NRCPD-OUAVM Joint Research Report

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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)

From 4/1/2020 to 03/31/2021 (The research project is continuing in FY2022)

5. Purposes and objectives

This study aimed to study pathogenicity of *Plasmodium bubalis* infection in buffaloes and its mosquito vector.

6. Outline of research process

A total of 110 blood samples were collected from the jugular vein of buffaloes and kept in ACD solution for futher blood smear examination and PCR diagnosis. Among these, 45 samples were collected during June to July of 2020 from Chachoengsao and 65 samples were from Nan province in December 2020. Nested PCRs using *Plasmodium* cytochrome b specific primers DW2-DW4 were performed to investigate the presence of *Plasmodium bubalis* in the buffalo's blood samples. At least two thin blood smears per buffalo were made on-site, fixed with methanol, and stained by 10% (v/v) Giemsa. Co- infection with piroplasms and *Anaplasma* spp. was evaluated using PCR assays, follow by sequencing. Body temperature was monitored twice a day in the morning and evening for 14 consecutive days. Blood sample collection were conducted daily for 14 consecutive days and continued every week until 56 days in Chachoengsao Buffalo farm (two infected buffaloes). Blood samples from infected buffaloes were examined by nested PCR and qPCR for each day. 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. We collected 55 female anopheline mosquitoes

from infected buffaloes' barn using either CDC light traps and aspirators for 14 nights. Female anopheline mosquitoes were initially identified based on morphology according to the mosquito identification key in Thailand (Rattanarithikul, 2006). Species of anopheline mosquitoes was confirmed by PCR and sequencing using cytochrome c oxidase I (COX1) and internal transcribed spacer 2 (ITS2) genes. Nested PCRs targeting cytochrome b, COX1, and 18S rRNA genes of *Plasmodium* genus was carried out to detect the presence of malaria parasite DNA from mosquitoes' pool samples. The positive PCR products were further subjected to sequencing.

7. Outline of research achievements

Natural infection of *P. bubalis* was detected in 2 out of 45 buffaloes (4%) from Chachoengsao (buffalo names hereafter referred to as Muk and Spa). A total of 6 out of 65 (9%) buffaloes from Nan province were PCR positive. No follow up was made for malaria infected buffaloed in Nan due to logistic reason. Two malaria infected buffaloes in Chachoengsao provinces were observed for 14 consecutive days. Afterwards, the infected buffaloes were followed up on a weekly basis until day 56. Buffalo Muk was a 6 years and 9 months old female with estimated body condition score (BCS) of 2 (1=skinny, 5= fat) and approximately 300 Kg body weight. Spa was a 8 years and 8 months old female with 4 months of her pregnancy (BCS = 2.5 and approx. 350 Kg body weight). No fever or other clinical signs were observed in both infected buffaloes within the entire observation period. Both buffaloes were clinically healthy during observation period and body temperature values were in the normal ranges. From day 1 to day 49, the malaria parasite in buffalo Spa was PCR positive and remained undetectable by PCR until day 56. *P. bubalis* in buffalo Muk was detected from day 1 to day 35, continuously and remained undetectable by PCR at days 42, 49, and 56. Parasitemia levels were counted on every day basis. We observed low parasitemia (<0.01-0.10%) in both infected buffaloes for the entired observation period. The highest parasitemia level was observed at day 9 at 0.09% and 0.1% in Muk and Spa, respectively (see Figures 1 and 2). It should be noted that only trophozoite stage was found in the thin blood smears during the observation days. Neither gametocyte nor schizont was observed. Co-infection with *Theileria* sp. was detected in buffalo Spa, while *Theileria* sp. and *Anaplasma marginale* were detected in buffalo Muk. Real-time quantitative PCR results showed that number of malaria parasite was from 26 to 140,506 copy per microlitre of genomic DNA in buffalo Spa and 14 to 3,516 copy per microlitre in buffalo Muk with standard curved R^2= 0.9656. The highest copy number of malaria parasite was measured at day 6 and day 8 for Spa and Muk, respectively (see Figures 3 and 4). Female anopheline mosquitoes were identified as *Anopheles barbirostris* (27/55 or 49.10%), *A. vagus* (13/55 or 23.64%), *A. peditaeniatus* (12/55 or 21.81%), *A. varuna* 1/55 or 1.81%), and unidentifiable anopheline mosquitoes in Maculatus group (probably *A. stephensi*) (2/55 or 3.64%). It is important to note that anopheline mosquitoes in Maculatus group has only 91% similarity to *A. stephensi* according to a BLASTn search. We detected *Plasmodium* positive results in 3 pools of mosquitoes based on cytb, cox1, and 18S rRNA genes. Sequencing results of cytb, cox1 genes showed 98% similarity to *P. bubalis*, while 18S rRNA gene showed 90% match with *P. falciparum*. *P. bubalis* DNA was detected in *A. barbirostris* mosquitoes (Table 1).

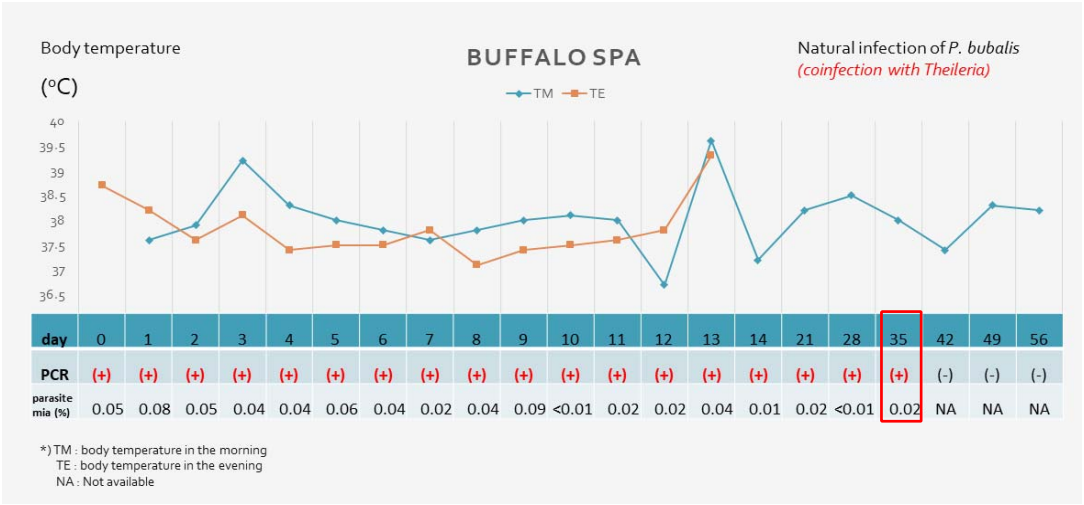

Figure 1. Observation of body tempurature and positivity of malaria parasites in buffalo Spa. Red box indicate last day of PCR positive for malaria parasite during the observation period.

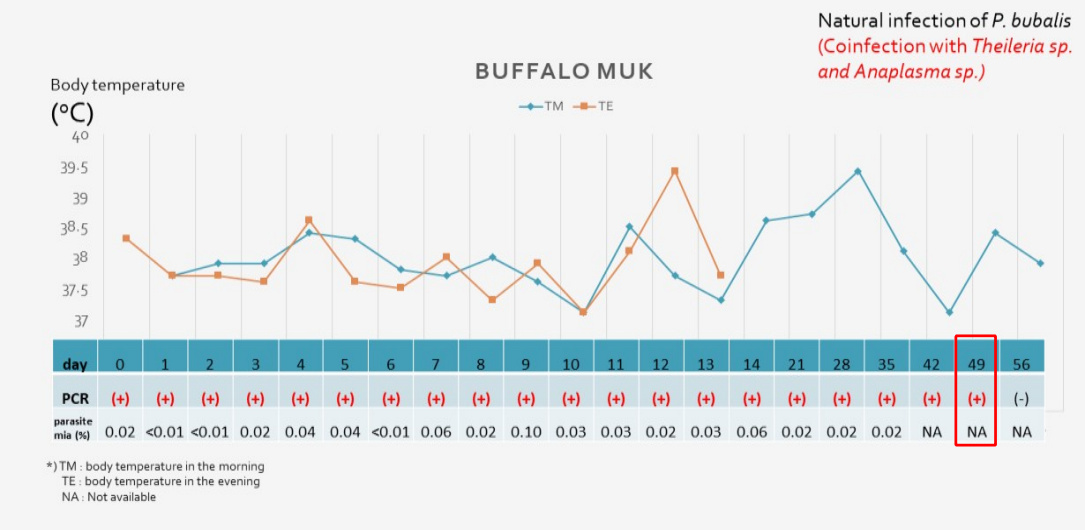

Figure 2. Observation of body tempurature and positivity of malaria parasites in buffalo Muk. Red box indicate last day of PCR positive for malaria parasite during the observation period.

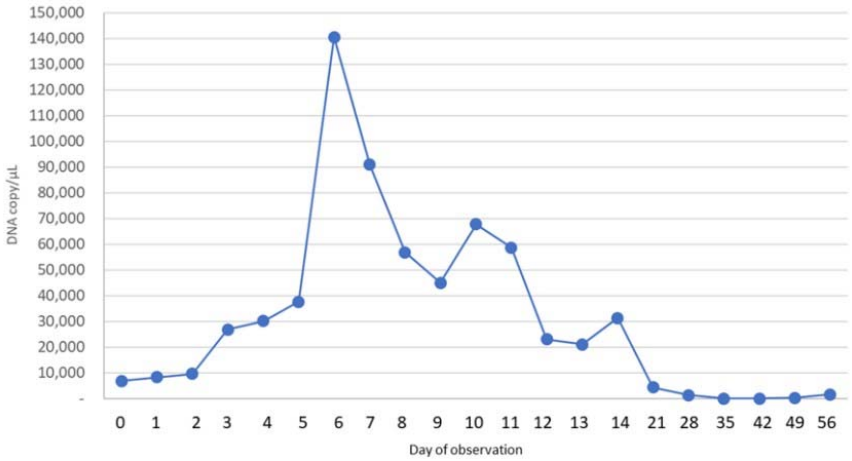

Figure 3. Malaria parasite's copy nuber in buffalo (Spa) during observation period

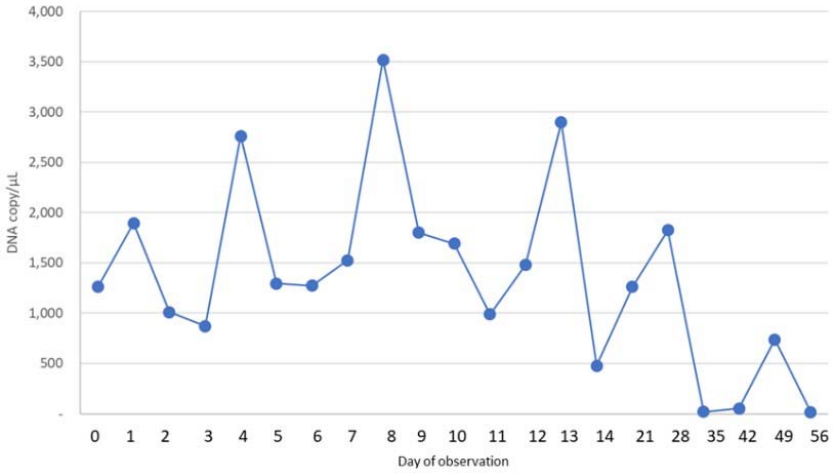

Figure 4. Malaria parasite's copy nuber in buffalo (Muk) during observation period

Table 1. Species confirmation of anopheline mosquitoes and *Plasmodium bubalis* detection in infected mosquitoes

No. of mosquitoes	Sample ID	BLASTn search result		Plasmodium positive by PCR		
		Cox1	ITS2	cytb	cox1	18S rRNA
3	THMos_Buff_BarP2	*A. barbirostris**	*A. barbirostris***	+	+	+
2	THMos_Buff_BarP4	NA	*A. barbirostris***	+	+	-
3	THMos_Buff_UmbP4	NA	NA	+	-	-
1	TH_Mos_Buff_2	*A. barbirostris**	*A. barbirostris***	-	-	-
1	TH_Mos_Buff_5	*A. vagus**	*A. vagus**	-	-	-
1	TH_Mos_Buff_7	*A. barbirostris**	NA	-	-	-
1	TH_Mos_Buff_8	*A. varuna**	*A. varuna**	-	-	-
3	TH_Mos_Buff_Bar_P3	NA	NA	-	-	-
3	TH_Mos_Buff_Pyr_P1	*A. vagus**	*A. vagus**	-	-	-
2	TH_Mos_Buff_Pyr_P2	NA	NA	-	-	-
2	TH_Mos_Buff_Pyr_P3	NA	NA	-	-	-
1	TH_Mos_Buff_Cul1	*A. barbirostris**	NA	-	-	-
3	TH_Mos_Buff_Cul_P2	NA	NA	-	-	-
3	TH_Mos_Buff_Cul_P3	NA	NA	-	-	-
3	TH_Mos_Buff_Cul_P4	NA	NA	-	-	-
2	TH_Mos_Buff_Cul2	NA	NA	-	-	-
1	TH_Mos_Buff_Umb1	*A. peditaeniatus**	*A. peditaeniatus**	-	-	-
3	TH_Mos_Buff_Umb_P2	NA	NA	-	-	-
3	TH_Mos_Buff_Umb_P3	NA	NA	-	-	-
1	TH_Mos_Buff_Umb5	NA	NA	-	-	-
2	TH_Mos_Buff_TesP1	*A. vagus**	*A. vagus**	-	-	-
2	TH_Mos_Buff_NeocP1	*A. stephensi**	NA	-	-	-
1	TH_Mos_Buff_Koc1	*A. vagus**	*A. vagus**	-	-	-
1	TH_Mos_Buff_Aitk1	*A. barbirostris**	NA	-	-	-
1	TH_Mos_Buff_Lin1	*A. barbirostris**	NA	-	-	-
2	TH_Mos_Buff_NeomP1	*A. vagus**	*A. vagus**	-	-	-
1	TH_Mos_Buff_Myzom1	*A. peditaeniatus**	*A. peditaeniatus**	-	-	-
1	TH_Mos_Buff_Myzor1	*A. barbirostris**	NA	-	-	-
2	TH_Mos_Buff_FunP2	*A. barbirostris**	NA	-	-	-
Total 55						

* Sequencing result, ** PCR result with specific primer, NA: Not available/not sequenced

8. Publication of research achievements

None (manuscript in preparation).