COLONIZATION AND MAINTENANCE OF *ANOPHELES BELENRAE* AND *ANOPHELES PULLUS* FROM THE REPUBLIC OF KOREA

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ABSTRACT. The *Anopheles* Hyrcanus Group in the Republic of Korea (ROK) consists of 5 morphologically indistinct species that can only be identified with certainty by polymerase chain reaction (PCR). A total of 86 bloodfed *Anopheles* spp. were collected from a cow barn located in the village of Tongilchon near the demilitarized zone in the ROK on June 13, 2016, and sent to the Armed Forces Research Institute of Medical Sciences in Bangkok, Thailand, where they were identified to species by PCR. The 1st shipment contained 15 *An. belenrae* and 37 *An. pullus* females that were used to start the colonies. Parent females that oviposited were identified by PCR for colonization. A higher proportion of F1–F4 females of *An. belenrae* than *An. pullus* bloodfed when provided both blood meals on human arms and using a membrane feeding system with human blood. Following blood meals, the females were forced mated for colony maintenance. The mean numbers of eggs oviposited per female for *An. belenrae* was 127.7 ± 19.3 and for *An. pullus* was 136 ± 23.6. On average, at 25°C (±2°C) *An. belenrae* and *An. pullus* took 15.1 and 16.1 days to develop from egg to adult, respectively. A 2nd group of bloodfed *Anopheles* spp. was collected at the same location in the ROK on June 24, 2017. This group contained 13 *An. belenrae* and 27 *An. pullus*. Similarly, eggs were obtained and adults identified by PCR and then reared to adults and subsequent generations forced mated to members of each of the existing colonies to increase genetic diversity. The colonies were established to evaluate their susceptibility to vector vivax malaria, which is essential to better understand the epidemiology of malaria transmission in Korea. This is the 1st report of colonization of both *An. belenrae* and *An. pullus*.

KEY WORDS *Anopheles belenrae*, *Anopheles pullus*, colonization, Republic of Korea

INTRODUCTION

The colonization of members of the *Anopheles* Hyrcanus Group in the Republic of Korea (ROK) is essential for evaluating their susceptibility to *Plasmodium vivax* (Grasso and Feletiti) due to its recent introduction along the demilitarized zone (DMZ) in the ROK (Chai et al. 1994, Klein et al. 2008, Park et al. 2009, Kim et al. 2016). *Anopheles sinensis* Wiedemann was previously reported to be the primary vector since it was commonly collected in areas of malaria transmission and *P. vivax* sporozoites were detected in captured specimens (Lee et al. 1998, Burkett et al. 2001, Han et al. 2006). However, in 2005 *An. sinensis* was recognized as a member of species complex comprising 5 species—*An. sinensis* s.s., *An. kleini* Rueda, *An. belenrae* Rueda, *An. pullus* Yamada (= *An. yatsushiroensis* Miyazaki), and *An. lesteri* Baisas and Hu (= *An. anthropophagus* Ma)—that were morphologically indistinguishable (Wilkerson et al. 2003, Hwang et al. 2004, Ree 2005, Rueda 2005). The members of the *Anopheles* Hyrcanus Group in the ROK could only be distinguished with certainty by polymerase chain reaction (PCR) techniques because their distributions overlapped in areas where malaria transmission occurs raised questions about the usefulness of earlier vector competence studies that considered only *An. sinensis* s.l. as the potential malaria vector and highlighted the need to evaluate each of the cryptic species for their ability to vector *P. vivax* (Joshi et al. 2009, 2011; Phasomkusolsil et al. 2014; Chang et al. 2016; Ubalee et al. 2016). Establishing colonies of mosquitoes in countries, such as Thailand where *P. vivax* is endemic, is important as it provides for mosquito species to be evaluated for susceptibility to malaria by collecting blood samples from infected patients, feeding infectious blood aliquots to multiple batches of mosquitoes, and then measuring infection rates, numbers of oocysts, and numbers of salivary gland sporozoites. A detailed account is offered to assist researchers who might need to know about the provenance of these colonies, and for future attempts to colonize these and other mosquito species.

MATERIALS AND METHODS

On June 13, 2016, resting bloodfed *Anopheles* females were captured from the barn of a beef farm located in Tongilchon, ROK (37°54′32.18″N, 126°44′01.88″E) (Paju-si, Gyeonggi Province), a small village located approximately 3 km south of the DMZ. The collection was conducted at night and resting females were observed with a flashlight and then mouth aspirated and transferred to screw-topped pint cartons. After collections, a cotton ball...
saturated with a 10% sucrose solution was placed on the screened top and covered with a petri dish lid to maintain humidity. The mosquitoes were then placed in an insulated container and transported to the Entomology Section, Force Health Protection & Preventive Medicine, 65th Medical Brigade/MEDDAC-Korea, Seoul, ROK, where they were kept overnight at room temperature. The following morning, the screen-topped cartons containing the collected mosquitoes were placed in secure and temperature-controlled containers and airmailed to Armed Forces Research Institute of Medical Sciences (AFRIMS), Bangkok, Thailand, arriving the following day, or approximately 48 h after the collections.

Upon arrival at AFRIMS, mosquitoes were maintained in a secure mosquito-rearing insectary and maintained at 25°C (±2°C) and 80% RH (±10%). The day after arrival, female mosquitoes were given an opportunity to take another blood meal in case they were not fully engorged when collected. This was performed using a standard membrane feeder (Rutledge et al. 1964), with sausage skin casings as the membrane, and human blood purchased from the Thai Red Cross. Mosquitoes were allowed to feed uninterrupted for 1 h, and then removed from the screen-topped pint cartons and put individually into glass vials (2 cm diam, 6 cm high). The vials contained 3 ml of filtered water for oviposition and 3 ml of filtered water for oviposition and maintained at 80°C. Also, for the 1st 4 generations (F1–F4), the opportunity to feed on a human host was provided. This was done to increase the percentage of mosquitoes taking a blood meal since the populations were small to start with and the desire was to have as many females as possible take a blood meal and develop eggs. An arm from one of the workers in the insectary was placed into the cage for 20 min after the membrane system had been used, to provide females that did not initially feed on the sausage casing another opportunity to feed.

Engorged females of both An. pullus and An. belenrae were forced mated according to standard procedures (Yang et al. 1963, Bryan and Southgate 1978). This was done to ensure that a bloodfed female would be mated and lay eggs. Once the colonies had grown in size after the F4 generation, a subset of 100 male and 100 female mosquitoes were placed into a cage to determine if they were capable of mating naturally in the laboratory. This was done 3 times for each species.

On June 24, 2017, a 2nd group of wild mosquitoes was captured from the same location at Tongilchon, ROK. They were sent to AFRIMS in Thailand and reared by the same methods. Once again, only An. pullus and An. belenrae egg batches were saved and reared to adults. The adult wild mosquitoes were forced mated with the established colonies for each species (wild male × colony female and wild female × colony male) as a method to increase genetic diversity and prevent any founder effects caused from starting colonies with low initial populations.

RESULTS

A total of 86 Anopheles spp. were collected on June 13, 2016, from Tongilchon, ROK, and 78 remained alive when they arrived at AFRIMS on June 15, 2016. There were 15 An. belenrae females and 37 An. pullus females (Table 1). Upon arrival the surviving females were given the opportunity to take an additional blood meal using an artificial membrane feeding system and 6/15 (40%) of the An. belenrae bloodfed while 22/37 (59%) of the An. pullus bloodfed (Table 2). From these, 8 (53%) of the An. belenrae oviposited and 30 (81%) of the An.
pullus oviposited (Table 1). The subsequent 4 generations (F1–F4) were given the opportunity to feed on a human arm in addition to the artificial membrane feeder. The bloodfeeding rates of An. belenrae were greater when both human arms and membrane feeders were used (range 80–93%) compared to An. pullus (range 37–64%). However, the feeding rates for the 2 species were similar once human arms were no longer provided after the F4 generation (Table 2).

Once the colonies had become established after the F10 generation, several additional characteristics were measured. The wing lengths of 30 male and female mosquitoes were measured and An. pullus was slightly larger (4.7 ± 0.1 mm males, 5.0 ± 0.2 mm females) compared to An. belenrae (4.5 ± 0.2 mm males, 4.9 ± 0.3 mm) (Table 3). Anopheles pullus oviposited a mean of 136.0 ± 23.6 eggs per female compared to An. belenrae with 127.7 ± 19.3 eggs per female. The development time from egg to adult was about 1 day longer for An. pullus (16.1 ± 0.7 days) compared to An. belenerae (15.1 ± 0.9 days) (Table 4). Three attempts were made with approximately 100 males and 100 females from each species to determine if these species were capable of self-mating and ovipositing eggs in standard laboratory cages. No eggs were observed for either species. All subsequent matings were performed by the forced mating technique.

A 2nd group of Anopheles spp. was collected from Tonglichon, ROK, on June 24, 2017. A total of 92 females were collected and 81 were alive upon arrival. Table 2. Number of Anopheles belenrae and An. pullus that were provided blood meals on an artificial membrane feeder and number (%) of engorged females. F1–F4 generations1 were provided blood meals on both human arms and artificial membrane. F5–F14 generations were only provided blood meals on an artificial membrane.

| Generation | No. provided blood meals | An. belenrae | An. pullus |
|------------|--------------------------|--------------|------------|
|            | No. (%) bloodfed         |              |            |
| Wild caught| 15                       | 6 (40.0)     | 37         |
| F1         | 120                      | 100 (83.3)²  | 1,000      |
| F2         | 190                      | 160 (84.2)²  | 1,000      |
| F3         | 300                      | 280 (93.3)²  | 1,500      |
| F4         | 520                      | 420 (80.8)²  | 1,500      |
| F5         | 2,100                    | 1,350 (64.3) | 1,500      |
| F6         | 600                      | 223 (37.2)²  | 1,500      |
| F7         | 2,400                    | 1,200 (50.0) | 600        |
| F8         | 2,400                    | 1,112 (46.3) | 2,400      |
| F9         | 3,000                    | 1,693 (57.1) | 2,400      |
| F10        | 2,600                    | 1,450 (55.7) | 3,000      |
| F11        | 2,700                    | 1,726 (64.0) | 2,600      |
| F12        | 2,600                    | 1,540 (59.4) | 2,700      |
| F13        | 2,000                    | 1,282 (64.1) | 2,600      |
| F14        | 2,100                    | 1,669 (78.9) | 2,000      |

1 Number of specimens that were alive or dead after oviposition.
2 Number of specimens that were alive or dead after 7 days that did not oviposit.
3 AFRIMS, Armed Forces Research Institute of Medical Sciences.
arrival at AFRIMS. In this group there were 13 An. belenrae and 27 An. pullus (Table 1). The offspring were reared to adults and then forced mated with the existing colony. No differences in development time, oviposition, or feeding rates were observed after the new mosquitoes were introduced.

**DISCUSSION**

This is the 1st colonization of An. pullus or An. belenrae. The colonies are now well established and >2,000 females are produced each generation. The techniques required to rear these mosquitoes in the laboratory are very similar to the colonization of An. kleini and An. sinensis s.s. at AFRIMS that originated from wild-caught specimens from Tongilchon, ROK (Phasomkusolsil et al. 2014). Anopheles pullus and An. belenrae oviposited, on average, 136 and 128 eggs per female, respectively, which is similar to An. kleini and An. sinensis s.s. that oviposited 146 and 157 eggs per female, respectively. The length of time from egg to adult was similar to the previous study and all 4 species blooded on a membrane feeding system with sausage skin casings and human blood. However, when allowed to feed on the arms of a human volunteer, An. belenrae fed more frequently compared to An. pullus (Table 2). It is unclear whether this represents a preference of An. belenrae to feed on humans or a general willingness to feed on any warm-blooded animal in comparison to a membrane feeder.

Similar to the previous members of the Anopheles Hyrcanus Group, both An. pullus and An. belenrae did not mate in sufficient numbers in standard 2-ft² screened cages to prevent a bottleneck for colony maintenance and therefore required forced mating to reproduce under laboratory conditions. Alternate types of cages, which may have increased self-mating behavior, were not used since the primary purpose was to rear sufficient quantities for evaluating them for malaria vector potential. Many anophelines are considered eurygamic and require swarming to induce mating behavior (Wharton 1953, Wijit et al. 2016). Such species will not mate in confined standard laboratory cages and copulation must be artificially induced. While it appears that both An. pullus and An. belenrae share this characteristic, different type of cages that allow swarming may have resulted in a “self-mating” colony. Artificial mating techniques are manpower intensive and for long-term colonization, alternate methods should be evaluated.

The colonization of An. pullus and An. belenrae in Thailand will allow these species to be tested for their vector competence and ability to transmit P. vivax. A previous study in Thailand found that An. kleini was a competent vector for P. vivax, while An. sinensis failed to develop sporozoites and is considered a poor vector (Ubalee et al. 2016). Studies such as this are important and should continue so the epidemiology of malaria transmission in Korea can be more fully understood. This study and the previous study (Phasomkusolsil et al. 2014) describe colonization of 4 out of the 5 members of the Anopheles Hyrcanus Group in Korea. The species that has yet to be colonized is An. lesteri, a malaria vector in China. This species was not colonized in the present study because it was not collected in sufficient numbers to start a colony. The collection in June 2016 only yielded 2 female An. lesteri and in June 2017 only 1 female was collected. Based on previous studies, An. lesteri is more commonly collected along the northwest coastal area and islands of the ROK. Thus, field collections for this species may have to be conducted at a different location to collect enough females. The numbers of adult bloodfed adults for oviposition was limited. To reduce the potential for “bottleneck” effect, a 2nd shipment of bloodfed adults was provided to AFRIMS to increase genetic diversity among the colonized specimens (data not shown).

**Table 3.** Mean wing length (mm), numbers of eggs oviposited per female, and numbers of larvae hatched per egg batch.

| Anopheles spp.          | Wing length ± SD (mm) | Mean no. eggs oviposited/female ± SD (n = 50) | No. hatched larvae/female ± SD (n = 50) |
|-------------------------|-----------------------|-----------------------------------------------|----------------------------------------|
|                         | Male (n = 30)         | Female (n = 30)                               |                                        |
| An. belenrae            | 4.5 ± 0.2             | 4.9 ± 0.3                                     | 127.7 ± 19.3                           | 102.8 ± 15.2                           |
| An. pullus              | 4.7 ± 0.1             | 5.0 ± 0.2                                     | 136.0 ± 23.6                           | 118.2 ± 24.1                           |

**Table 4.** Development time from egg to adult for 10 groups of 150 larvae.

| Mosquito species         | Eggs (n = 10) | L1 (n = 10) | L2 (n = 10) | L3 (n = 10) | L4 (n = 10) | Pupa to adult | Total days from egg to adult |
|-------------------------|--------------|-------------|-------------|-------------|-------------|---------------|-------------------------------|
| Anopheles belenrae      | 2.0 ± 0      | 2.3 ± 0.5   | 2.9 ± 0.3   | 3.0 ± 0     | 2.9 ± 0.6   | 2.0 ± 0       | 15.1 ± 0.9                   |
| An. pullus (n = 10)     | 2.0 ± 0.5    | 2.1 ± 0.3   | 3.0 ± 0.0   | 3.1 ± 0.3   | 3.4 ± 0.5   | 2.5 ± 0.5     | 16.1 ± 0.7                   |
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