0                   | 6<br>(1.5)           | 570         | 25   | 0                   | 0                    | 25          |
| Total                        | 1727  | 17                  | 48                   | 1662        | 507  | 10                  | 29                   | 468         |

{MV= main vector, SV=suspected vector, MBR= Man biting rate (bite/man/night)}

Indoor and outdoor man biting rate of main vector *An.minimus* were found 1.25 and 1.5 bite/man/night in Bawbin and 1.25 and 1.00 bite/man/night in Maypinthar. The man-biting rate of *An.culicifacies* was 1.25 and 1.25 bite/man/night in indoor and 3.00 and 5.25 bite/man/night in outdoor in Thayetchaung village and Maypinthar villages respectively.

The sibling species composition of the *An.culicifacies* complex in the study areas as revealed by cyto-taxonomy using ovarian nurse cell chromosome study is given in Table 2. *Anopheles culicifacies* sibling species B was present in both study villages and species A, C, D and E were absent. Species C is xab/2+g<sup>1</sup>h<sup>1</sup> standard arrangement of chromosome and a vector of malaria and B is xab2g<sup>1</sup>+h<sup>1</sup> standard arrangement of chromosome and a poor vector of malaria. Species B was predominantly present in Maypinthar village of Myothit Township (61.24%), and (38.76%) in Thayetchaung village, Bawbin dam area. Table 3 shows that only one well of pool samples of *An.minimus* was found harboring circumsporozoite antigen of *Pv*<sub>247</sub> with a sporozoite rate of

1/31(3.226%) in Thayetchaung village Bowbin dam area. None of the specimens belonging to *An.culicifacies* B was found with circumsporozoite antigen of any of the *Plasmodium* species in both areas.

**Table 2:** *Anopheles culicifacies* sibling species composition in Thayetchaung and Maypinthar villages

| Study areas                   | Total collection | Cyto-taxonomic identification | Relative proportion |
|-------------------------------|------------------|-------------------------------|---------------------|
| Thayetchaung village (Bowbin) | 150              | Species B                     | 38.76%              |
| Maypinthar village            | 237              | Species B                     | 61.24%              |
| Total                         | 387              | Species B                     | 100%                |

**Table 3:** Detection of Circumsporozoite (CS) antigens of *Plasmodium* species in the main vector (MV) and suspected vector (SV) of malaria by ELISA method from Thayetchaung and Maypinther villages

| Species                      | Thayetchaung village Ghobinkauk Township Bago Division (Bowbin dam areas) |           |                          |                          |                   | Maypinther village, Myothit Township Magway Region |           |                          |                          |                   |
|------------------------------|---|-----------|--------------------------|--------------------------|-------------------|--|-----------|--------------------------|--------------------------|-------------------|
|                              | Total collected mosquitoes  | <i>Pf</i> | <i>Pv</i> <sub>210</sub> | <i>Pv</i> <sub>247</sub> | Sporozoite + rate | Total collected mosquitoes                         | <i>Pf</i> | <i>Pv</i> <sub>210</sub> | <i>Pv</i> <sub>247</sub> | Sporozoite + rate |
| <i>An. minimus</i> (MV)      | 31  | -         | -                        | 1                        | 3.226%            | 24   | -         | -                        | -                        | 0                 |
| <i>An. annularis</i> (SV)    | 430   | -         | -                        | -                        | 0                 | 60   | -         | -                        | -                        | 0                 |
| <i>An. aconitus</i> (SV)     | 20  | -         | -                        | -                        | 0                 | 34   | -         | -                        | -                        | 0                 |
| <i>An. barbirostris</i>      | 73  | -         | -                        | -                        | 0                 | 37   | -         | -                        | -                        | 0                 |
| <i>An. culicifacies</i> (SV) | 150   | -         | -                        | -                        | 0                 | 237  | -         | -                        | -                        | 0                 |
| <i>An kochi</i>              | 27  | -         | -                        | -                        | 0                 | 0  | -         | -                        | -                        | 0                 |
| <i>An. karwari</i>           | 4   | -         | -                        | -                        | 0                 | 0  | -         | -                        | -                        | 0                 |
| <i>An. maculatus</i> (SV)    | 164   | -         | -                        | -                        | 0                 | 42   | --        | -                        | -                        | 0                 |
| <i>An.philippnensis</i> (SV) | 238   | -         | -                        | -                        | 0                 | 25   | -         | -                        | -                        | 0                 |
| <i>An. stephensi</i> (SV)    | 14  | -         | -                        | -                        | 0                 | 21   | -         | -                        | -                        | 0                 |
| <i>An. tessellates</i> (SV)  | 0   | -         | -                        | -                        | 0                 | 2  | -         | -                        | -                        | 0                 |
| <i>An. vagus</i> (SV)        | 576   | -         | -                        | -                        | 0                 | 25   | -         | -                        | -                        | 0                 |
| Total                        | 1727  | -         | -                        | 1                        | 0.058%            | 507  | -         | -                        | -                        | 0                 |

MV= main vector, SV=suspected vector, *Pf*= *Plasmodium falciparum*, *Pv*<sub>210</sub>= *Plasmodium vivax*<sub>210</sub>, *Pv*<sub>247</sub>= *Plasmodium vivax*<sub>247</sub>,

## DISCUSSION

In both study areas over 85% of the total population are farmer, remaining are woodcutter, charcoal baker, hunter, and fisher man and about 60% of the geographical areas is forested foot hill areas and malaria is endemic. Study carried out in different areas reported *An. dirus* and *An. minimus* are the major vectors of malaria although *An.culicifacies*, *An.vagus*, *An.maculatus*, *An.annularis* and *An.philippinensis* are the secondary or suspected vectors in Myanmar [3]. Present study found that high prevalence and dominantly present of *An. vagus* and *An.annularis* in both areas followed by *An.maculatus* and *An.culicifacies* although main malaria vector *An.minimus* found moderately distributed in both areas. This is due to present of paddy fields, slow running water streams, sand pools, water pools in stream bank, and channels around the villages are the referred breeding sites of above *Anopheles* mosquitoes [19, 20, 34]. Although other researcher revealed that *An.minimus* was caught high number in Laikkyi village, Taikkyi Township, Yangon Region which area is a foothill area of Bago mountain Range [35]. Analysis of sibling species composition of *An.culicifacies* showed that species B were abundantly present in both areas. The prevalence of species B is not equal proportion. *An.culicifacies* of both areas were found to be exclusively zoophilic as revealed by catching high number of *An.culicifacies* adult by animal bait K net. It is the first time report on the sibling species composition of *An.culicifacies* in both areas. Same sibling species B was observed in Paukkaung Township and Madaya Township [21] although MyatMyat Thu et al., [8] identified as three sibling species of *An.culicifacies* A, B, and D in Myanmar. Recently high number of *An.culicifacies* B was found in KatineHtit and Kaine Taw villages Kamamaung Township Kayin State [9]. High number of *An.culicifacies* B was collected from animal bait than indoor and outdoor human bait collection and in all head and thorax samples found to be no naturally infected with *Plasmodium* species. *An.culicifacies* has been reported to be more exophilic than endophilic with low man biting densities in outdoor and indoor collection and found highly zoophilic in nature [36]. Very low number of *An.culicifacies* B was collected resting indoors in human dwellings in morning collection. Similar result has been found in Chhattisgarh and Orissa in India, *An.culicifacies* and *An.fluviatilis* were found resting in human dwelling as the cattle sheds were open type without walls [37, 38].

A study of Yesitkan village also found sporozoite positive in salivary gland of *An.minimus* and suspected vector *An.maculatus* by ELISA test [39]. *Anopheles nuneztovari* is an important vector of Vivax malaria in other parts of the South America [40] and was incriminated as a vector of *Pv210* in the Brazilian amazon [41]. In Pakistan high number of *An.culicifacies* B was found sporozoite positive by vector incrimination study in laboratory [42] but the species B is a main vector of malaria in Sri Lanka [43]. Vector incrimination study of other pools samples of suspected vector of *Anopheles* mosquitoes as *An.maculatus*, *An.vagus*, *An.annularis* and *An.philippinensis* were found negative for circumsporozoite antigen of *Pf*, *Pv210* and *Pv247* by ELISA method. *An. maculatus* was recently reported as vector of malaria from research conducted Bokpin Township, Tanintharyi Region

near the Thai border (Unpublished data 2013). Maung Maung Mya and his associates observed that *An.minimus* was 1.07% *Pf* circumsporozoite antigen positive and *An.dirus* was found 3.7% *Pv210* circumsporozoite antigen positive in Kamamaung Township, Kayin State [9]. Tun Lin et al., [34] revealed that *An. vagus* and *An. maculatus* collected from Thabyewa village Oktwin Township were found circumsporozoite antigen positive by vector incrimination study using ELISA technique.

Study revealed that suspected vectors are predominantly present in both study areas of Thayetchaung village Bawbin dam area, Ghobinkauk Township Bago Region and Maypinthar village Myothit Township Magway Region, and all collected *An.culicifacies* from both areas was found sibling species B by polythene chromosome study and also vector incriminated study showed they are non-vectors of malaria in these areas. In conclusion all four malaria species, *P. vivax* was circulating in the *An.minimus*. *Anopheles minimus* was the most important malaria vector in Bowbin dam areas of BagoYoma foothill area in Ghobinkauk Township Bago region due to lack of *An.dirus* in this area in the study period. The collected mosquitoes samples from Maypinthar were found negative for circumsporozoite antigen, it is suggested that they are not important vectors at the study area.

## COMPETING INTEREST

The authors have declared that no competing interests exist.

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