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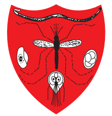

RESEARCH ARTICLE

Development and validation of a PCR-based method to differentiate human sex in blood-fed *Aedes aegypti* (Diptera: Culicidae)

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ABSTRACT

Monitoring mosquito host choice to identify high-risk groups for different vector-borne diseases is important to devise vector control strategies and disease management. The present study was conducted to develop and validate a PCR-based method to identify human sex in blood-fed *Aedes aegypti* mosquitoes. Several human genes present in both the X and Y chromosomes were screened and diagnostic PCR primers were successfully designed and amplified for the human *STS* gene. The limit of detection of this PCR assay was carried out on *Ae. aegypti* fed with human blood up to 5 days (120 hours) post blood-meal under laboratory condition. The efficiency of this PCR assay was evaluated in field-collected *Ae. aegypti* mosquitoes and compared with other existing methods. The developed PCR primers can successfully amplify and distinguish human sex in mosquitoes up to 72 hours after a blood meal, with an amplified product of 627bp and 298bp for male (XY) and 627bp for female (XX) blood-fed mosquitoes. Further, validation of this assay in field-collected *Ae. aegypti* mosquitoes revealed that this assay could detect human sex in mosquito blood meal substantially more efficiently ($\chi^2 = 4.5$, $p = 0.034$) than other PCR based assay. The newly developed PCR assay highly specific to human DNA and can distinguish male and female DNA for up to 72 hours. This assay can be is used for identifying high-risk groups and extended to other medically important hematophagous insects to assess their role in disease transmission and epidemic preparedness.

Keywords: Vector-borne diseases; blood feeding; epidemiology; polymerase chain reaction; STS locus.

INTRODUCTION

Mosquitoes are hematophagous insects that require blood for development and reproduction. This characteristic of mosquitoes makes them epidemiologically significant because during blood feeding, they can take up pathogens responsible for diseases from infected hosts and spread them to other hosts when the mosquito feeds again. Thus, mosquitoes are effective vectors for a variety of infectious diseases, collectively known as vector-borne diseases (VBDs), which imposes a major socioeconomic and public health burden and account for more than 17% of all infectious diseases worldwide (WHO, 2020). Understanding mosquito blood-feeding behavior is critical for improving VBD surveillance and prediction as well as developing efficient vector control methods (Swei *et al*., 2020).

While the prevailing notion characterizes mosquito biting as stochastic, instances of divergent feeding patterns related to the host's gender have been reported (Michael *et al*., 2001). Mukabana *et al*. (2002) observed a distinct inclination for male blood among *Anopheles gambiae* mosquitoes. Blood meal preferences for young adults and males have also been observed in *Aedes aegypti* collected in Florida and Puerto Rico (De Benedictis *et al*., 2003). Notably, there exists an epidemiological discrepancy wherein the Zika virus infects a higher proportion of women compared to men (Jimenez Corona *et al*., 2016). Furthermore, pregnant women exhibit more susceptibility to mosquito bites and vector-borne diseases (Lindsay *et al*., 2000). This differential sex preferences of mosquitoes for human blood meal could be attributed to the immune response of the host (Klein & Roberts, 2015), pregnancy (Lindsay *et al*., 2000), parasite infection (Pathak *et al*., 2012), ABO blood group (Anjomruz *et al*., 2014), skin microbiome (Verhulst *et al*., 2011), exhaled levels of CO2, and other chemicals such as volatile organic compounds (VOCs) (De Obaldia *et al*., 2022). Determining human gender from mosquitoes' blood meals aids in understanding disease transmission dynamics based on gender-related behaviours or preferences and to identify high-risk groups for mosquito bites. This knowledge in epidemiology helps tailor targeted prevention strategies and better comprehend the impact of diseases on different demographics. Therefore, it is imperative to develop and use sensitive tools that not only identify human blood in mosquito blood meals but also differentiate the sex of the human host.

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Although it is possible to determine the sex of the host from the blood meal using short tandem repeat (STR) or variable number tandem repeat (VNTR) profiling, this method is intricate due to its high cost, limited scalability, and the need for DNA profiles of all individuals in a given area to match with human DNA found in mosquitoes (Teltscher *et al*., 2021). Consequently, focus has shifted towards devising a singular PCR-based method capable of targeting universally conserved genes on both the X and Y chromosomes of humans. A notable example involves utilizing the *amelogenin* gene (AMEL), which exists on both the X (AMELX) and Y (AMELY) chromosomes, to differentiate the sex of the human host in the blood meal of *Ae. aegypti* (Harrington *et al*., 2014). However, challenges such as low resolution (6bp) between the amplification products of the X and Y chromosomes, along with the potential for typing errors due to AMELY primer binding site mutations or deletions limits its application in public health (Harrington *et al*., 2014; Garcia-Rejon *et al*., 2020). To overcome this limitation, the method was modified further by integrating primers targeting the SRY gene on Y chromosome (Garcia-Rejon *et al*., 2020) or adopting different sets of primers for the AMEL gene (Teltscher *et al*., 2021), or employing nested PCR approaches (Talebzadeh *et al*., 2023). However, in both instances, the assay was not entirely human specific and demonstrated cross-species amplification (such as with chimpanzees). This present study introduces a novel PCR-based technique tailored for human specificity, designed to discern the gender of the human host within blood-engorged mosquitoes. The efficacy of this method was validated using field-collected *Ae. aegypti* mosquitoes.

MATERIALS AND METHODS

Primer design and sequence retrieval

Several human genes from both the X and Y chromosomes of the human genome were initially screened for the development of sex-specific diagnostic PCR primers using the NCBI human genome and NCBI BLAST tool. The sequences of the human *steroid sulfatase* (STS) gene (GenBank accession no: NG_021472; location: Xp22.31) and its Y chromosome homolog STSP1 (GenBank accession no: NG_001197; location: Yq11.221) were retrieved in the FASTA format. The sequence alignment program MAAFT (multiple alignment using fast Fourier transform) was used to align the downloaded sequences. Appropriate sequences, based on the conserved region of the alignment and fulfilling primer design criteria, were selected to design both forward and reverse primers. Other standard primer characteristics, such as primer-dimer formation, primer melting temperature (Tm), and GC content, were checked using the online oligoanalyzer tool (https://eurofinsgenomics.eu/en/ecom/tools/oligo-analysis/). The sequences of the designed primers used for the developed PCR assay were mentioned in Table 1. These primers were expected to yield a product size of 627 bp and 298 bp for male (XY) and 627 bp for female (XX) human DNA, respectively (Figure 1a).

Table 1. PCR primers used in this study

	Primer Name	Sequence (5' to 3')	Reference
Blood meal identification PCR	UniR	GRGCNAGDACDCCTCCTAGTTTRTTWGG	Field *et al*., 2020
	AvF	CHCTMATAGCAACYGCCTTCGTAGG	
	NHF	GDGGSTTYTCAGTNGACAAAGC	
Human sex PCR	MF_F	CTGATGGTTGGCCTCAAGCCTGTG	Nakahori *et al*., 1991
	MF_R	TAAAGAGATTCATTAACTTGACTG	
Human sex PCR	STS_F	GATCATTCAGCAGCCCATGTCC	This study
	STS_R	GTCTCTTTGCCAACATTCCC	

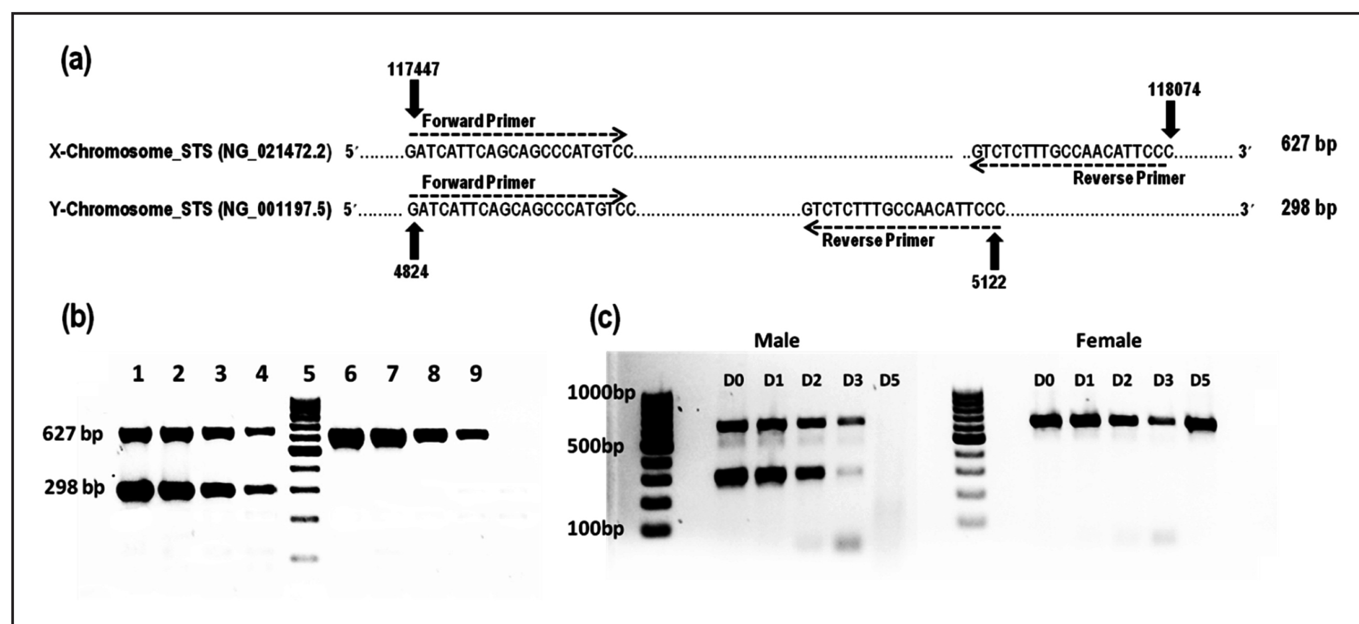

Figure 1. (a) PCR primer design based on the STS region of the human genome's X and Y chromosomes; (b) Amplification efficiency of the designed primers on different concentrations of human male and female DNA (Lane 1 to 4: Male DNA with concentration 100ng, 10ng, 1ng and 0.1ng; Lane 5: 100bp DNA marker; Lane 6 to 9: Female DNA with concentration 100ng, 10ng, 1ng and 0.1ng; (c) Amplification of STS loci in laboratory fed mosquitoes after different days post blood meal.

Standardization of PCR method

The PCR assay using the designed primers was first standardized using DNA extracted from blood samples donated by self-consenting donors. 100µl of blood samples, donated by one self-consented male and female donor, were used for DNA extraction using the DNeasy Blood & Tissue kit (Qiagen, cat no: 69504), following the manufacturer's instructions. Qubit 4.0 (ThermoFisher Scientific) was used to quantify the extracted DNA. DreamTaq Green PCR Master Mix (2X) (ThermoFisher Scientific, cat no: K1081) was used to perform PCR amplification of the STS locus using the designed primers to distinguish the human sex. A reaction volume of 10µl was set up with 0.5 µM forward and reverse primers and 1µl of DNA template (10ng/µl). The PCR cycling conditions were as follows: initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 55°C for 30 s, and extension at 72°C for 45 s, with a final extension at 72°C for 7 min. A 1.5% agarose gel was used to visualize the PCR amplification products. After standardizing the PCR method (named as STS_PCR), the limit of detection in terms of DNA concentration was determined by using different concentrations (100ng, 10ng, 1ng and 0.1ng) of both male and female human template DNA.

Establishment of *Ae. aegypti* colony

Ae. aegypti colonies were established in the laboratory from field-collected larvae to evaluate the assay in mosquitoes. Field-collected larvae from a single larval source were transported to the laboratory and reared to the adult stage in an insect growth chamber (Percival, USA) maintained at 28°C, 70-80% relative humidity, and a 12-hour light and dark cycle. Newly emerged adults were given a 10% sucrose solution using cotton pads. Four-day old female *Ae. aegypti* mosquitoes (approximately 50 numbers) were deprived of sugar for 24 hours prior to the blood-feeding. Blood donated by one male and one female self-consenting co-author was used for the blood feeding experiment. Mosquitoes were allowed to feed on male and female blood via a membrane-feeding apparatus in separate cages. Blood meal was maintained at 37°C by circulating warm water through an external jacket. Mosquitoes were allowed to feed for 30 minutes. Fully fed mosquitoes were maintained in the insectary at room temperature and supplied with a cotton pad containing 10% sucrose solution as a carbohydrate source.

Evaluation of STS_PCR assay

Blood-fed mosquitoes were collected at different time interval including, <1 hour, 24 hours, 48 hours, 72 hours and 120 hours post blood-feeding and DNA was extracted from the whole mosquito using DNeasy Blood & Tissue kit (Qiagen, cat no: 69504), according to the manufacturer's instructions, and eluted in 40µL elution buffer. These DNA samples were used to evaluate the efficacy of the newly developed STS_PCR assay for detecting and distinguishing human sex in terms of post blood-feeding time.

Validation in field collected mosquitoes

Further to validate the STS_PCR assay in field collected mosquitoes, adult *Ae. aegypti* were collected using aspirator from different areas of Bhopal city (Table 2). Mosquitoes were brought to the laboratory, frozen, and morphologically identified. DNA from individual whole mosquitoes were extracted as mentioned above. To avoid the digestion of blood and further degradation of host DNA, DNA was extracted on the same day of collection. The STS_PCR assay was performed for all *Ae. aegypti* mosquitoes using the reagent concentrations and PCR cycling conditions described above. Similarly, all the field collected mosquitoes were used for human sex determination by using already published method developed by Nakahori *et al*. (1991) and modified by Teltscher *et al*. (2021) for comparative purposes. DNA extracted from whole blood obtained from male and female donors was used as a positive control, whereas DNA of an unfed lab-grown mosquito was used as a negative control. The amplified PCR products of the newly developed STS_PCR assay was confirmed by sequencing.

Statistical analysis

McNemar's test and Cohen's kappa statistic test were used to determine agreement between the two PCR assays.

RESULTS

The developed PCR assay (STS_PCR), which used primers designed for the STS gene located on both the X and Y chromosomes of the human genome, successfully amplified DNA from both male and female blood donors. As expected, product size of 627bp and 298bp for male (XY) and 627bp for female (XX) were observed. Furthermore, the developed assay has been standardized with wide range of DNA template concentrations (100ng, 10ng, 1ng and 0.1ng). The assay is sensitive enough to detect as low as 0.1ng of DNA template in both the gender effectively (Figure 1b). The effect of blood meal digestion on the newly designed PCR assay was also investigated. Amplification was performed in duplicate on male and female blood-fed *Ae. aegypti* mosquitoes collected on <1 hour, 24 hours, 48 hours, 72 hours and 120 hours in the laboratory. The developed assay was found to successfully amplify and differentiate the human sex in mosquitoes taking blood meals up to D3 (72 hours)

Table 2. Details of the *Ae. aegypti* collection sites along with the number of mosquitoes collected

Name of the sampling localities	Latitude (N)	Longitude (E)	Number of *Ae. aegypti* collected
Bairagarh	23.263901	77.33795601	33
GandhiNagar	23.29835	77.34246003	57
Sanjiv Nagar	23.301451	77.37383902	25
Lalghati	23.279299	77.373246	54
Bhanpur	23.298558	77.43066204	16
Gupta Nagar	23.287376	77.42342996	18
Shaid Nagar	23.258597	77.39650402	26
NITTR	23.238759	77.38967001	17
Banganga	23.23677	77.392333	23
Jamalpura	23.27725	77.39863797	41
Kotra Sultanabad	23.222493	77.40425703	53
Subhash Nagar	23.248515	77.43477303	50
Shankar Nagar	23.225866	77.43378597	117
Anna Nagar	23.233465	77.449629	31
Nizamuddin Colony	23.259392	77.46734502	28
Awadhpuri	23.238052	77.48943804	30
NLU	23.186125	77.36367303	12
Katara Hills	23.191271	77.48870697	13
Mahabali Nagar	23.179906	77.41403201	30
BHEL Area	23.25216	77.44726298	12
Priyanka Nagar	23.155584	77.416094	20
Gulmohar	23.19573	77.43356	21
Khanugaon	23.262072	77.36878599	16
Shakti Nagar	23.220199	77.45783198	17
Bheem Nagar	23.233587	77.41728402	22
Kolar Road	23.185363	77.41319097	60
Misroad	23.159524	77.46932399	17
Karond	23.297507	77.40782403	62
	Total		**921**

(The *Ae. aegypti* mosquitoes were collected from September 2022 to June 2023).

Table 3. Summary of comparative results of the newly developed PCR assay with that of Nakahori *et al.* (1991)

AMEL_PCR	Number of samples with STS_PCR			McNemer's Test (p value)	Cohen's kappa (95% CI)
	Positive	Negative	Total		
Positive	22	1	23	4.5 (0.033)	0.743 (0.58–0.91)
Negative	7	34	41		
Total	29	35	64		

post meal in both males and females while up to D5 (120 hours) post meal in females (Figure 1c). To validate this assay in field-collected mosquitoes, 921 adult *Ae. aegypti* female mosquitoes were collected from different parts of Bhopal city using an aspirator (Table 2). DNA was extracted from the mosquitoes, and blood meal identification PCR, as described by Field *et al*. (2020), was carried out to identify the source of blood meals in the field collected mosquitoes. Of the 921 field-collected mosquitoes, 64 mosquitoes were found to have engorged with human blood (6.94%) based on the PCR method of Field *et al*. (2020). These 64 samples with human blood were further tested with the newly developed PCR assay (STS_PCR) and already existing PCR assay (Teltscher *et al*., 2021) to differentiate the human sex in the mosquito blood meal. The newly developed PCR was able to differentiate sex in 29 samples (45.31%) while the old assay developed by Teltscher *et al*. (2021) could differentiate human sex in 21 samples (32.81%). 35 samples were not amplified by the newly developed assay, while 43 samples could not be amplified by the assay developed by Teltscher *et al*. (2021). This might be due to the differential amplification efficiency of PCR primers targeting different genomic regions. For example, Field *et al*. (2020) designed the PCR primers targeting the mitochondrial cytochrome-b region to distinguish human, bird, and mammalian host blood meals, while Teltscher *et al*. (2021) developed the PCR primers targeting the amelogenin locus (AMEL) located on X and Y chromosome of human genome. McNemer's test statistics revealed a significant discordance between these two results (χ^2 = 4.5, p = 0.03), while the Cohen's kappa statistic was 0.743 with a lower 95% confidence interval of 0.58 (Table 3) indicates a substantial agreement in between these two assays. To confirm the identity of the amplified products, the male and female amplification products of STS_PCR from five randomly chosen samples were sequenced. The sequencing results of the PCR products (GeneBank accession ID OQ818305 and OQ818306) were matched using the NCBI BLAST algorithm. The BLAST findings revealed that the amplified products were from the human genome and matched 100% with E-values of 0.0 and $1e^{-145}$ for both X and Y chromosomes. This indicated the specificity of amplification of only human samples using the developed primer sets.

DISCUSSION

Understanding the dynamics of mosquito vectors feeding on vertebrate host blood is critical for identifying potential vector species, evaluating disease risks, and establishing vector-borne disease pathogens. Different approaches, from serological techniques (Washino & Tempelis, 1983) to more recently developed PCR-based amplification of host DNA (Kent, 2009), have been used to accurately identify the vertebrate hosts of mosquitoes at the species level or, even, at the individual level, providing information on the relative importance of different mosquito species in the transmission of particular pathogens. Many mitochondrial DNA genes such as *cytochrome c Oxidase* Subunit I (COI), *cytochrome b* (Cyt b), 12S, 16S Mitochondrial Ribosomal DNA (rDNA), *NADH Dehydrogenase* Subunit I (ND1), and nuclear genes (nuclear ribosomal DNA, *Prepronociceptin* gene, and *Alu* Transposable Elements) have been targeted to develop genetic markers for identification of host blood meal in mosquitoes (Kent, 2009; Borland & Kading, 2021). However, in most cases, these assays identify the source of the blood meal up to the species level and do not differentiate between the sexes of the human host. A few forensic entomology approaches have been used to identify the sex of human hosts in mosquito blood meals using STR loci (De Benedictis *et al*., 2003; Harrington *et al*., 2014), *amelogenin*, and *SRY* genes (Garcia-Rejon *et al*., 2020; Teltscher *et al*., 2021). By identifying the sex of the human host through the analysis of mosquito blood meals, researchers can gain valuable insights into the epidemiology of vector-borne diseases. This information helps in tracking how diseases spread within human populations, especially when certain diseases show sex-specific prevalence or severity. Besides, knowing the sex of humans in areas with a high risk of disease transmission allows for the development of more targeted and effective vector control strategies. For example, if mosquitoes exhibit a preference for biting females, who might be pregnant, this knowledge can be used to implement protective measures for high-risk groups and develop tailored interventions. In the present study, a specific PCR-based assay was developed to identify the DNA of male and female in mosquito blood meals by targeting the *steroid sulfatase* (STS) region of the human genome.

The developed PCR assay can successfully amplify as little as 0.1ng DNA and can differentiate both male and female human DNA in mosquitoes up to three days (72 hours) and five days (120 hours) post blood-meal respectively. The sensitivity of detection of human DNA in the mosquito gut depends strongly on the degree of blood digestion, which again depends on the storage conditions from the time of mosquito collection to processing for DNA extraction in the laboratory (Santos *et al*., 2019), and on the quality of primers and primer targets and mosquito species (Teltscher *et al*., 2021). Human blood DNA was detected in live fully fed mosquitoes until three days after blood feeding and for four weeks when stored at -20°C in field-collected *Ae. aegypti* (Siriyasatien *et al*., 2010), whereas Garcia-Rejon *et al*. (2020) detected human DNA for up to 36 h in laboratory-reared *Ae. aegypti*, using *amelogenin* and *SRY* gene based PCR assays. Similarly, Teltscher *et al*. (2021) differentiated male and female human DNA in 93.3-100% of *Ae. aegypti* and *An. coluzzi* mosquitoes after 24 hours of blood feeding using the *amelogenin* gene based PCR assay. A gradual decline in DNA amplification was also observed in this study (Teltscher *et al*., 2021), with amplification of only 3.3% of samples from *Ae. aegypti*, and no amplification from *An. coluzzi*, after 60 hours post blood-feeding. A recently developed nested PCR-based assay using the AMEL gene reported detection of human DNA in female *An. stephensi* up to 60 hours after a blood meal (Talebzadeh *et al*., 2023).

CONCLUSION

The PCR-based assay developed in this study can successfully amplify both male and female human DNA up to three days (72 hours) after a blood meal in laboratory conditions. However, this could not be tested in field-collected samples as the time of

blood intake in field-collected samples is difficult to infer, and thus represents a different degree of blood meal digestion. Furthermore, the assay lacked the ability to differentiate the host's sex in mixed blood-fed mosquitoes, where DNA from both male and female hosts was present, as the primers were designed to target X and Y chromosomes simultaneously and can be considered as a limitation of the study. Nonetheless, the specific detection of human DNA in mosquito blood meals, as demonstrated by the sequencing results, and the low detection limit of this PCR assay compared to other PCR based methods, could be useful in understanding mosquito biting behavior in large epidemiological studies. This developed PCR assay can be effectively used to identify high-risk groups and can be applied to other hematophagous insects of medical importance to evaluate their role in disease transmission, and epidemic preparedness. This assay has the potential to yield valuable insights into monitoring sex-specific disease transmission and its severity. Epidemiological data derived from this assay could enhance comprehension of vector behavior, facilitating the implementation of precise vector control measures for high-risk groups via targeted strategies.

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Conflict of interest

All authors have declared no conflicts of interest.

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