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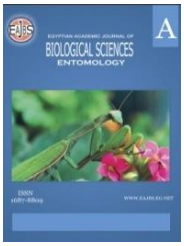

Identification of Human DNA from Degraded Mosquito Blood Meals Over Time

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ABSTRACT

Hematophagous mosquitoes are considered among the biological evidence that can be collected at the crime scene to identify missing persons, perpetrators, or victims. This study is to simulate what might be expected at a crime scene with an insect that took a human blood meal and then died after various time intervals of post-feeding and remained at the crime scene for more periods. It aimed to analyze the DNA profiling of the *Culex pipiens* human blood meal stored at room temperature for a week after 24-, 48-, 72- and 96-hours of post-feeding. The two hypervariable regions (HV1 and HV2) of the mtDNA D-loop region were also used to define the possible period for identifying a person at the crime scene. The DNA concentration showed degraded amounts between 8.9 to 0.00 pg/µL due to the long storage. A complete STR profile was possible by direct PCR at 48 hours of post-feeding, while a partial profile was obtained 72 hours of post-feeding. It was possible to amplify HV1 & HV2 up to 96 hours of post-feeding. A gradual decrease of STR allele appearance and mtDNA with time course of post-feeding was recorded. MtDNA is more efficient than STR as HV1 & HV2 regions were possible to be amplified up to 96 hours blood meal of post-feeding. Thus, we conclude that mtDNA is an efficient molecular marker for time course identification of human DNA from blood meals taken by the mosquito. Examination of the blood meal of insects collected from the crime scene over a sufficient period, potentially aids in the identification of suspects and victims.

INTRODUCTION

Adult female mosquitoes of *Aedes*, *Culex*, and *Anopheles* (Order: Diptera) are among the hematophagous insects sucking human and animal blood (Ortega-Insaurralde & Barrozo, 2022). Because of their specific feeding habit, mosquitoes may provide valuable evidence if collected at the crime scene since their blood meal may be regarded as a human trace sample (Ahmed *et al.*, 2023). Several studies investigated the possible analysis of DNA profiling from human blood meals taken by mosquitoes. A complete profile of 15 STR loci was successfully used to identify a suspect from the minute blood meal taken by a mosquito

in the vicinity of his killing, even though the DNA was degraded (Spitaleri *et al.,* 2006). Rabelo *et al.* (2015) analyzed the human blood taken by hematophagous mosquitoes and correlated the obtained profiles with the reference swab samples indicating the possibility of using mosquitoes as biological evidence in closed environments. Human DNA retrieved from mosquito blood meals decreased over time and was greatly affected by temperature and the insect species (Ibrahim *et al.,* 2015). The studies conducted so far revealed that blood meals taken by mosquitoes can produce complete STR profiles necessary for human identification up to 48 hours post-feeding (Gray *et al.,* 2020) and a complete digestion of the blood meals was found at 72 hours post-feeding. However, a recent study (Ahmed *et al.,* 2023) identified persons from *Culex piepins* (Family: Culicidae) blood meals up to 36 hours post-feeding, even when the blood is mixed with another human or non-human blood. The time of post-feeding was estimated by calculating the relative peak heights (rfu) of the STR alleles and the DNA concentrations within a half-day accuracy for the first 2 days (Hiroshige *et al.,* 2017). *C. piepins* has been chosen in this study, as it is a widespread insect inhabiting urban and suburban environments including Saudi Arabia and can occasionally bite humans. In some cases of human identification, autosomal DNA is either completely absent or severely damaged. The analysis of mitochondrial DNA (mtDNA), becomes, therefore, standard procedure for human identification. Moreover, in low copy number DNA samples such as degraded mosquitoes blood meal, mtDNA is efficient in identifying these caseworks (Ambers *et al.,* 2016). The mtDNA control region (D-loop) shows point mutations among humans and therefore it is the only region of this genome used for human identification in forensic cases. Based on the revised Cambridge Reference Sequence (rCRS), which assigns nucleotides in the mtDNA, the control region is located between sites 16024 and 576. Three segments inside the control region are referred to as the hypervariable (HV) regions that acquire most of the sequence variability in the mtDNA genome. The HV1 region spans from 16024 to 16365, the HV2 region spans from 73 to 340, and the HV3 region is found between position 438 to 574. HV1 and HV2 exhibit greater variability than HV3, which explains why the former two regions are the most often sequenced (Greenberg *et al*., 1983). Several studies used to analyze HV1 and HV2 for human identification from different biological traces in forensic purposes (El Ossmani *et al.,* 2009; Kavitha *et al.,* 2013; Amer *et al.,* 2017; Syndercombe Court, 2021; Connell *et al.,* 2022; Sturk-Andreaggi, 2022).

The aim of this study was to examine the effect of time course on human identification by analyzing the STR profiling of human blood meals obtained from the midgut of *C. pipiens* at one week after 24-, 48-, 72- and 96-hours of post-feeding. Additionally, HV1 and HV2 were examined to support this objective.

MATERIALS AND METHODS

Experimental Design:

Thirteen male Swiss White mice (*Musculles domesticus*) weighted approximately 30 -35 g were tested and bred in accordance with In Vivo Experiments (REAC) and Federal Animal Care guidelines. Mice were housed in a rearing room designed at a constant room temperature (20–22°C) and located in the Animal House, Department of Zoology, College of Science, King Saud University. The cages were designed with metal wires and transparent walls that enabled observe, in addition to holes equipped for water and food (25, 22, 30 cm^3). Its photoperiod was regulated as 12 hours under light.

A stock colony of *C. pipiens* L. mosquitoes was raised in the insectary of the Zoology Department, College of Science, King Saud University, under controlled circumstances in accordance with (Ahmed *et al*., 2023). Six days old mosquitoes were starving for a period of 12 hours prior to blood feeding on mice blood. Mice was

intraperitoneally injected with a pre-prepared anesthetic including (1 ml of ketamine, 0.5 ml of xylene, and 8.5 ml of sterile water for injection), according to its weight. It was placed in the starved mosquito's cage for feeding for 20-30 minutes and the adult female mosquitoes produced eggs after three days. Cups with distilled water were placed inside the cages for egg laying, and the newly hatched larvae were distributed in plastic trays filled with distilled water (34 x 24 x 10 cm^3 each). The larvae were fed on crushed goldfish flake food and the growing pupae were separated and transferred to polystyrene cups filled with distilled water and were kept inside the breeding cages (30 x 30 x 30 cm^3) until maturation. Adult mosquitoes were fed on 10% sugar solution (w/v) (Ahmed *et al.,* 2023).

After obtaining a signed informed consent form 10 Saudi adult healthy volunteers in addition to oral swabs, 10 ml fresh blood samples were collected and transferred directly into 4 ml lithium heparin tubes (BD Vacutainer®, USA), placed in a water bath at 37°C for 5 minutes before use (Boix *et al.,* 2013).

Two groups of 6-day-old reared adult mosquitoes were used in this experiment with each containing approximately 100 adult samples. Both groups were fed, using Hematok membrane feeder (Hemotek, Blackburn, UK (Atoni *et al*., 2019)), on the human blood for 20-30 minutes. Feeding was stopped and 20 fully engorged mosquitoes were randomly collected, isolated into 4 groups (5 samples for each) and placed in suitable plastic cups. The four isolated fully engorged groups were allowed to feed on 10% sugar solution for 24, 48, 72 and 96 hours, respectively. Mosquitoes were stored for one week at room temperature and their abdomens were isolated by hand for further use. Volunteers' blood was also used as controls.

Genetic Study:

Using QIAamp® DNA mini kit (Qiagen, London, UK), DNA was extracted from mosquito samples according to the manufacturer's protocol with some modifications as follow: 200 µL ATL was taken with 20 µL proteinase K in 1.5 ml tube, vortexed for 15 seconds and incubated at 56 °C for 1hr with continuous shaking. Carrier RNA (1 µL) was added, and the mixture was vortexed shortly. 200 µL AL buffer was added, then vortexed for 15 seconds and incubated for 10 min at 70 °C with mixing. 500 µL ethanol 96-100% was added, vortexed for 1 min then centrifuged. The ppt was allowed to dry for 2 minutes. 20 µL AE was added for dissolving the DNA for further use.

Meanwhile, 3 mosquito samples fed on the blood of the same person were pooled and their abdomens were separated and crushed on a filter paper by a sterilized scissor. The filter paper was cut into small 3 pieces and incubated in 1 mL distilled water for 30 min at room temperature and gently mixed. Floating supernatant was carefully removed and the rest 30 µL was slowly sucked and added to 200 µL 5% Chelex and 10 µl proteinase K. The mixture was incubated at 56 °C for 30 min and mixed for 10 sec at high speed, then incubated in a water bath at 95 °C for 8 min, and again mixed for 10 sec and centrifuged at 15.000 x g for 3 min and the sample was finally ready for DNA quantification. Using Quantifiler® Trio DNA Quantification Kit (Applied Biosystems, Foster City, CA, USA) and following the manufacturer's instructions, the extracted DNA was quantified via ABI 7500/ABI7000 real time PCR.

Human blood was adsorbed from mosquito s` abdomen into FLOQSWAB and the swab was incubated in 35 µL low TE-buffer at 70 °C for 30 min and the PCR reaction mixture (7.5 µL master mix+ 2.5 µL primer set + 15 µL lysate buffer) was added. The reaction was conducted on Applied Biosystems Veriti™ Thermal Cycler (Thermofisher Scientific, Waltham, MA, USA) for 30 cycles with an initial incubation for 1 min at 95 C, denaturation at 94 °C for 10 sec and annealing was at 59 °C for 90 sec. The final extension was at 60 °C for 10 min and the final hold was at 4 °C. The amplified loci were analyzed

using capillary electrophoresis in an ABI 3500 genetic analyzer (Qiagen, Manchester, UK) and Hi-Di formamide (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

The hypervariable regions of mtDNA were amplified using L15997 forward primer (5'-CACCATTAGCACCCAAAGCT-3`) and H16401 reverse primer (5'-TGATTTCACGGAGGATGGTG-3`) for HV1 and L29 forward primer (5'-GGTCTATCACCCTATTAACCAC-3`), H408 reverse primer (5'-CTGTTAAAAGTGCA TACCGCCA-3`) for HV2 (Sullivan *et al.,* 1992). Using Platinum™ Hot Start PCR Master Mix (2X), half reaction volume was used to amplify the 2 mtDNA HVs. The PCR mixture was prepared by adding12.5 µL master mix, 0.5 µL of each primer and 9.5 µL H_2O, then was vortexed and placed in the well of the Micro Amp Optical 96-Well Reaction Plate and a 2 µL DNA template was added. The plate was sealed with MicroAmp™ Clear Adhesive Film, vortexed, centrifuged and was placed for amplification. The reaction was conducted for 35 cycles with an initial denaturation of 2 min at 94 °C. The PCR cycle was adjusted for denaturation at 94 °C for 30 sec., annealing at 59 °C for 30 sec and final extension at 72 °C for 1 min and finally was held at 4 °C. Agarose gel (2%) was prepared with 10X TAE Buffer. SYBER Safe DNA gel stain (6 µL) was added to the heated gel. 5 µL DNA ladder with 1 µL orange/ blue Load dye (6X) was mixed and injected into the first well. 3 µL amplified DNA was mixed with 2 µL dye and injected into the other wells. The amplified PCR products were visualized and photographed under UV light.

GeneMapper® IDX v. 1.5 software (Applied Biosystems, Foster City, CA, USA) was used to analyze STR profiles while IBM SPSS Statistics v.21 (Armonk, NY: IBM Corp, 2012) was used for other statistical analysis.

RESULTS

DNA Quantification:

Table 1, indicates the mean DNA concentrations (pg/µL) and degradation index (D-index) that were obtained from individual mosquitoes and stored for a week after different time intervals of post-feeding. DNA and degradation index showed a gradual decrease exhibiting a reverse correlation with time course (*r* coefficient = 0.95). It ranged between 2.38 ± 3.1 pg/µL at 24 hours to 0.004 ± 0.00 pg/µL at 96 hours of post-feeding. The DNA exhibited a gradual degradation over time with standard deviations greatly fluctuated (Fig. 1).

Table 1. Mean ±SD DNA concentration (pg/µl) extracted from blood meals at different time interval of post-feeding. The DNA degradation index and DNA quality are also shown.

Post-feeding interval	DNA Con. (pg/µl)	D-Index	DNA Quality
24hrs	2.38 ± 3.1	13.7 ± 14.4	Slightly degraded
48 hrs	1.10 ± 2.8	5.1± 7.5	Moderately degraded
72 hrs	0.22 ± 0.4	4.4± 5.8	Degraded
96 hrs	0.004 ± 0.00	2.1± 2.5	Highly degraded

Fig. 1. Box and whisker plot of the average DNA concentration (ng/µL, N =10 at each time interval post-feeding). Whiskers indicate the range of the observed data. The values which are extreme from the mean or outliers are shown as middle lines in the boxes.

STR Profiling:

Damaged STR profiles were obtained in most cases of post-feeding intervals (data not shown) as the DNA was highly degraded. We selected a sample that showed gradual decrease in the DNA concentration over time and compared its STR profile obtained by the extracted DNA to that obtained by direct PCR (Table 2). A complete STR profile was possible by direct PCR at 48 hours of post-feeding (Figure 2). At the same time interval, the input of the extracted DNA was 0.063 pg/µl and the obtained STR profile was damaged.

Table 2. STR allele data for the selected sample (no. 5) at different time intervals post-feeding. Direct PCR amplified loci were compared to that obtained from the extracted DNA at the 48 hrs post-feeding interval (blue shaded columns).

Loci	Reference	Extracted DNA				Direct PCR
		24 hrs	48 hrs	72 hrs	96 hrs	48 hrs
D3S1358	16, 18	16, 18	-	-	-	16, 18
VWA	14, 16	14, 16	-	-	-	14, 16
D16S539	10, 11	10, 11	-	-	-	10, 11
CSF1PO	10, 12	12	-	8, 7	-	10, 12
TPOX	9, 11	9, 11	-	-	-	9, 11
Y-ind	2	2	2	1, 2	2	2
D8S1179	11, 13	11, 13	-	-	-	11, 13
D21S11	29, 32.2	Multi alleles	-	-	-	29, 32.2
D18S51	15, 17	15, 17	-	-	-	15, 17
DYS391	11	11	8, 11	-	-	11
D2S441	11.3, 14	11.3, 14	-	-	-	11.3, 14
D19S433	14, 16	14, 16	-	-	-	14, 16
TH01	6, 9.3	6, 9, 9.3, 13.3	-	-	-	6, 9.3
FGA	22, 27	22, 27	14	-	-	22, 27
D22S1045	15, 16	15, 16	-	-	-	15, 16
D5S818	12, 13	12, 13	17	-	-	12, 13
D13S317	11, 12	11, 12	-	-	-	11, 12
D7S820	8, 9	8, 9	-	8, 12	8, 9	8, 9
SE33	17, 33.2	Multi alleles	-	-	-	17, 33.2
D10S1248	14, 16	14, 16	-	-	-	14, 16
D1S1656	16, 18	16	-	9, 12, 16	-	16, 18
D12S391	23, 24	23, 24	-	-	-	23, 24
D2S1338	17, 20	17, 20	-	20, 22	-	17, 20
AMEL	X, Y	X, Y	X	X, Y	X, Y	X, Y

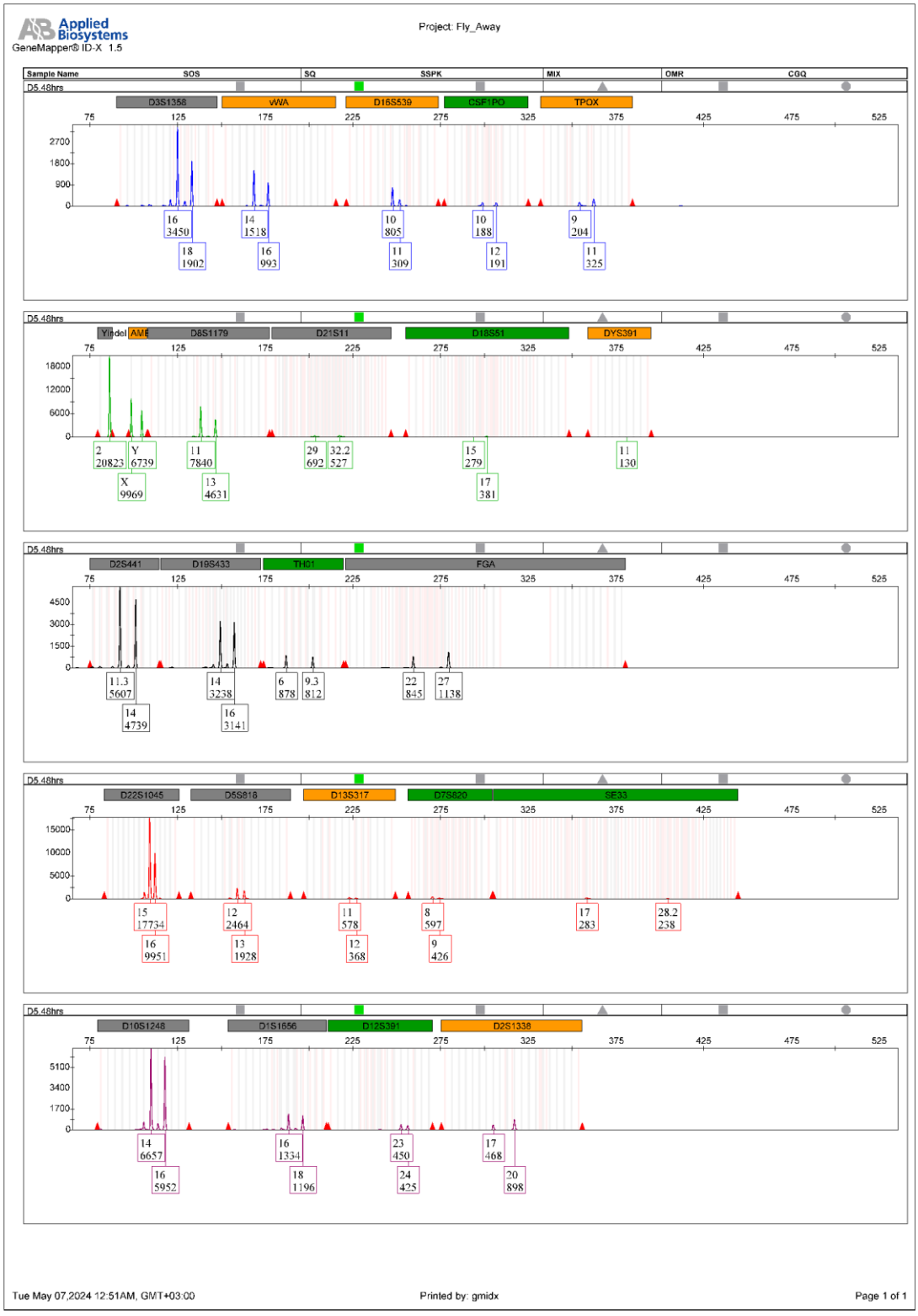

Fig. 2. The electropherogram of the STR profile obtained by direct PCR (Global Filer) at 48 hours of post-feeding. The values at each peak indicate the allele (upper) and the rfu value (down).

PCR was conducted to 6 samples (3 pooled females/each). The individual and average allele appearance of the amplified loci showed a gradual decrease with time course of post-feeding (Table 3 and Fig. 3).

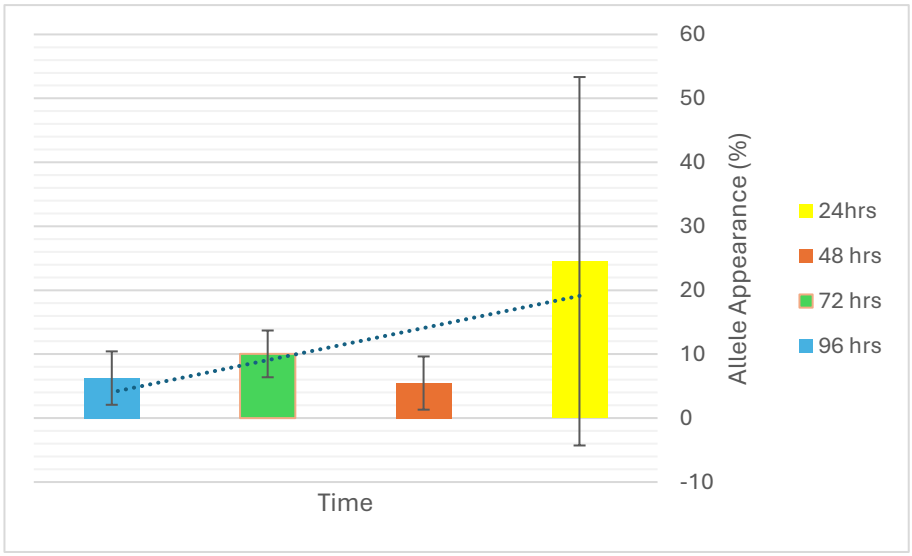

Fig. 3. Box and whisker plot of the average DNA allele appearance (N =6 pooled blood meal samples at each time interval post-feeding). The outlier's values are shown as middle lines in the boxes.

Table 3. Allele appearance % of the amplified loci for pooled blood meal samples.

Pooled blood meal samples	Allele Appearance %			
	24hrs	48 hrs	72 hrs	96 hrs
S3	21	7	7	5
S5	82	2	7	7
S6	10	5	12	10
S7	7	12	7	12
S8	20	7	11	5
S9	7	0	16	0
Average	25	5	10	6
±SD	28.81	4.16	3.66	4.18

MTDNA:

It was possible to amplify HV1 and HV2 regions of the D-loop at different post-feeding intervals with a significant gradual decrease in the product quantities (Table 4 and Fig. 4**).** The amplified products showed inverse correlation with post-feeding time course.

Table 4. The percentage of amplification success of HV1 and HV2 at different post-feeding intervals. "*r*" refers to the correlation coefficient values that indicates the inverse relationship between DNA concentration and post-feeding intervals.

24 hrs	HV 1	100	***r** for HV1= -0.95**
	HV 2	100	***r** for HV2= -0.94**
48 hrs	HV 1	83.33	
	HV 2	100	
72 hrs	HV 1	83.33	
	HV 2	66.67	
96 hrs	HV 1	66.67	
	HV 2	33.33	

**Pearson Correlation Coefficient*

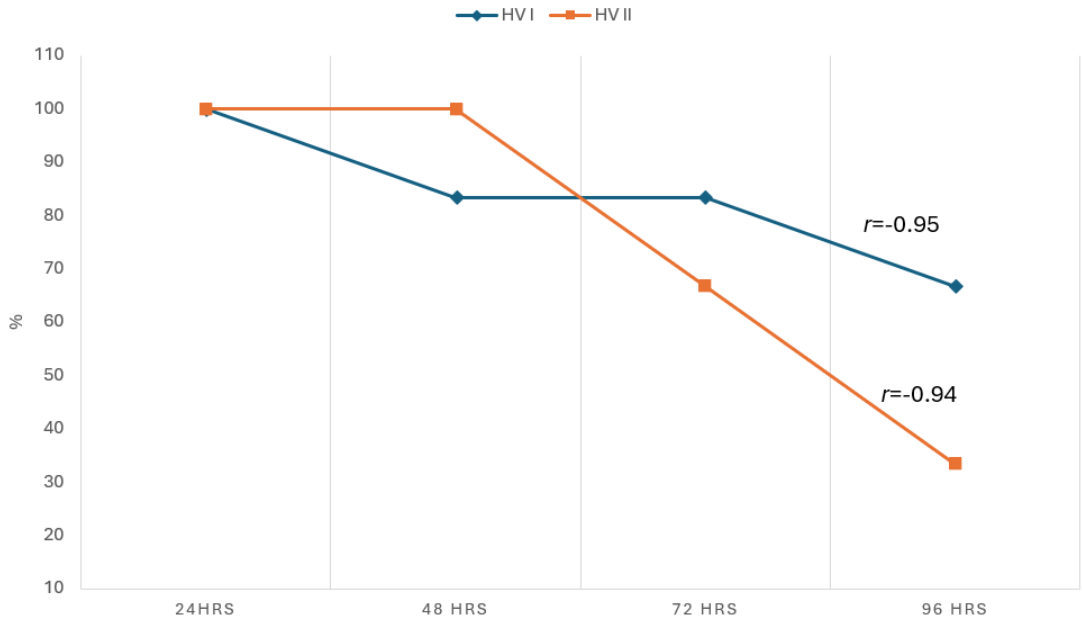

Fig. 4. Linear Pearson Correlation Coefficient indicates the significant negative correlation of the amplification of HV1 and HV2 mtDNA regions to the post-feeding intervals. The "Y" axis refers to the percentage of samples successfully amplified, while the "X" axis refers to the post-feeding intervals.

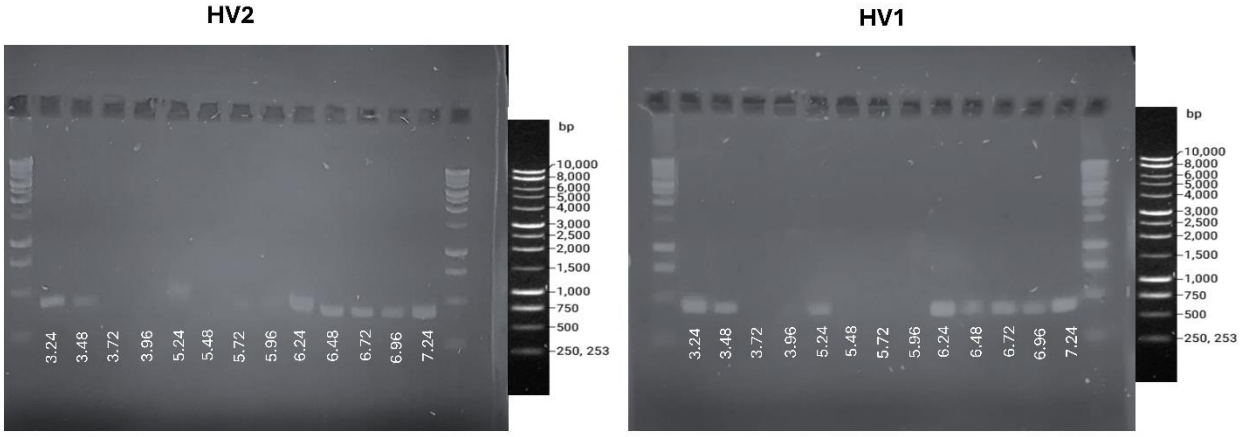

Fig. 5. Agarose gel (2%) profile shows the amplified HV1 and HV2 of blood meals taken from 6 individuals at different post-feeding intervals. Numbers in the "X" axis refer to the sample number and post-feeding interval (3.24, 3.48, 3.72, 3.96, etc..). The lane to the right side of the gel image refer to the DNA size marker.

Table 4 and Figure 5, show the PCR products of the 6 blood meal samples selected at different post-feeding intervals. HV1 and HV2 regions were positively amplified in most cases. At 24 hours of post-feeding, all samples showed positive amplifications (100%) for HV1 and HV2. The amplification showed a gradual decrease with post-feeding intervals. At 48 hours of post-feeding, HV1 region was amplified for 83.33 % of the samples. HV2 was amplified for all samples at the same time interval. At 72 hours of post-feeding, HVI was amplified for 83.33% of the samples, while HV2 was successfully amplified for 66.67% of the samples. HV1 of 66.67 of the samples was positively amplified at 96 hours of post-feeding, while HV2 was amplified for only 33.33 % of the samples.

DISCUSSION

The mosquito *C. pipiens*, either dead or alive, is considered a valuable piece of forensic evidence (Curic *et al.*, 2014) and a good source for human identification at the crime scene up to 3 days of post-feeding. It is also well-known that DNA profiling of the blood meal isolated from the mosquito abdomen is inversely correlated with the time interval of post-feeding. A full DNA profile was likely from Culicidae mosquitoes up to 48 hours and the probability of successful profiling is decreased by 15.5% every 8 hours more in post-feeding interval (Curic *et al.*, 2014). Ahmed *et al.* (2023) also explained that the delay for more than 48 hours post-feeding decreases the possibility of obtaining full STR profiles as the blood is completely digested at this interval and beyond it the DNA degradation might increase. Although the present study was conducted under severe conditions of DNA degradation because of the storage conditions, it was possible to obtain a full STR profile by direct PCR using Global Filer at 48 hours post-feeding. The average DNA concentration indicated a gradual decrease over time of post-feeding; however, the standard deviation is greatly fluctuated due to the storage conditions or because not all individual female mosquitoes took the maximum amount of blood. This amount was estimated to be 0.68 mg/mosquito (Alharbi *et al.*, 2024).

Direct PCR showed promising ability to produce STR profiles from very small DNA quantities (Swaran *et al.*, 2012). It has been proven, in the present study, that using a single individual mosquito is possible to produce a full STR profile at 48 hours post-feeding with few picograms DNA inputs, particularly, when Global Filler kit was used. This kit is considered to have high sensitivity and discrimination power in forensic caseworks as has been revealed by Singh *et al.* (2021). On the other hand, using Mini Filer kit, direct PCR was not able to produce even a partial STR profile at 48 hrs post-feeding interval from the same DNA input.

Chelex is known for its effectiveness in binding and extracting DNA by chelating metal ions that may stimulate the breakdown of DNA. It is effective to yield a good DNA quantity with high purity from minute quantities of tissues and blood (Snigh *et al*., 2018). The pooled samples extracted herein by Chelex showed higher allele appearance than those extracted individually by QIAamp® DNA mini-DNA extraction kit.

The data obtained from HV1 and HV2 support the inverse relationship between time course and human DNA identification obtained by STR. As the post-feeding interval increases, the possibility of obtaining readable DNA markers (either STR or mtDNA) decreases. The present study revealed that it was possible to amplify mtDNA from human blood meals up to 96 hours post-feeding and beyond. There are limited studies on the effect of the different post-feeding intervals on mtDNA of human or mammalian blood meals taken by the mosquito. The cytochrome b gene of the mtDNA obtained from mammalian blood meals including human was examined at different post-feeding intervals (Kent & Norris, 2005). The authors were able to amplify this mtDNA gene up to 72 hours post-feeding. Talebzadeh *et al*. (2023) were able to detect human DNA (including 16S rRNA and cytb mtDNA genes) up to 113 hours post-blood-feeding. The authors used *Anopheles stephensi* mosquitoes in their study, but the present study used *C. pipiens* mosquito. Despite the time necessary to digest the blood meal does not exceeding 48 hours post-feeding (Reeves *et al.*, 2016; Lee *et al.*, 2015), still there is a non-significant variation in the average time to digest the blood meal in *Ae. aegypti* and *Cx. pipiens* (Al-Rashidi *et al.*, 2022). For *Anopheles*, the situation is little bit different as the complete digestion of human blood meal was recorded up to 72 hours post-feeding (Jeyaprakasam *et al*., 2022). Some characteristic features enable mtDNA to be obtained from degraded biological samples compared to nuclear DNA. Among

these characteristics are that mtDNA exhibits high copy number per cell and higher resistance to degradation (Bhoyar *et al.*, 2024).

Despite the obtained results, this study was subjected to several limitations, namely the limited timetable of the laboratory part, the bad freezing conditions of the collected mosquitoes engorged by blood meals, and the inability to estimate the size of the blood meal taken by the adult females (Alharbi *et al.*, 2024), and the selection criteria of the filled-abdomen mosquitoes after taking the blood meal. Other environmental factors (temperature and humidity) are also considered among the challenges that could not be controlled. Further study with more samples, enough timetable and more rearing mosquitoes could define the effect of time interval of blood meal post-feeding on the DNA genotyping necessary for human identification. Insect abdomen which did not take blood meal and equivalent to the size of a blood meal and a human blood sample could be used as controls.

In this study, mosquito blood meals are considered as potential evidence for human identification at crime scenes. STR profiling for up to 48 hours of post-feeding, and mtDNA for up to 96 hours of post-feeding gives a potential identification of victims or suspects, particularly in scenarios where traditional forensic samples are limited or unavailable (Ahmed *et al.*, 2023). Further studies are required to optimize DNA extraction and amplification methods for this unique source of biological evidence to ensure its admissibility and evidentiary value in legal proceedings. In conclusion, mtDNA is the most efficient molecular marker for time course identification of human DNA from blood meals taken by the mosquito.

Declarations:

Ethical Approval: This study was approved by the Ethics Committee of Naif Arab University for Security Sciences (code: Nauss-Rec-24-06).

Authors Contributions: Prof. Dr. Sayed Amer and Prof. Dr. Ashraf Ali designed the experiments, reviewed drafts of the article, and approved the final draft. Hadeel Alaswad, Heba Alharbi and Abrar Alsaleh performed the experiments, analyzed the data, prepared figures and/or tables. All authors reviewed drafts of the article and approved the final draft.

Competing Interests: The authors declare that they have no competing interests.

Availability of Data and Materials: The data supporting the study findings are available from Hadeel Alaswad and Heba Alharbi upon reasonable request.

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