News Release

Title

A Human Genotyping Trial to Estimate the Post-Feeding Time from Mosquito Blood Meals

Key Points

- Using a commercially released multiplex typing kit for 15 STRs in the world at present
- To estimate the time that elapsed since feeding from the degree of human DNA digestion
- Systematic experimental procedures to control the post feeding time without infection
- Possible to estimate the post-feeding time to about half a day between 0 and 2 days

Summary

Mosquitoes occur almost worldwide, and females of some species feed on blood from humans and other animals to support ovum maturation. In warm and hot seasons, such as the summer in Japan, fed mosquitoes are often observed at crime scenes. The current study attempted to estimate the time that elapsed since feeding from the degree of human DNA digestion in mosquito blood meals and also to identify the individual human sources of the DNA using genotyping in two species of mosquito: *Culex pipiens pallens* and *Aedes albopictus*. After stereomicroscopic observation, the extracted DNA samples were quantified using a human DNA quantification and quality control kit and were genotyped for 15 short tandem repeats using a commercial multiplexing kit. It took about 3 days for the complete digestion of a blood meal, and genotyping was possible until 2 days post-feeding. The relative peak heights of the 15 STRs and DNA concentrations were useful for estimating the post-feeding time to approximately half a day between 0 and 2 days. Furthermore, the quantitative ratios derived from STR peak heights and the quality control kit (Q129/Q41, Q305/Q41, and Q305/Q129) were reasonably effective for estimating the approximate post-feeding time after 2–3 days. We suggest that this study may be very useful for estimating the time since a mosquito fed from blood meal DNA, although further refinements are necessary to estimate the times more accurately.

Research Background

The mosquito, one of the most common and widespread insects, belongs to the order Diptera and up to about 3,600 species in 37 genera are found worldwide mainly in temperate and tropical regions. Only the females consume blood to support the maturation of ova. Mosquitoes, mainly *Culex pipiens pallens* (*CPP*) and *Aedes albopictus* (*AA*), are common outdoors and indoors in Japan from early summer (around May) to late fall (around November); in winter, they may occasionally be seen indoors. Both dead and live mosquitoes are often observed at crime scenes in Japan, especially indoors in summer.

We wondered whether it is possible to identify the bitten individuals and estimate the post-feeding (PF) time using the gut contents of the mosquitoes. Many previous studies have

investigated fed mosquitoes. Some have reported species identification using immunoprecipitation with antisera from various animals, classical blood typing, serum protein typing, and red cell enzyme typing. Other studies used DNA-based techniques such as identifying the host species using the mitochondrial DNA (mtDNA) by direct sequencing and polymerase chain reaction with the restriction fragment length polymorphisms (PCR-RFLPs). Some studies have investigated human personal identification using variable number tandem repeats (VNTRs) and/or short tandem repeats (STRs) genotyping with gel electrophoresis followed by silver staining, and two multiplex STR genotyping studies with capillary electrophoresis have been published. The former also referred to the time limits of qualitative band detection, or the time period within which the bands could be detected macroscopically. In the latter study, the study would not be able to follow because of the ethical issues; specifically, only one donor among the co-authors was fed blood to mosquitoes, which were doubted to be infected or uninfected, more than 150 times in an uncontrolled open field. Methodologically, a less accurate quantification kit based on slot blot detection was used and less accurate genotyping was performed by reducing the PCR reaction volume and the number of PCR cycles not according to the manual.

Currently, multiplex STR typing kits for more than a dozen human loci with a high discrimination power are commonly used, and the data can be digitized using computationally detected peak numbers, peak heights (PHs), peak areas, and the ratios of those data. A new commercial human DNA quantification kit that can assay the degree of DNA fragmentation to assess DNA quality has also been released. Furthermore, the availability of some mosquito species that are maintained under controlled conditions in exclusive cages has allowed procedures to be developed that keep blood-fed mosquitoes for several days, from the time of feeding until death.

The current study examined the extent to which DNA in a blood meal can be identified over post-feeding (PF) time using multiplex STR genotyping, according to the manufacturer's protocol. Furthermore, the time that elapsed since feeding was estimated by assaying the DNA quantity and integrity using quantitative PCR for three sizes of PCR amplicons, as well as the number and height of the signal peaks from the STR loci detected during genotyping. As this study was performed using two species of mosquitoes found both in Japan and extensively across multiple continents, it is likely that the same procedures will be applicable to other mosquito species in other countries. Therefore, a novel systematic and controllable procedure is proposed for genotyping multiple STRs and estimating the time since blood feeding more accurately. With ethical approval, it will be possible for all researchers to follow or improve this procedure.

Research Results

Stereomicroscopic examinations were performed using photographs of mosquitos taken just after sacrifice at each timepoint after feeding. Representative images showing changes in the color of the abdomen and the morphological alterations caused by blood digestion and ovum

maturation. The abdomen color varied from red to white as the blood meals were digested and the ovaries matured. Although it was confirmed macroscopically that all the mosquitoes had taken in a significant blood meal, several of the fed mosquitoes appeared similar to unfed controls upon stereomicroscopic examination immediately after sacrifice. This was observed at earlier times PF, especially before 12 h. They had presumably released the blood meal at some point without our noticing.

The concentrations of the Q41, Q129, and Q305 DNA samples extracted from both mosquito species (*CPP* and *AA*) at each PF time point were quantified using a KAPA Human Genomic DNA Quantification and QC Kit (Kapa Biosystems) and converted to common logarithms. The mean logarithmic DNA concentrations just after feeding (at 0 h) for CPP at Q41, Q129, and Q305 were 3.24 (1,750 pg/μL), 3.14 (1,370 pg/μL), and 3.09 (1,230 pg/μL), respectively. The amounts decreased gradually over time, but varied widely. At 72 h after feeding, DNA was undetectable in most of the samples. Regression curves were obtained from the correlation between the logarithmic DNA concentrations and PF time at Q41, Q129, and Q305, with coefficients of determination (R^2) of 0.92, 0.93, and 0.89, respectively. The mean logarithmic DNA concentrations just after feeding for *AA* at Q41, Q129, and Q305 were 3.03 (1,070 pg/μL), 3.05 (1,110 pg/μL), and 3.03 (1,070 pg/μL), respectively. These values also decreased gradually but varied less than did samples from *CPP*. At 72 h after feeding, DNA was undetectable in most samples, as with *CPP*. Regression curves were obtained from the correlation between the logarithmic DNA concentrations and PF time for all three Qs, with R^2 values of 0.97. All R^2 values for *AA* were higher than those for *CPP*. Human DNA was not detectable in negative control samples from either species.

T-tests were performed to assess the pairwise statistical differences in the DNA concentrations at Q41, Q129, and Q305 among PF times; the results are summarized on Table A in S2 Table. The DNA concentrations at all amplicon sizes tended to differ significantly before and after 18 h for AA, but not for CPP.

The Q-ratios (Q129/Q41, Q305/Q41, and Q305/Q129) were calculated from the DNA concentrations for each amplicon size (41 bp [Q41], 129 bp [Q129], and 305 bp [Q305]) for both species (*CPP* and *AA*) at each PF time. For *CPP*, the mean Q129/Q41 was almost constant (about 1.0) from 0 to 48 h PF, whereas Q305/Q41 and Q305/Q129 were about 0.7. For *AA*, the mean Q129/Q41 was almost constant (about 1.0) from 0 to 48 h PF, as in *CPP*. However, Q305/Q41 and Q305/Q129 decreased gradually from about 1.0 at 0 h to 0.7 at 36 h PF and from about 1.0 at 0 h to 0.8 at 36 h, respectively. Both ratios reached about 0.4 at 48 h. At 72 h after feeding, no ratio could be calculated because most values were below the level of detection in both species. Based on Q-ratios of 0 at 72 h PF, regression curves were obtained from the correlation between Q-ratios and PF time at Q129/Q41, Q305/Q41, and Q305/Q129, with values for R^2 of 0.88, 0.69, and 0.77 for *CPP*, and 0.84, 0.92, and 0.96 for *AA*, respectively. Generally, the mean ratios for *CPP* between 0 and 6 h PF fluctuated, whereas those for *AA* were comparatively stable.

T-tests were performed to assess the pairwise statistical differences between the Q-ratios

at Q129/Q41, Q305/Q41, and Q305/Q129 among PF times. There were few significant differences between the Q-ratios at all amplicon sizes and all PF times in both species.

Each sample was PCR-amplified using AmpFℓSTR® Identifiler® Plus PCR Amplification Kit (IDPlus) (Life Technologies) and 1 µL of DNA extract in 28 cycles. All the alleles detected originated the from volunteers' genomic DNA, as determined by comparisons with samples extracted from buccal swabs. No alleles were detected from the unfed negative control samples from either mosquito species.

The mean number of alleles detected for each sample at each PF time was counted as homozygotes were presumed to generate a single peak of twice the heterozygote peak height (PH) for a single allele. On average, most human alleles present (32 alleles) were detected in *CPP* samples from 0 to 24 h PF. Then, the allele number decreased gradually over time; several alleles were detectable until 48 h and none were detected at 72 h PF. In contrast, on average, most alleles could be detected from 0 to 18 h PF in *AA* samples. Subsequently, the number decreased gradually and no alleles were detected at 72 h, as with *CPP*. Sigmoid regression curves were obtained from the correlation between the mean number of alleles detected and PF time for *CPP* and *AA*, with an R^2 of about 0.99 for both species.

T-tests were performed to assess the pairwise statistical differences between the number of alleles detected among PF times. The number of alleles detected tended to differ significantly before and after 36 h for *AA*, but not for *CPP*.

The mean PHs were calculated as the sum of the PHs for the alleles detected in each sample divided by the number of alleles detected. The PHs were calculated as half the observed height in cases of homozygous alleles. The mean PHs were converted to common logs for each PF time. The log mean for PH just after feeding in CPP was 3.48 (3,600 RFU). The log averages decreased gradually, but varied widely. No alleles were detected 72 h PF, and the mean PH was 0. A regression curve was calculated from the correlation between the log mean PH and PF time, with an R^2 of 0.9. For *AA*, the log mean PH was 3.45 (3,300 RFU) at 0 h. Subsequently, the log average decreased gradually and was 0 at 72 h PF, as in *CPP*. If the log average at 72 h was 0, the correlation between log mean PH and PF time generated a regression curve with an R^2 value of 0.99 for AA.

T-tests were performed to assess the pairwise statistical differences in PHs among PF times. The number of alleles detected tended to differ significantly between 8 and 12 h for *AA*, but not for *CPP*.

The relative PHs were calculated as follows: assuming the PH of allele X for each DNA sample was 1.0, the ratios of the PHs for all other alleles detected in the DNA sample were calculated relative to the PH of allele X. For *CPP*, the mean of the relative PHs was almost constant (about 1.0) from 0 to 48 h PF. For *AA*, the mean of the relative PHs also remained almost constant (about 1.0) from 0 to 36 h PF; however, the mean gradually decreased to about 0.4 by 48 h. At 72 h after feeding, relative PHs could not be calculated as no alleles were detected in either species.

T-tests were performed to assess the pairwise statistical differences between the mean

relative PHs among PF times. There were few significant differences between all mean relative PHs and all PF times in both species.

Research Summary and Future Perspective

The current study attempted to estimate the time that elapsed since feeding from the degree of human DNA digestion in mosquito blood meals and also to identify the individual human sources of the DNA using genotyping in two species of mosquito: *Culex pipiens pallens* and *Aedes albopictus*. The extracted DNA samples were quantified using a human DNA quantification and quality control kit and were genotyped for 15 short tandem repeats using a commercial multiplexing kit. It took about 3 days for the complete digestion of a blood meal, and genotyping was possible until 2 days post-feeding. The relative peak heights of the 15 STRs and DNA concentrations were useful for estimating the post-feeding time to approximately half a day between 0 and 2 days. Furthermore, the quantitative ratios derived from STR peak heights and the quality control kit (Q129/Q41, Q305/Q41, and Q305/Q129) were reasonably effective for estimating the approximate post-feeding time after 2–3 days. We suggest that this study successfully developed a basic estimation of PF time, especially for *AA*. More accurate estimations should be possible after improvements in the quantitation method, resolution of the blood-meal release issue, and increasing the number of samples.

Publication

Yuuji Hiroshige[1], Masaaki Hara[2], Atsushi Nagai[3], Tomoyuki Hikitsuchi[4],
Mitsuo Umeda[4], Yumi Kawajiri[4], Koji Nakayama[4], Koichi Suzuki[5], Aya Takada[2],
Akira Ishii[1], Toshimichi Yamamoto[1]

[1] Department of Legal Medicine and Bioethics, Graduate School of Medicine, Nagoya University
[2] Department of Forensic Medicine, Saitama Medical University
[3] Department of Legal Medicine, Graduate School of Medicine, Gifu University
[4] Research & Development Laboratory, Dainihon Jochugiku Co., Ltd.,
[5] Department of Legal Medicine, Osaka Medical College

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