NRCPD-OUAVM Joint Research Report

Date: June 2, 2022

Project no: 2021-joint-7

1. Principal investigator

Name: Morakot KAEWTHAMASORN

Position: Associate professor

Affiliation: Faculty of Veterinary Science, Chulalongkorn University

2. Project title:

Pathogenicity of the buffalo malaria parasites

3. Collaborating research group members at NRCPD

Name: Masahito Asada

Position: Associate Professor

4. Research period (in mm/dd/yyyy, and total number of years)

April 1, 2021–March 31, 2022 (1-year continuing project)

5. Purposes and objectives

This study aimed to study on pathogenicity of *Plasmodium bubalis* infection in buffalo calves and its vector.

6. Outline of research process

We carried out several surveys in Thailand i) from Asian water buffaloes in smallholder farms in Nakhon Ratchasima in December 2019 (n = 22); ii) from Murrah dairy buffaloes in Chachoengsao in June 2020 (n = 45); iii) in November 2021 (n = 45) and iv) from Asian water buffaloes in smallholder farms in Nan in December 2020 (n = 65). All blood samples (n=177) were collected from the jugular vein of buffaloes and kept in ACD tubes. DNA samples from buffalo blood underwent nested PCR screening for *Plasmodium* using primers DW2 and DW4 targeting cytochrome b (*cytb*) gene as the outer primers and NCYBINF and NCYBINR for the inner primers. Subsequently, *Plasmodium*-positive samples were further confirmed using primer sets targeting the cytochrome c oxidase subunit 1 (*cox1*) and *18S* ribosomal RNA (*18S*) genes.

Naturally infected buffalo, which has already been confirmed by microscopic examination and molecular diagnostic, was followed up for 14 consecutive days. Afterward, the infected buffalo was monitored weekly until day 56. During the observation period, the following data were collected: body temperature, clinical signs, anemia, and appetite. Every day for 14 consecutive days, one ml of blood samples was drawn from the jugular vein and kept in a tube containing ACD solution (two infected buffaloes). Blood samples from infected buffaloes were examined by nested PCR and qPCR for each day. Parasite burden was measured by real-time quantitative PCR. Real-time quantitative PCR (qPCR) was performed using PbubCox1-F32 and PbubCox1-4B3 primers. Parasitemia levels

were counted from thin blood smears under a microscope.

A total of 1,652 female mosquitoes were collected from two sampling sites Chachoengsao (n = 1,571) and Ratchaburi (n = 81) (Table 5). Anopheline mosquitoes and non-Anopheline mosquitoes were screened for the presence of malaria parasites. Adult female mosquitoes were grouped based on their morphology. A total of 268 female mosquitoes (214 *Anopheles* spp., 24 *Culex* spp., 24 *Aedes* spp., and 6 *Mansonia* spp.) were examined. Among 214 *Anopheles* spp., 133 were collected from a buffalo farm where concurrently water buffalo samples were shown to be *P. bubalis* positive). Anopheline mosquitoes were carefully dissected within three days after collection to obtain the salivary glands of each mosquito. Mosquito pools were made (head and thorax including dissected salivary glands) following morphological identification and were subsequently confirmed by molecular identification. Each pool was made up of one to three mosquitoes from the same groups depending on sample availability. DNA samples extracted from mosquitoes were subjected to PCR screening for *P. bubalis* based on *cytb*, *cox1*, and *18S* rRNA genes. *Plasmodium'*s-positive samples that underwent PCR confirmation were subsequently subjected to sequencing. Anopheline mosquito species were conducted by sequencing the PCR products targeting *cox1*, cytochrome c oxidase subunit 2 (*cox2*), and internal transcribed spacer 2 (*ITS2*) markers.

7. Outline of research achievements

7.1 Detection of malaria parasite in blood samples

A total of 177 buffalo blood samples were collected and underwent screening for *P. bubalis* infection. We found that 2 out of 45 buffaloes in Chachoengsao collected in 2020 (THBuff20_37 and THBuff20_39) tested positive for the malaria parasite from day 1 after observation until day 49, being undetected. Parasite burden was measured by real-time quantitative PCR. Parasite burden in both buffaloes was detected as low as 15 x 10^4 copies per microliter of blood sample until day 28, as shown in Figure 1. Co-infection was detected in both infected buffaloes. *Theileria* sp. and *Anaplasma* sp. were detected in THBuff20_37, while THBuff20_39 was co-infected with *Theileria* sp only. All co-infection were tested by PCR using published primers PanPiroF1 and PanPiroR1 for piroplasma infection and EhrliAnaplas16SR5U-EhrliAnaplas16SR3U/ EhrliAnaplas16FWD-EhrliAnaplas16REV for *Anaplasma* infection. We recorded that the buffalo which were infected by *P. bubalis* had no symptoms. Body temperature in both infected buffaloes showed no significant deviation from normal ranges during the observation period (Figure 2). During the infection, the trophozoite stage was predominated. Evaluation of parasitemia levels Giemsa-stained blood smears were recorded as low parasitemia (<0.01-0.09%) for 14 consecutive days.

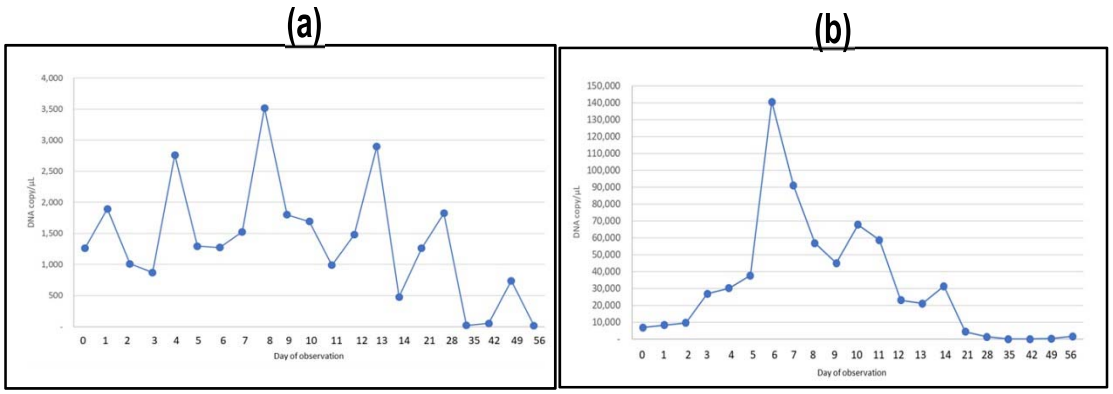

Figure 1. Malaria parasite's copy number measured by qPCR in buffalo during the observation period (a) THBuff20_37, (b) THBuff20_39

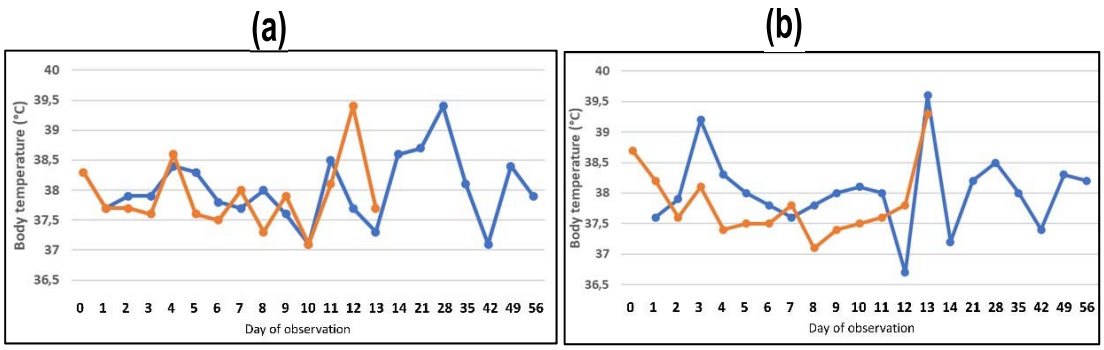

Figure 2. Body temperature malaria-infected buffaloes during observation period (a) THBuff20_37, (b) THBuff20_39. Blue and orange lines indicate the observed temperature in the morning and evening, respectively.

7.2 Morphological identification of mosquitoes' samples

Among collected mosquitoes, anopheline mosquitoes accounted for 13.01%, 70.94% for *Culex* spp., 12.41% for *Aedes* spp., 0.36% for *Mansonia* spp., and 3.27% were unidentifiable due to body part's destruction. Among 214 identified anopheline mosquitoes, nine different *Anopheles* spp. were identified belonging to Barbirostris, Hyrcanus, Funestus, Ludlowae, and Jamesii groups. *An. peditaeniatus*, which is a member of the Hyrcanus group, was predominant in the buffalo farm in Chachoengsao, while *An. aconitus* was the most common mosquito on the Ratchaburi buffalo farm, as depicted in Figure 3.

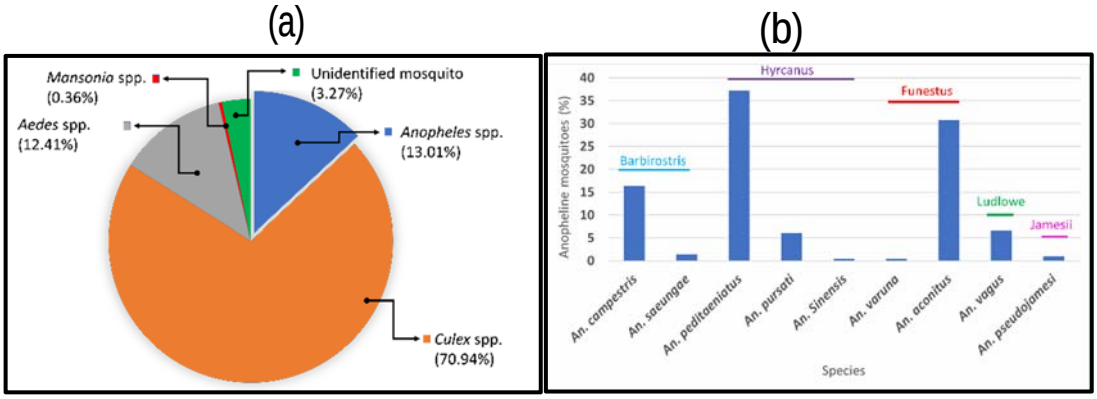

Figure 3. Chart illustrating the number of mosquitoes, according to morphological identification. (a) Percentages of each genus collected in this study as shown above. (b) *Anopheles* mosquitoes collected from buffalo farms based on group and species.

7.3 Molecular identification of anopheline mosquitoes

Among collected anopheline mosquitoes, 128 (59.81%) were selected for DNA extraction, which was made up of 102 pools per species depending on the availability of mosquitoes (1-3 individuals per pool). The number of each pool was subjected for sequencing and the results as follows: *An. wejchoochotei/An. campestris* (n = 35, 23 pools), *An. peditaeniatus* (n = 80, 46 pools), *An. vagus* (n = 14, 7 pools), *An. varuna* (n = 1, 1 pool), *An. pseudojamesi* (n = 2, 1 pool), *An. saeungae* (n = 3, 3 pools), *An. pursati* (n = 12, 12 pool), and *An. aconitus* (n = 66, 8 pools) *An. sinensis* (n = 1, 1 pool) (Table 1).

Table 1. Summary of *P. bubalis*'s PCR screening results of anopheline mosquitoes collected from the buffalo farm

Sampling sites	Group	No. collected	No. of pools	No. of positive pools			No. of pools sequenced for mosquito genes and determined species name
				cytb	*18S rRNA*	*cox1*	
Chachoengsao	Barbirostris	35	23	2	2	2	17 (*An. wejchoochotei* or *An. campestris*)
	Hyrcanus	80	46	1	1	1	15 (*An. peditaeniatus*),
		1	1	0	0	0	1 (*An. sinensis*)
	Funestus	1	1	0	0	0	1 (*An. varuna*)
	Ludlowae	14	7	0	0	0	6 (*An. vagus*)
	Jamesii	2	1	0	0	0	1 (*An. pseudojamesi*)
Ratchaburi	Barbirostris	3	3	0	0	0	1 (*An. saeungae*)
	Hyrcanus	12	12	0	0	0	5 (*An. pursati*)
	Funestus	66	8	0	0	0	8 (*An. aconitus*)
Total		**214**	**102**	**3**	**3**	**3**	**55**

7.4 Molecular detection of *P. bubalis* in mosquito samples

Out of 79 pools of anopheline mosquitoes originating from Chachoengsao, three pools were PCR positive for *Plasmodium*. These samples were *An. wejchoochotei/An. campestris* (IDs THMosqBuff20_P6_3, THMosqBuff20_P8_2), and *An. peditaeniatus* (ID THMosqBuff20_P20_3) (Table 6). The MIR (%) was calculated at 5.7 (0.015-0.186) in *An. wejchoochotei/An. campestris* and 1.9 (0.003-0.101) in *An. peditaeniatus* (Table 2). None of the 54 non-anopheline mosquitoes from the buffalo farm were PCR positive for *Plasmodium*.

Table 2. Minimum infection rates of *Plasmodium bubalis* from collected mosquitoes

Species	**Total no. mosquitoes**	**Pool size (range)**	**No. tested**	**No. positive pools**	**MIR (%) (95%, CI)**
An. campestris or *wejchoochotei*	35	1-3	35	2	5.7 (0.015-0.186)
An. peditaeniatus	80	1-3	52	1	1.9 (0.003-0.101)

8. Publication of research achievements

Nugraheni YR, Arnuphapprasert A, Nguyen TT, Narapakdeesakul D, Nguyen HLA, Poofery J, Kaneko O, **Asada M** and **Kaewthamasorn M** 2022. Myzorhynchus series of Anopheles mosquitoes as potential vectors of *Plasmodium bubalis* in Thailand. Sci Rep. 12(1): 5747. https://doi.org/10.1038/s41598-022-09686-9