Universidade Federal de Pernambuco
Centro de Ciências Biológicas
Laboratório de Imunopatologia Keizo Asami
Programa de Pós-Graduação em Biologia Aplicada à Saúde

IDENTIFICAÇÃO DE DNA HUMANO ENCONTRADO EM TRATO DIGESTÓRIO DE CULICÍDEOS HEMATÓFAGOS PARA FINS FORENSES

KAYNARA CECÍLIA NERY RABÊLO

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Tese submetida ao Programa de Pós-Graduação em Biologia Aplicada à Saúde do Laboratório de Imunopatologia Keizo Asami, do Centro de Ciências Biológicas, da Universidade Federal de Pernambuco, para defesa de doutorado.

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KAYNARA CECÍLIA NERY RABÊLO

IDENTIFICAÇÃO DE DNA HUMANO ENCONTRADO EM TRATO DIGESTÓRIO DE CULICÍDEOS HEMATÓFAGOS PARA FINS FORENSES

A comissão examinadora, composta pelos professores abaixo, sob a presidência do primeiro, considera a candidata como:

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Recife, 30 de junho de 2015.

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RESUMO

Insetos e outros artrópodes quando em locais de crime podem servir como vestígio criminal. O advento da genética forense pode auxiliar na obtenção do DNA humano a partir destes insetos, podendo relacionar o suspeito a cena do crime. Desta forma, esta pesquisa objetivou a obtenção e a comparação do perfil genético humano extraído de mosquitos hematófagos com diferentes metodologias para a extração de DNA, analisando os seguintes fatores: variação temporal para obtenção dos perfis genéticos após a hematofagia; a obtenção e a comparação dos perfis de DNA humano do sangue proveniente do trato digestivo dos mosquitos hematófagos com as amostras referências (saliva) de voluntários; influência da amônia, ácido lático e tipo sanguíneo, além da temperatura corporal dos voluntários e a relação na atratividade dos mosquitos e consequente obtenção do material genético; avaliou-se também a mistura de perfis genéticos provenientes de um único mosquito e o intervalo temporal após a hematofagia. Para a análise da comparação das extrações foi utilizado o kit DNA IQ^{TM} ,a resina Chelex® 100 e extração com NaOH; e para as outras variáveis em estudo utilizou-se somente o DNA IQ^{TM}. A quantificação foi realizada com o Quantifiler® Duo e a amplificação com o kit AMPF*l*STR Identifiler® Plus® PCR, que analisou 15 *loci* STR e amelogenina. A quantificação para o estudo das misturas de DNA nos mosquitos foi realizada com PowerPlex 16HS System. Os dados foram analisados através do programa estatístico PATCAN v. 1.2 software e para a análise das misturas foi utilizado o programa DNA MIX v. 3.2 software. Os resultados demonstraram que o uso do DNA IQ^{TM} foi melhor quando comparado a resina Chelex® 100, com obtenção de perfis viáveis em até 72h após a refeição sanguínea. Não foi obtido perfil de DNA quando utilizado NaOH. Os resultados demonstraram também uma confrontação positiva entre o sangue encontrado no trato digestivo dos mosquitos e o material genético cedido pelos voluntários, como amostra referência. As análises bioquímicas demonstraram que o tipo sanguíneo com maior número de obtenção de perfil genético foi o tipo O; além disso, foi constatado valores de normalidade para o exame de lactato, mas para a análise de amônia foi obtido DNA também com valores maiores que o padrão de referência para este tipo de exame, tanto em homens quanto em mulheres. Houve obtenção de DNA nas temperaturas corporais registradas entre 36ºC a 37º C. Foi observado também que mistura de DNAs humano pode ser detectado a partir de um único mosquito hematófago. Desta forma, os resultados demonstraram que os mosquitos hematófagos quando encontrados em cenas de crimes tem efetivo valor forense.

Palavras-chave: DNA forense; Entomologia Forense; Mosquitos hematófagos.

ABSTRACT

Insects and others arthropods can be used as traces when located in the crime scene. The advent of forensic genetic can assist in obtaining human DNA from these insects, relating the suspect to the crime scene. So, this research aimed the obtainment and the comparison of the human gene profile extracted for the hematophagous mosquitoes with different methodologies to the DNA extraction, analyzing the following factors: temporal variation to the obtainment of the genetic profile after the hematophagy; obtainment and comparison of the human gene profile from the hematophagous mosquitoes´ digestive tract with the volunteers´ samples (saliva); influence of ammonia, lactic acid and blood type, besides the volunteers' body temperature and the relation on mosquitoes´ attractiveness and genetic material obtainment; the compound of the genetic profile from one mosquito and temporal intermission after hematophagy was also evaluated. DNA IQ^{TM}, resin Chelex® 100 and extraction with NaOH was used to the analyses of the extract comparison. The quantification was held with Quantifiler® Duo and the amplification with AMPF*l*STR Identifiler® Plus® PCR kit, that analyzed 15 loci STR and amelogenin. The quantification to study the compound of DNA in the mosquitoes was held with PowerPlex 16HS System. Data were analyzed through statistic program PATCAN v. 1.2 software and to analyze the profiles mixtures DNA MIX v. 3.2 software program was used. The results showed that the use of DNA IQ^{TM} was typical when compared with Chelex® 100, and success in gene amplification with obtainment of viable profiles up to 72 hours after blood meal. DNA profile was not obtained when used NaOH. The results also showed a positive confrontation between blood found in the mosquitoes´ digestive tract and the material assigned by the volunteers, with reference sample. Biochemical analysis demonstrated that the blood type with bigger obtainment number obtained profile gene was type O; besides that the human DNA profiles were achieved from hematophagous mosquitoes when compared with the correspondent biochemical analysis of the volunteer found normal values to lactate exam, but to ammonia analysis was obtained DNA with higher values than the reference standard to this type of exam, in both gender. There was DNA obtainment from body temperatures registered between 36°C to 37°C. It was also observed that human DNA compound can detected through a only single hematophagous mosquito. With that the results showed hematophagous mosquitoes when found in the crime scene have effective forensic value.

Keywords: Forensic DNA; Forensic entomology; Hematophagous mosquitoes.

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LISTA DE ABREVIATURAS E SIGLAS

AFLP	Polimorfismo de comprimento de fragmento amplificado
CODIS	Sistema Indexado Combinado de DNA
DNA	Ácido Desoxirribonucléico
FBI	Serviço Federal de Investigação
HVII	Região de hipervariedade
IPM	Intervalo pós-morte
LPPGF	Laboratório de Perícia e Pesquisa em Genética Forense
LR	Razão de verossimilhança
PCR	Reação em cadeia da polimerase
RAPD	Perfil da amplificação randômica de DNA
REP1R	Sequência palindrômicas extragênicas repetitivas
RFLP	Polimorfismo de tamanho de fragmento de restrição
SDS	Secretaria de Defesa Social de Pernambuco
STRs	Repetições consecutivas curtas

1.INTRODUÇÃO

1. INTRODUÇÃO

A Entomologia Forense é o estudo de insetos e outros artrópodos com aplicabilidade em procedimentos legais (Amendt et al., 2004; Oliveira-Costa, 2011). Nesse contexto, artrópodes que se alimentam de sangue, como os mosquitos, tem se apresentado como uma ferramenta entomológica potencial aplicada na resolução de casos criminais (Kreike e Kampfer, 1999). Em meados dos anos 80, outra ciência fundamental na investigação forense, a genética, apresentou seu ponto culminante, colaborando atualmente com a identificação humana, em investigações criminais (Benecke, 1998). A união da Entomologia e da Genética Forense, a Entomogenética Forense, vêm auxiliando na busca de suspeitos, no exoneramento de inocentes, na demonstração da culpabilidade de criminosos, ou até mesmo ajudando a convencer o pronunciamento da confissão (Butler, 2005; Pena, 2005), fortalecendo de modo decisivo a busca da evidência criminal.

A prova entomológica utilizada pela criminalística como evidência forense no cometimento de um crime ainda é considerada uma nova generalidade em procedimentos operacionais como vestígio criminal no âmbito nacional (Oliveira Costa, 2011). No caso de insetos hematófagos usados com essa finalidade são realizadas análises genéticas comparativa do sangue humano encontrado no trato digestivo do animal coletado em locais de crime, sendo possível se obter subsídios que podem servir como prova forense (Scott et al., 1993; Kreike; Kampfer, 1999; De Benedicts, 2003). Nxo entanto, a base das investigações forenses depende da habilidade do perito criminal em reconhecer no local do crime, a possibilidade de utilização e a importância de diferentes tipos de evidências físicas (Silva e Passos, 2002).

Dados criminais, emitidos pela Secretaria de Defesa Social do Estado de Pernambuco/Brasil demonstram a ocorrência crescente de crimes de sequestro/cárcere privado em Pernambuco/Brasil. Entre 2008 e 2011, por exemplo, o número passou de 155 para 238 ocorrências. Nestes tipos de crimes, ocorridos em ambientes fechados, pode ainda ocorrer mistura de perfil genético (vítima e dos suspeitos) no sangue de mosquitos presentes no local.

Comumente, quando interferida a refeição sanguínea, a fêmea continuará o repasto no mesmo ou em outro indivíduo, podendo assim obter sangue de fontes diferentes e indicar DNA de mais de um indivíduo num único mosquito. Através das análises e dos cálculos estatísticos já é possível a interpretação dos perfis de DNA complexos – que apresentam

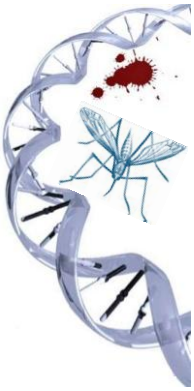
misturas de perfis genéticos - em casos forenses (Voskoboinik e Darvasi, 2011; Haned et al., 2015).

Desta forma, mosquitos hematófagos poderiam servir como vestígios adicionais coletados em locais de crime. A prática de sequestro e cárcere privado, dentre vários outros delitos que podem ocorrer em lugares fechados, torna este tipo de local favorável a coleta dos mosquitos e a possibilidade de ser elencado como indício forense a partir da análise do DNA humano proveniente do repasto sanguíneo, podendo assim interligar o suspeito a cena do crime. Contudo, como se trata de um organismo vivo e que ao realizar o repasto já começa a consumir o sangue para a maturação dos ovários e consequente desenvolvimento dos ovos (Forattini, 2002) necessita de serem analisadas as mais diversas variáveis que poderiam implicar em alteração na emissão de perfis genéticos viáveis como prova judicial. Tais implicações foram o alvo de estudo desta pesquisa.

Sendo assim, os objetivos deste estudo consistiram em: (1) verificar qual o melhor método de extração de DNA humano do sangue proveniente do trato digestivo dos mosquitos hematófagos; (2) subsidiar a relação da qualidade do perfil genético humano obtido e o maior intervalo temporal pós-hematofagia que o material sanguíneo foi amplificado; (3) analisar a quantificação do DNA e a qualidade do perfil genético obtido; (4) relacionar e estimar o grau de correspondência entre o perfil de DNA humano encontrado nos mosquitos hematófagos e as amostras referências de voluntários; (5) comparar se fatores como amônia, ácido lático, tipo sanguíneo e temperatura corporal interferem na atração dos mosquitos hematófagos; (6) verificar a presença de misturas de DNA humano num único mosquito e até que intervalo de tempo há qualidade na emissão dos perfis de DNA.

Além do mais, com a inserção do Laboratório de Perícia e Pesquisa em Genética Forense no Estado de Pernambuco e a criação do Banco de Dados que obriga criminosos que cometeram atos delituosos dolosamente, com violência grave, e os crimes hediondos, de fornecerem material biológico para extração de DNA, embasam a importância dessa pesquisa científica. Em consequência, quando os mosquitos hematófagos forem coletados em local de delito e obtido o perfil genético do repasto sanguíneo, e com as investigações policiais discernirem a questão do suspeito para comparação do perfil obtido, poderá inclusive comparar aos perfis já armazenados no Banco de Dados e verificar se há reincidência do delito cometido.

2. REVISÃO DE LITERATURA



2. REVISÃO DE LITERATURA

2.1 ENTOMOLOGIA FORENSE: O ESTADO DA ARTE

O conceito de Entomologia Forense é originário da união das palavras gregas *Enton* e *Logos*, que significam respectivamente, inseto e estudo, ou seja, é o estudo dos insetos, além de outros artrópodes, aplicados a procedimentos criminalísticos (Byrd e Castner, 2000; Pereira, 2015). Os insetos são os primeiros a chegarem ao local da morte, como no caso das moscas varejeiras, atraídos pelos gases desprendidos pelo processo de decomposição, já que possuem órgãos sensoriais altamente especializados na detecção de odores, tendo sido relatado casos de encontro de oviposições inclusive em vítimas que estavam ainda agonizando (Mason, 1980; Catts, Goff, 1992; Campobasso et al., 2001).

Os primeiros casos que utilizaram o conhecimento da Entomologia Forense foram relacionados com moscas (Diptera). No século XIII, foi relatado o primeiro caso documentado com o uso da Entomologia Forense no Manual de Medicina Legal Chinês e no século XV tal ciência foi retratada com figuras em talhas de madeira em a *Dances of the Death* (Benecke, 1998; Benecke, 2004). Mas somente no final do século XX, principalmente na América do Norte e na Europa, a Entomologia Forense passou a ser considerada uma ferramenta com importância forense (Goff et al., 1991a; Catts e Goff, 1992).

Apenas no ano de 1855 houve a primeira estimativa de intervalo pós-morte baseada no estudo de insetos, realizada pelo médico francês Bergeret. Ele relatou a descoberta de um cadáver de uma criança dentro de um gesso de uma residência, o qual foi constatado um intervalo pós-morte extenso com a associação da fauna encontrada e o estágio de decomposição do corpo, e desta forma, os recentes moradores do imóvel foram inocentados de tal crime, recaindo as suspeitas aos habitantes anteriores da casa. E somente após 39 anos que foi lançado o primeiro livro sobre este tema, intitulado *La faune de cadavres*, do autor Mégnin, contendo fundamentação teórica, descrições dos insetos e casos reais com a aplicabilidade desta ciência (Pujol-Luz et al., 2008; Oliveira-Costa, 2011).

A aplicabilidade da Entomologia Forense é muito diversificada, sendo mais aplicada em casos criminais. Muitas dúvidas podem ser solucionadas nesses casos através do estudo de insetos, sendo mais divulgada na área da medicina legal. Pode promover respostas como o intervalo de morte, se o cadáver foi removido do local original do crime, se a morte foi natural

ou violenta, identificação do cadáver através da extração do material genético humano encontrado no interior de larvas necrófagas, além de como, quando e onde a morte ocorreu (Oliveira-Costa, 2011).

Além disso, crimes como tráfico de entorpecentes podem ser solucionados através da identificação dos insetos prensados na droga, como a maconha (*Cannabis sativa)*, podendo-se estabelecer a rota do tráfico por meio da distribuição geográfica destes insetos (Crosby et al., 1986). O consumo de drogas ilícitas e o envenenamento por parte da vítima também podem ser detectados através da análise de larvas encontradas no corpo (Oliveira-Costa, 2011).

Outras formas de crimes, como maus tratos, também podem ser constatados com a presença e a análise de insetos, através da ocorrência de miíase que é uma afecção causada pela presença de larvas de moscas, demonstrando a consequente privação de cuidados de higiene (Lord e Rodriguez, 1989; Goff et al., 1991b; Fares et al., 2005). Crimes, principalmente os cometidos em locais fechados, onde este tipo de local pode limitar o forrageio do inseto, podem ser solucionados a partir da análise comparativa do material biológico humano encontrado ou deixado pelos insetos, como piolhos, larvas de moscas e mosquitos (Repogle, 1994; Lord et al., 1998; Spitaleri et al., 2006; Oliveira-Costa, 2011; Li et al., 2011).

Pesquisas entomológicas com finalidade forense em parceria com instituições de perícias e faculdades já constituem uma realidade mundial. Inclusive, embasa esta afirmação o FBI nos Estados Unidos da América que possui uma linha de pesquisa na área de perícia entomológica (Gredilha et al., 2015). No Brasil, a entomologia forense ainda é incipiente, mas já tendo incentivo no setor deste tipo de pesquisa (Oliveira-Costa, 2011).

As pesquisas no Brasil, foram iniciadas com os estudiosos pioneiros Edgard Roquette Pinto e Oscar Freire, em 1908, que registraram a diversidade da fauna de insetos necrófagos na região da Mata Atlântica que servem até hoje como parâmetros de novos estudos realizados nesta área (Pujol-Luz et al., 2008). Desde então, simpósios, congressos e até associação, cursos interligados com Universidades e Institutos de Criminalística foram organizados e realizados no Brasil como forma de divulgação desta ciência (Pujol-Luz et al., 2008).

Apesar disto, ainda há escassez de entomólogos forenses e consequentemente de uma maior divulgação desta ciência no âmbito jurídico (Pujol-Luz et al., 2008). O conhecimento da aplicabilidade de insetos que podem conter em seu trato digestivo DNA humano como piolhos (Repogle, 1994), mosquitos (Kreike e Kamper, 1999; Hawley e Budowle,2000) e califorídeos (Clery, 2001) ainda é uma realidade não muito divulgado e aplicada na área

criminal, havendo ainda poucos trabalhos realizados com estes potenciais insetos. Nessa área, os projetos mais financiados são aqueles voltados ao interesse médico ou veterinário (Pujol-Luz et al., 2008).

O conhecimento da identificação, da distribuição geográfica, tipos de insetos encontrados em decorrência dos estágios de putrefação do cadáver e hábitos das espécies de insetos colonizadores é fundamental para auxiliar nas respostas de questionamentos básicos na cena criminal, como: quem é o cadáver, como a morte ocorreu, onde tal crime ocorreu, quando ocorreu e, se foi natural, acidental ou criminal (Catts e Haskell, 1991; Campobasso e Introna, 2001; Introna et al., 2001; Barbosa et al., 2009). Desta forma, estudos com modelo animal é fundamental para melhor conhecimento à procura da acurácia nas respostas supramencionadas, sendo o porco (*Sus scrofa*) um animal bastante utilizado como forma de simular situações de crimes (Campobasso e Introna, 2001; Introna et al., 2001; Barbosa et al., 2009).

A utilização do porco se fundamenta na semelhança com a estrutura orgânica humana, no que diz respeito à anatomia interna, distribuição de gordura, falta de pelagem espessa, além de serem onívoros. Com estas características torna-se um excelente modelo de estudo e observação da fauna de díptero e coleóptero associada à carcaça suína que participam no processo de colonização e decomposição cadavérica (Catts e Goff 1992; Campobasso et al. 2001). Contudo, tais estudos necessitam de ser otimizados ao máximo, gerando um maior volume de resultados e evitando repetições, afim de evitar o sacrifício de animais, e quando o fizer tomar o cuidado de ser de forma rápida e menos indolor possível. Além disso, o modelo animal tende a outras dificuldades como a obtenção do mesmo, quem e como sacrificá-lo e a onerosidade na compra (Teixeira et al., 2015).

Outros ramos mais específicos na área forense, com pesquisas relacionadas ao estudo da bionomia e dietas sintéticas de dípteros necrófagos também foram realizadas no intuito de fornecer subsídio na tentativa de substituir o alimento natural, ou seja, carne putrefeita, por outro sintético que seja mais propício tanto técnica como economicamente em laboratório (Cohen, 2005). Dietas a base de carne e dietas semi-sintéticas com inclusão de sardinha, rúmen ou ovo de galinha foram testadas por Rabêlo et al. (2011) apresentando sucesso na criação de *Chrysomya megacephala* e *Chrysomya putoria* em laboratório, espécies comuns no Brasil, inclusive a espécie *C. megacephala* já foi encontrada em cadáveres humanos em Pernambuco, como comprovado no estudo de Oliveira e Vasconcelos (2010) que identificaram esta e outras espécies como *C. albiceps, C macellaria, O riograndensis* e *R. belforti* coletadas em cadáveres no Instituto de Medicina Legal localizado em Pernambuco.

Outrossim, tais dietas servem como substrato também para outros tipos de pesquisas forenses, como os de natureza sexual. Por exemplo, Clery (2001), utilizando Y-STR, detectou perfil genético humano no trato digestório de larvas de califorídeos que foram alimentados com dieta artificial adicionada a sêmen humano, a fim de utilizar a técnica em crimes sexuais seguidos de morte.

2.2 TÉCNICAS USUAIS DE ANÁLISE GENÉTICA APLICADAS AOS ESTUDOS DA ENTOMOLOGIA FORENSE EM INVESTIGAÇÕES CRIMINAIS

"A individualidade biológica é uma característica única, de pessoa para pessoa. Qualquer pessoa, desde o seu nascimento, até à altura da sua morte, possui características genéticas, as quais são imutáveis ao longo da vida e estão expressas logo após o nascimento. A identificação biológica pressupõe o princípio da individualidade biológica de cada ser humano" (Moisan, 1996).

A primeira utilização da análise do DNA relacionado à identificação de uma pessoa foi realizada na década de 80 por Sir Alec Jeffreys (Jeffreys et al., 1985). Após este fato, a análise do DNA passou a ser considerada como sendo uma nova forma de evidência científica (Primorac e Schanfield, 2014). Fato corroborante no que concerne de aproximadamente 99,9% do genoma de um indivíduo ser idêntico a um outro, mas ainda restando a diferença crucial de 3,2 bilhões de pares de bases de nucleotídeos que compõem o genoma, ou seja, quando comparamos uma pessoa a outra, esta diferença é o suficiente para a identificação já que não há duas pessoas com a mesma sequência de bases no seu DNA, excetuando-se gêmeos idênticos (Ojopi et al., 2004).

A escolha da técnica a ser usada na análise genética está relacionada ao tipo de material biológico envolvido, influenciando diretamente no sucesso da análise (Leite et al., 2013). O uso de técnicas apropriadas para o isolamento, amplificação e caracterização do material genético humano a partir do material genético extraído do trato digestivo de insetos hematófagos, e também de insetos necrófagos, é possível para que se possa usar esse material como ferramenta na resolução de casos criminais (Repogle, 1994; Lord et al., 1998; Spitaleri et al., 2006; Oliveira-Costa, 2011; Li et al., 2011).

Em geral as técnicas de obtenção do DNA humano de insetos tem como base a extração do DNA, seguida da amplificação através da PCR e posterior comparação dos

resultados obtidos nos eletroferogramas (Primorac e Schanfield, 2014). Alguns dos métodos comumente utilizados para a realização da extração do DNA são: fenol/clorofórmio, brometo de cetiltrimetilamonio, resina Chelex 100 ou a utilização de um dos mais variados kits comerciais disponíveis no mercado (Primorac e Schanfield, 2014). Outras análises genéticas também podem ser aplicadas à procura do material genético, como por exemplo, a utilização do mtDNA, que também pode ser utilizado em casos do material biológico degradado, podendo-se proceder seu uso na entomologia forense de forma similar ao DNA barcoding (Holland; Parsons, 1999; Morley et al., 1999; Hebert et al., 2003), além do uso de outras técnicas como PCR-RFLP, RAPD e AFLP (Benecke, 1998; Schroeder et al., 2003; Picard e Wells, 2012).

Alguns casos criminais que resultaram das vicissitudes e dos benefícios da união de algumas técnicas de biologia molecular aplicadas na entomologia forense podem ser relatados através da resolução de casos reais. Segundo Chávez-Briones et al. (2013), relataram que foram os primeiros a reportarem no México um caso de análise de DNA humano isolado proveniente do trato gastrointestinal de larvas para a identificação de uma vítima. Um cadáver foi encontrado severamente queimado, com ausência de mãos e pés e desta forma com a impossibilidade de identificação biométrica, colonizado por larvas de moscas (Diptera: *Calliphoridae e Sarcophagidae*). Apenas um anel de formatura foi recolhido como pertence da vítima. Tecidos moles não estavam disponíveis devido ao estado adiantado de putrefação. Com as investigações, foi relatado um caso de desaparecimento e o provável pai reconheceu o anel de formatura, mas não teve condições de reconhecer o corpo pelo estado de decomposição. Desta forma, foi recolhido DNA do pai e do trato gastrointestinal das larvas de moscas, e realizada a extração de DNA com a resina Chelex100. O material foi amplificado com o kit comercial AmpF*l*STR® Identifiler (Applied Biosystems). O mesmo perfil de DNA foi obtido das três larvas coletadas do cadáver. Tal resultado de perfil de DNA foi comparado com o do provável pai da vítima (Tabela 1), tendo-se a probabilidade de 99,685% de paternidade, com pelo menos um alelo correspondente entre a vítima e o pretenso pai. O mesmo teste de DNA foi posteriormente realizado e confirmado a partir da análise dos ossos da vítima, após inúmeras tentativas.

Locus	Vítima	Pai
Amelogenin	XX	XY
D8S1179	**13/15**	**13/15**
D21S11	29/**30.2**	**30.2**/33.2
D7S820	**10**/10	**10**/11
CSF1PO	n/r†	10/11
D3S1358	14/**17**	16/**17**
TH01	**7**/7	**7**/9.3
D13S317	12/**13**	9/**13**
D16S539	10/**11**	**11**/12
D2S1338	24/**25**	17/**25**
D19S433	**15**/16	13/**15**
vWA	**16**/18	15/**16**
TPOX	**8**/8	**8**/8
D18S51	n/r	14/15
D5S818	**12**/12	11/**12**
FGA	n/r	18/26

* associação dos alelos correspondentes marcados em negrito

† resultados não obtidos

Tabela 1 – Comparação dos resultados da análise do DNA humano pela técnica do STR a partir do material genético encontrado nas larvas e o suposto pai da vítima (Chávez-Briones et al., 2013).

Um outro exemplo ocorreu em março de 2008, na cidade de Changsha, na China. Li et al. (2011) reportaram que foi encontrado um cadáver sem cabeça, e a 500 metros do local sua provável cabeça (Figura 1). A identificação não foi possível devido ao estado adiantado de putrefação, mas foi observada presença de bastante larvas que foram coletadas para posterior análise molecular. A partir da análise do DNA mitocondrial e do STR de fragmentos de HVII, provenientes do conteúdo do trato digestivo encontrado em larvas de moscas varejeiras da espécie *Aldrichina grahami* (Figura 1)**,** inseto necrófago, coletadas tanto no corpo quanto na cabeça, foi possível a associação das partes separadas do corpo, fornecendo a matéria forense necessária para a identificação do cadáver a partir da análise do eletroferograma (Figura 2).

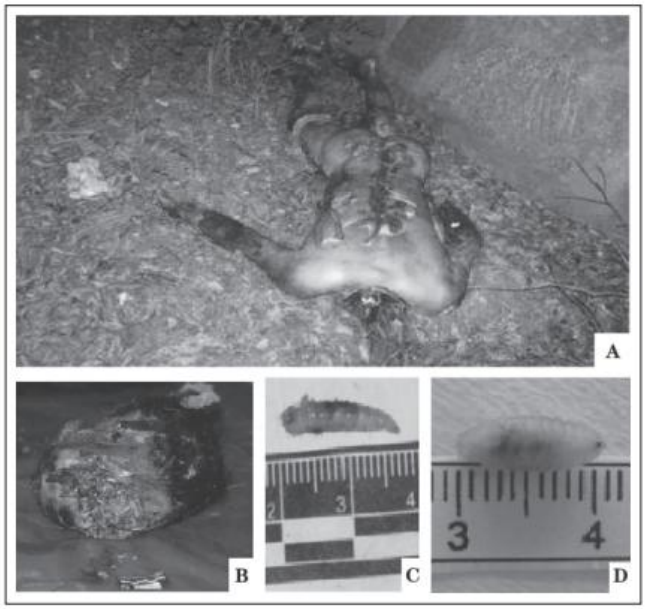


Figura 1 - (A) O cadáver sem cabeça do sexo masculino no local do crime; (B) O crânio separado do corpo; (C) larvas de terceiro ínstar da espécie *Aldrichina grahami* e a medição do comprimento (entre 1.1cm e 1.6cm); (D) Observação de uma parte escura na larva (Li et al., 2011).

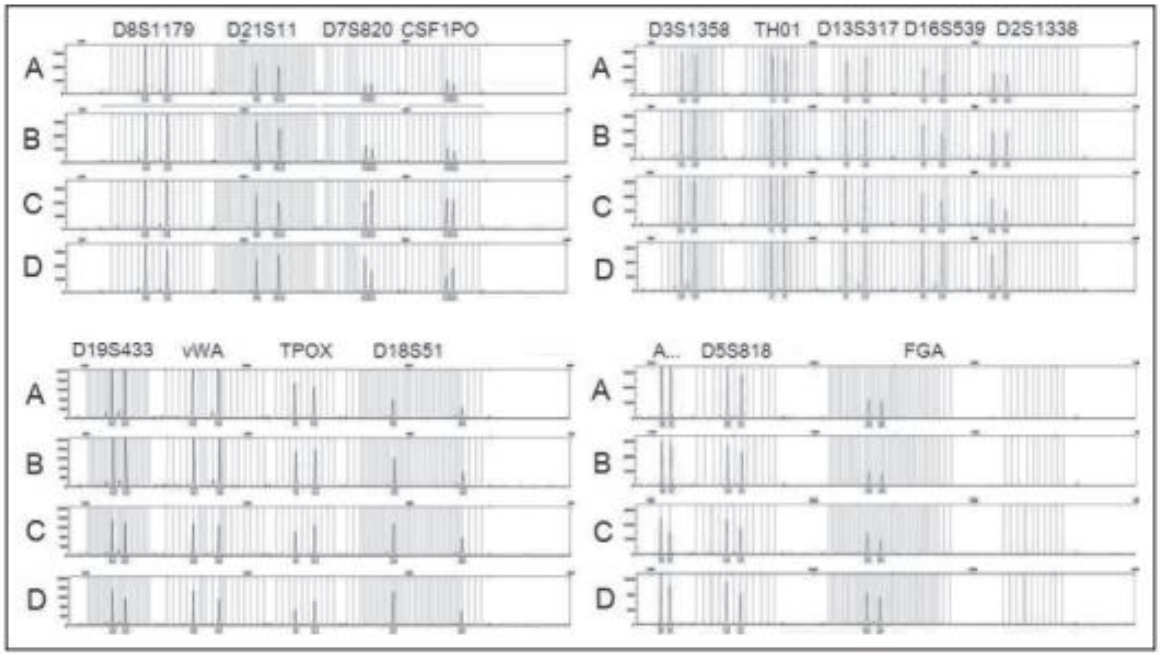


Figura 2 – Eletroferograma demonstrando o perfil completo com os 16 *loci* STR. (A) larvas obtidas do corpo; (B) larvas obtidas da cabeça; (C) cadáver sem cabeça; (D) crânio (Li et al., 2011).

Um outro caso resolvido com o advento da entomologia e genética forense, foi relatado por Benecke (1998), com ocorrência em Outubro de 1997, onde insetos foram coletados como evidência de um corpo que se achava no Instituto de Medicina Legal, na Alemanha. O corpo encontrava-se coberto com saco e em avançado estado de decomposição. As larvas foram coletadas tanto do lado de fora do saco quanto da parte de dentro onde estava o cadáver humano, e mediam aproximadamente 9mm. A questão era se as larvas encontradas eram ou não da mesma espécie, remetendo ou não a uma segunda oviposição para a distinção do IPM. Pupas também foram encontradas embaixo do cadáver, sem ter a certeza se pertenciam ao corpo em questão ou de outro cadáver do necrotério. A dificuldade para estabelecimento do IPM dá-se por larvas em estágios iniciais, diferentes tempos de desenvolvimento de espécimes encontrados e não conhecimento de todas as diferentes espécies que podem ser encontradas. Sendo assim, a técnica do RAPD foi utilizada no intuito de uma rápida identificação genética. A extração foi realizada com proteinase K seguida da extração com fenol (sem clorofórmio). Amostras de quatro larvas foram comparadas com as de pupas, além de duas moscas varejeiras e um besouro (controle negativo). Os resultados demonstraram que o perfil de DNA analisado das larvas pertencia ao mesmo gênero; que o perfil de DNA da pupa também pertencia ao mesmo gênero quando comparado com das larvas; que não houve similaridade entre o perfil encontrado nas moscas e os demais perfis analisados nas larvas e por fim, que o perfil obtido do besouro não demonstrou similaridade com os perfis obtidos das moscas. Neste exemplo, através dos perfis obtidos do RAPD (Figura 3) foram distinguidas as seguintes espécies: *Lucilia spec.*, *Calliphora erythrocephala* e um besouro (Coleoptera: *Silphidae*). A figura 3 mostra a semelhança nos perfis obtidos das quatro larvas durante os 460 minutos da análise, demonstrou também a não identificação da pupa com respectivas larvas, além da não similaridade com as moscas. O perfil do besouro (controle negativo) não apresentou nenhuma semelhança com os demais perfis. Dos 11 iniciadores RAPD, um só foi suficiente para resolver uma situação prática forense. Este é o primeiro relato de uma aplicação forense de tipagem de DNA RAPD.

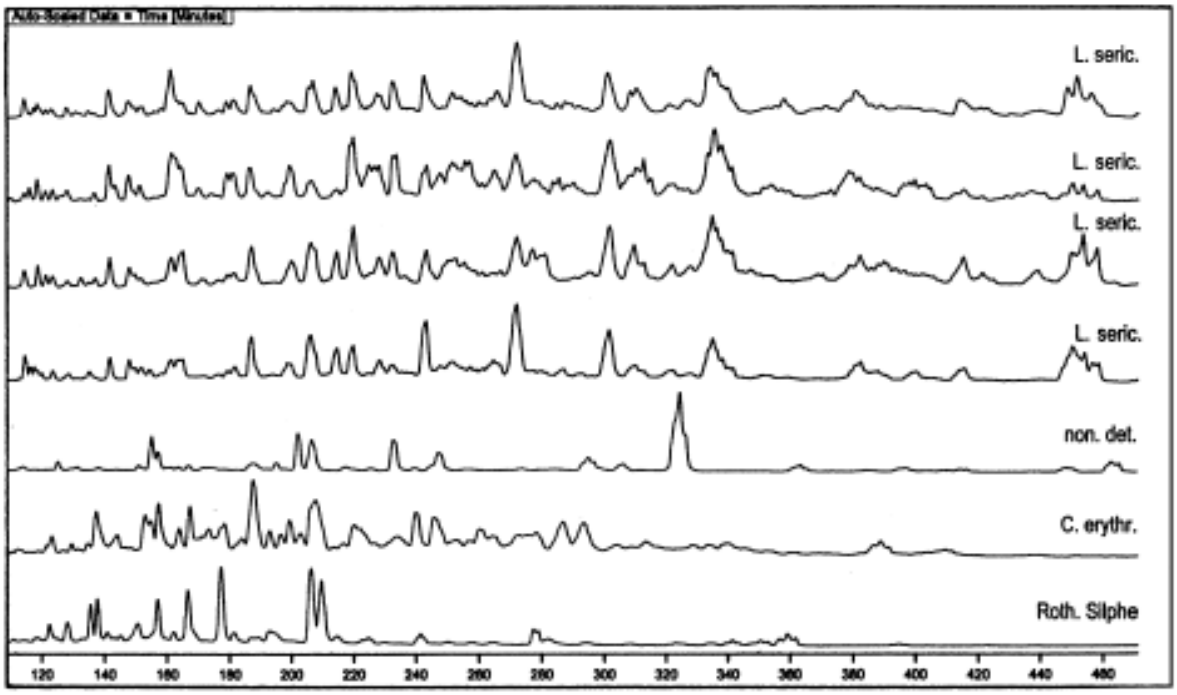


Figura 3 – Comparação do Perfil de DNA pela metodologia RAPD (primer REP1R) de quatro larvas encontradas no corpo e a pupa encontrada próximo ao cadáver, além de mosca varejeira e um besouro (Benecke, 1998).

Outros dois casos solucionados com o estudo da entomologia e genética forense, ocorreram em agosto de 2011, e tiveram a aplicabilidade do uso da Resina Chelex 100 (Bio-Rad) e do kit PrepFiler (Applied Biosystem), e foram relatados pelos pesquisadores Marchetti et al. (2013). Pupários vazios foram coletados dos dois casos investigados pelo Instituto Forense da Universidade Sacro Cuore, em Roma. O ‘’caso um’’ trata-se de um homem que foi encontrado pendurado na zona rural localizado em Roma, sendo a causa da morte por asfixia. Como estava em estado adiantado de putrefação foi requerida a identificação através da análise genética. O ‘’caso dois’’ trata-se de uma mulher encontrada nua num Parque próximo a Roma. Ela havia desaparecido há cerca de 7 dias e também apresentava sinais de avançada decomposição. A morte se deu por intoxicação aguda de drogas. Como havia suspeitas de crime sexual, uma investigação genética por provável abuso sexual foi requerida. Nos dois cadáveres havia larvas de moscas em diferentes estágios de desenvolvimento, que foram coletadas e preservadas em etanol a 80%. Pupas de cada corpo, identificadas como sendo da espécie *Lucilia sericata*, foram colocadas em criação até completar o seu desenvolvimento, o que ocorreu após cinco dias. Os pupários foram armazenados para posterior extração do DNA. Os pupários do caso 1 foram marcados como ‘’A’’ e ‘’B’’; e do caso 2 por ‘’C’’ e ‘’D’’. As amostras foram quantificadas utilizando-se para a PCR o kit Quantifiler Human (Applied Biosystem) e amplificadas com o kit AmpF*l*STR NGM^{TM}

SELect (Applied Biosystem). Os resultados demonstraram que a amostra ‘’A’’ (extraída com Chelex 100) do caso 1, não obteve perfil genético, contudo na amostra B (extraído com PrepFiler) do mesmo caso, a análise STR mostrou um perfil completo correspondente com o da vítima. Com relação ao caso 2, os perfis de STR (extraídos com PrepFiler), obtidos a partir destes dois pupários (C e D) combinam um com o outro e com o perfil da vítima (Figura 4). Desta forma, tal estudo possibilitou também observarmos a importância na escolha da técnica adequada na análise genética comparando métodos utilizados rotineiramente em investigações forenses.

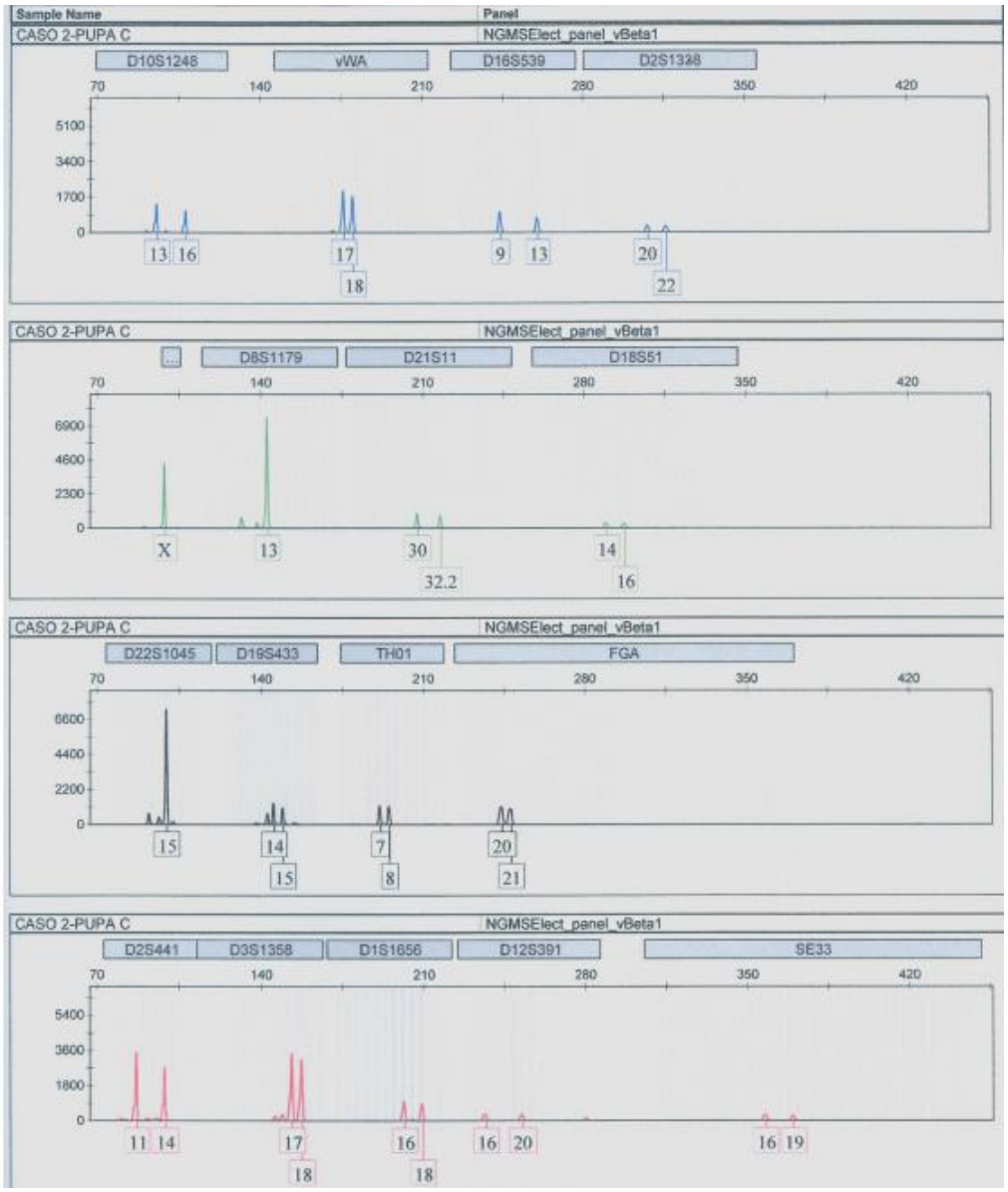


Figura 4 – Perfil STR obtido da vítima através do pupário ‘’ C’’ (Marchetti et al., 2013).

Em crimes sexuais ainda podemos salientar a importância da análise dos perfis genéticos obtidos dos vestígios biológicos apresentarem mistura de DNA de mais de um

indivíduo. A partir da análise com acurácia do perfil genético que apresenta mistura de mais de um contribuinte é possível identificar os indivíduos separadamente, principalmente com o auxílio de análises estatísticas (Isaacson et al., 2015). O trabalho de Durdle et al. (2013) demonstrou inclusive a possibilidade da análise genética de vestígios biológicos de sangue humano, sêmen e saliva a partir de adultos de moscas da espécie *Lucilia cuprina* realizando a extração com DNA IQ^{TM}, a quantificação utilizando o kit Quantifiler Human e a amplificação e a genotipagem usando AMPF*l*STR Profiler Plus. A presença de DNA humano em tal artefato tem significativo valor forense já que a presença de tal mosca pode ser encontrada em corpos em decomposição.

Com o fornecimento dos novos mecanismos na busca e obtenção de vestígios forenses, obteve-se uma forma de remodelamento do sistema de comparação entre vestígios e prováveis suspeitos. Sendo assim, surgiu a necessidade da implementação do Banco de Dados Forense (Koch e Andrade, 2008).

O primeiro Banco de Dados Forense foi originário na Inglaterra, contudo o mais importante e divulgado é oriundo dos EUA, criado pelo FBI, chamado de CODIS (Koch e Andrade, 2008). No Brasil, tal Banco de Dados começou sua vigência em 28 de maio de 2012, com a promulgação da lei nº 12.654, de 28 de maio de 2012, a qual obriga criminosos que cometeram atos delituosos dolosamente, com violência de natureza grave contra pessoa, e os crimes (consumados ou tentados) de fornecerem material biológico para extração de DNA, por técnica adequada e indolor. Desta forma, a biografia criminal genética do indivíduo pode ficar arquivada no Banco de Dados Forense para uma posterior comparação.

Ainda segundo esta lei, esses crimes incluem: 1. Homicídio (quando praticado em atividade típica de grupo de extermínio, ainda que cometido por um só agente); 2. Homicídio qualificado; 3. Latrocínio; 4. Extorsão qualificada pela morte; 5. Extorsão mediante sequestro e na forma qualificada; 6. Estupro; 7. Estupro de vulnerável; 8. Epidemia com resultado morte; 9. Falsificação, corrupção, adulteração ou alteração de produto destinado a fins terapêuticos ou medicinais; 10. Genocídio.

2.3 MOSQUITOS HEMATÓFAGOS COMO VESTÍGIOS BIOLÓGICOS FORENSES

Mosquitos quando capturados em locais de crime ocorridos em ambiente fechado, como os utilizados na prática de crimes como sequestros, roubos e cárcere privado, poderão

ser ferramentas na elucidação desses fatos criminosos (Oliveira-Costa, 2011). Já consta no artigo n° 158 do Código Penal que é indispensável à realização de exame de corpo de delito quando o crime deixar vestígios, não podendo suprí-lo a confissão do acusado, os quais, gradativamente, constituem o embasamento de cometimento de delitos, podendo os insetos representarem tais vestígios (Pereira, 2015). Os vestígios são como testemunhas mudas do crime (Locard, 1939) e desta forma são fundamentais e imprescindíveis quando pesquisados, coletados e analisados de forma correta.

A genotipagem do DNA humano encontrado em mosquitos na cena de um crime foi reportada por Spilateri et al. (2006) num caso ocorrido na Sicília. Um cadáver de uma prostituta transexual que apresentava sinais evidentes de estrangulamento foi encontrado parcialmente escondido por pequenos arbustos de uma planta endêmica de uma praia na Sicília. As investigações policiais na residência do suspeito, cujo carro foi visto na área na noite do assassinato, resultaram no encontro de um mosquito (Díptera) da espécie *Culex pipiens* parcialmente esmagado, no recolhimento de um par de tênis que parecia estar um pouco estragado com o contato com o solo e as vestimentas que apresentavam fragmentos de folhas. Após as análises genéticas no sangue obtido do mosquito parcialmente esmagado, com a extração de DNA realizada com o kit QIAmp (QIAGEN) e a amplificação com AmpF*l*STR Identifiler (Applied Biosystems), sendo o controle adicional da referência do DNA do inseto, amplificado com AmpF*l*STR Identifiler. Foi obtido o perfil genético com 15 *loci* STRs. O perfil de DNA humano obtido foi comparado e verificado que pertencia ao mesmo perfil da vítima, comprovando que a vítima esteve presente na residência do suspeito. Fato comprovado após exames entomológicos constatarem que tal inseto, o qual apresentava seus membros intactos, não teria viajado/voado entre a praia e a residência do suspeito. Em adição a esta evidência foi achado o mesmo fragmento da planta *Calendula maritima* (Asteraceae) no indumento encontrado no apartamento do suspeito e na vítima que foi encontrada na praia. Além disto, a microscopia eletrônica e a microanálise química da areia encontrada no tênis foram consistentes com os da amostra recolhida da praia. Desta forma, o significado científico destas evidências mostrou a possibilidade deste tipo de inseto ser considerado também parte de vestígio biológico encontrado na cena criminal (Primorac e Schanfield, 2014).

De acordo com Borror e Delong (1988), Díptera constitui uma das maiores ordens de insetos e seus representantes abundam em indivíduos e espécies em quase todos os lugares. Os Culicidae são dípteros, de corpo alongado, conhecidos vulgarmente por mosquitos. São capazes de detectar e podem ser atraídos por odores exalados pelos hospedeiros vertebrados,

como dióxido de carbono, amônia, octenol e ácido lático em humanos. Além disso, estímulos visuais (silhueta) e correntes de convecção (temperatura e umidade) são fatores que podem influenciar o sucesso da hematofagia (Forattini, 2002; Neves et al., 2000).

As fêmeas são hematófagas, com o volume de sangue no abdômen após o repasto sanguíneo em média de 3.0 a 3.5 mg, e muitas espécies são antropofílicas com tendência de permanecer no mesmo local se houver hospedeiros disponíveis (Oliveira-Costa, 2011; Forattini, 2002). O hábito hematofágico é devido à necessidade de pelo menos dez aminoácidos essenciais encontrados na dieta para que ocorra a produção normal de ovos: arginina, isoleucina, leucina, lisina, fenilalanina, treonina, triptofano, valina, estridina e metionina. Sendo que inclusive o tipo de sangue ingerido pode influenciar no tamanho da desova (Consoli e Oliveira, 1994).

A sucção do sangue (Figura 5) acontece pela ação dos dois êmbolos de sucção: bomba cibarial e a bomba faringeana. O estômago ou intestino médio é altamente elástico, adaptado à digestão e a absorção. Na parte posterior do estômago existe a válvula pilórica que se liga aos tubos de Malpighi, cuja estrutura e tamanho se mantêm praticamente intactos desde o último estágio larval, envolvidos na excreção e reabsorção de água (Consoli e Oliveira, 1994).

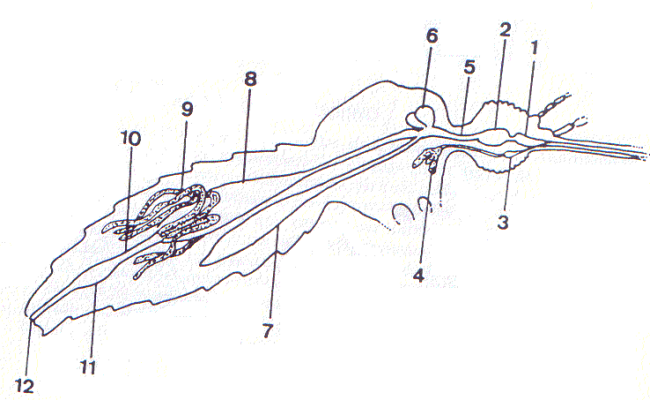


Figura 5 – Morfologia interna do mosquito *Aedes aegypti* – Sistema digestivo de adulto. 1) Bomba cibarial; 2) Bomba Faringeana; 3) Bomba salivar; 4) Glândula Salivar; 5) Esôfago; 6) Divertículos dorsais; 7) Divertículo ventral; 8) Estômago ou Intestino médio; 9) Tubos de Malpighi; 10) Íleo / cólon; 11) Reto; 12) Ânus (Consoli e Oliveira, 1994).

Mistura de perfil de DNA humano pode ser detectado em alguns tipos de casos, como os relatados em crimes sexuais (González-Andrade et al., 2006). A detecção de refeições

múltiplas realizadas por mosquitos hematófagos, e consequentemente mistura de perfis genéticos também é possível. A fêmea é muito ágil ao picar e sempre que perturbada durante a ingestão de sangue, interrompe o processo, voa e logo após, estará novamente apta a ser atraída ao mesmo, ou a outro hospedeiro, ocasião que deverá completar sua refeição sanguínea (Boreham et al., 1976; Scott et al., 1993). A análise e a interpretação em perfis de STR complexos, como os que ocorrem na mistura de DNA, podem ser solucionados com auxílio de cálculos estatísticos como o LR, auxiliando a separar tais perfis na interpretação das amostras questionadas e de referências (Haned e Gill, 2011).

Alguns trabalhos relacionados com o sucesso no isolamento, extração e amplificação do DNA em mosquitos podem ser elencados, mas ainda escassos na área forense. O estudo de Kreike e Kampfer (1999) relatou a importância do uso de mosquitos na resolução de casos criminais com o sucesso no isolamento qualitativo e quantitativo de DNA isolado dos mosquitos. Curic et al. (2014) também relataram a importância dos mosquitos no contexto forense avaliando o intervalo temporal pós-refeição sanguínea e a qualidade do DNA comparando-se kits da análise na amplificação de DNA (Identifiler, Minifiler e Quantifiler), tendo-se sucesso com perfis completos em até 48 horas após a hematofagia. Desta forma, o uso de culicídeos na área forense ainda é uma ferramenta inovadora, mas com grande potencial na resolução de determinados casos criminais.

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4. PRODUÇÃO CIENTÍFICA

ARTIGO 1

Human DNA present in digestive tract of hematophagous mosquitoes for forensic purposes: extraction methods comparison.

Status: a submeter

Human DNA present in digestive tract of hematophagous mosquitoes for forensic purposes: extraction methods comparison.

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ABSTRACT. A suspect identification from biological evidence of hematophagous mosquitoes have several contributors in solving crimes. The Human DNA profiles obtained from hematophagous mosquitoes can assist in solving crimes committed in closed environments. This study evaluated three different protocols for DNA extraction to compare the quality of the extracted genetic material from the digestive tract of female *Aedes aegypti* for forensic evidence use. The mosquito sample was assessed with the time interval between 24 hours until 120 hours after a blood meal in the arms until repletion. Was examined the allele frequencies of 15 STR *loci* and amelogenin. More concise genetic profiles were recorded using for DNA extraction Kit IQ^{TM} although intact DNA profiles have been obtained with the DNA IQ^{TM} and Chelex® 100 in blood up to 72 h after blood meal. Genetic profiles were not identified when we perform the extraction NaOH. These results suggest that the DNA kit IQ^{TM} is the most appropriate for human DNA extraction from mosquitoes allowing elucidating crimes indoors, also confirming the use of these insects as viable forensic evidence. The results suggest that the collection of mosquitoes in the suspected location can be held up to 72 hours to obtain mosquitoes with blood not yet fully degraded.

Key words: *Aedes aegypti*; DNA Kit IQ^{TM}; Chelex® 100 Resin; Forensic Entomogenetical.

1. INTRODUCTION

The physical evidence is the first step to be investigated at the crime scene as a possible form of evidence that links the suspect to the crime scene, being a crucial tool for the confession of the author (Fisher, 2003; Petherick and Rowan, 2015). The difficulty in obtaining such evidence in crimes committed indoor, such as kidnapping and or false private sequestration, led to the search for alternatives to solve in this problem. Among these alternatives, obtaining intact human genetic profiles found inside the hematophagous insects such as mosquitoes have been quite promising, suggesting the possibility of use of these insects as forensic evidence (Keh, 1985; Catts and Goff, 1992; Nuorteva, 1997; Butler, 2005; Spitareli et al., 2006).

Among the hematophagous insects, the family Culicidae stands out as a possible tool to aid forensics due to of their very relevant characteristics: as anthropophily, high reproductive rate and abundance of breeding sites in different types of environments (Maciel-de-Freitas, 2007; David et al., 2009;). Only females feed on humans since they require a blood meal for production of eggs (Rozendaal, 1997; Mehlhorn, 2008) and two to five hours after a full blood meal, if there is an attractive donor, a female will take another blood meal (Jones, 1973).

The forensic DNA typing is important by providing reliable evidence both for convicting the offenders and for exonerating the innocent suspects (El-Alfy and El-Hafez, 2012). Small quantities of biological material have been analyzed and successfully amplified in the study of microsatellite and short tandem repeat (STR), applying the technique of Polymerase Chain Reaction (PCR), common in the area related to forensic human identification (Hochmeister, 1997; Jobling and Gill, 2004).

In the present study, analysed the most efficient method for extracting DNA (DNA Kit IQ^{TM} ,Chelex® 100 Resin and extraction with NaOH) from human blood sucking mosquitoes and determined for how long after taking a blood meal the human donor could be identified. In addition the quantity of human DNA present in mosquitoes was investigated.

2. MATERIALS AND METHODS

2.2 Obtaining the mosquitoes and protocol of the experiment in the laboratory

The mosquitoes adults of *Aedes aegypti* were hatched from eggs collected in the archipelago of Fernando de Noronha (Pernambuco/Brazil) 3º 50 'and 3° 52' south latitude and 32º 24' and 32° 28' west longitude from Greenwich. The mosquitoes were reared in the laboratory (26°C temperature and 50% humidity and photoperiod of 12 hours). The identification of mosquitoes was performed in stereoscopic microscope (Olympus), using the taxonomic keys of Rueda (2004). Adult males and females were kept in a cage, which was offered 10% sucrose solution as food for 24 hours before the blood supply of females.

The biting was performed for three minutes for two volunteers separately who were previously informed about the procedures to be performed and on the research objectives, with their consent by signing the agreement form. Before the procedure, the power arm was cleaned using water and mild soap. For the DNA extraction, after feeding, groups were formed of 10 females for each conditions: extractions DNA IQ™ , Chelex® 100 Resin and sodium hydroxide (NaOH) and these extractions was evaluated in the following time intervals (24h, 48h, 72h, 96h and 120h after blood meal) totaling 100 females for each type of extraction analysis.

Mosquito's engorged female was anesthetized with chloroform and the abdomens was squashed on filter paper and left dry. The genetic was analysis in the Laboratory of Expertise and Research in Forensic Genetics of Pernambuco / Brazil. As a reference, was collected the saliva of volunteers using buccal swabs.

2.3 Extraction of human DNA using DNA IQ™ System Kit (Promega Corporation), Chelex® 100 Resin (Bio-Rad Laboratories) and NaOH.

The extracted DNA samples using the DNA IQ™ System kit (Promega, Madison, WI, USA) as recommended by the manufacturer, Chelex® 100 Resin (Bio-Rad Laboratories) was based on the method described by Lincoln and Thomson (1998) and the extraction using NaOH (sodium hydroxide) was based on the method described by Rudbeck and Dissing (1998).

2.4 Genetic Analysis

Each type of extraction (DNA IQ™ System Kit , Chelex® 100 Resin and NaOH) resulted in a final volume of 50µL. The quantity of human DNA was determined using Quantifiler® Duo DNA Quantification Kit (Applied Biosystems, New Jersey, USA) on ABI 7500 Platform. PCR of all samples to a final volume of 1 ng of DNA, was performed using ‘*’Identifiler® Plus’’* Kit (Life Technology) following the manufacturer's instructions, analyzing 16 *loci* STR (D8S1179, D21S11, D7S820, CSF1PO, D3S1358, TH01, D13S317, D16S539, D2S1338, D19S433, VWA, TPOX, D18S51, D5S818, FGA and amelogenin). The PCR perform the samples were first subjected for a period of 11 minutes at 95°C, and then 28 cycles of 94°C by changing for 20 second at 59°C for 3 minutes. After this, the end extension was for a period of 10 minutes at 60°C and hold in the end of 4°C up to 24 hours. Electrophoresis was performed using the ABI PRISM 3500HID Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

The protocol followed in this study was the same used by the Laboratory for Expertise and Research in Forensic Genetics of the State of Pernambuco/ Brazil in the routine genetic analysis relate to crime scenes. Blank controls were used to detect possible contamination.

2.5 Ethics released

The project was approved by the Ethics Committee of the Federal University of Pernambuco / Brazil (protocol number 462/11).

3. RESULTS AND DISCUSSION

3.1 DNA profiling and type of extraction of human DNA

Both DNA extractions using the DNA IQ™ kit as well as the Chelex ® 100 resin allowed the identification of genetic profiles until 72h after blood feeding practice by the mosquitoes. However genetic profiles showed a little better when the DNA was extracted with DNA IQ™ kit. After 24h of biting all profiles resulting from these two types of DNA extractions showed 100% amplification of the samples indicating that the sooner the better, blood meal quality of the amplified material (Figures 1 and 2). DNA extraction using sodium hydroxide (NaOH) had no success for obtaining genetic profiles in any of the time intervals under consideration, with the DNA quantification of 0.0 ng / for this type of extraction.

The DNA IQ™, using magnetic resin is the most commonly applied method for DNA extraction in forensic laboratories, and it is suitable for very small amounts of genetic material, being a very sensitive method. However, the disadvantages of this method include the fact that it is more expensive kit, and requires care in its methodology in the final part where you can't let the resin dry out too much, it can occur in the DNA bind irreversibly to it, and it can't be eluted later. Observed the tested Chelex® 100 resin since it is cheaper than DNA IQ™ and easier to be handled (Lincoln and Thomson, 1998). Despite the two methods gave similar results, allowing the identification of genetic profiles within 72 hours after the blood meal, the number of peaks in the electropherograms and the best quality were detected using the DNA™ IQ kit.

The short alkaline DNA extraction using NaOH, Tris-HCl and IQ PicoPure DNA extraction were methodologies described by Vandewoestyne et al. (2012) to test and optimize DNA extraction protocols for forensic STR typing after laser capture microdissection. It is no note that Vandewoestyne et al. (2012) just analyzed 4 loci in their study, while we considered 16 STR in our experimental procedure. Furthermore, the amount of tris-HCl used to neutralize more than twice then the one used in our experiments. We acknowledge the quantitative (in terms of Tris-HCl) and qualitative (number of loci analyzed) differences within our study and the one of Vandewoestyne et al. (2012), however we point out that, at least in our hands method using NaOH was not successful, providing poor quality STR genotyping.

Curic et al. (2014) observed the identify individuals through DNA from mosquitoes for up to 88 hours after the blood meal, testing Identifiler, Minifiler and Quantifiler PCR kits. Chow-Shaffer et al. (2000) demonstrated that the degradation caused by human DNA digestion by metabolism occurred in blood of hematophagous mosquitoes affect the success of amplification already 24 hours after the blood meal. In our work we can amplifications less time than that obtained by Curic et al. (2014), however in a time interval three times greater than that demonstrated in the studies by Chow Shaffer et al. (2000).

3.2 Quantity of human DNA

Both DNA kits resulted in sufficient amplification for determination of forensic DNA profile, the DNA IQ™ kit was more effective for quantify genetic material giving 0.77 ng/µL (±0.40) in the group analyzed 24 h after the blood meal, whereas in similar conditions to Chelex® 100 resin had 0.01 ng/µL (±0.0).

Hematophagous mosquitoes can be used with forensic evidence, as reported by Spitareli et al. (2006), but for being a living biological trace, blood in the digestive tract of mosquitoes is in constant consumption for egg production (Rozendaal, 1997), thereby being degraded every passing hour the day of hematophagy, making human DNA increasingly degraded. Gilbert (2010) discussed the possibility of success in the use of quantitatively minor criminal evidence. The authors reported that even if DNA can't be quantified in the routine forensic laboratory analysis, it is possible to obtain DNA profiling suitable for forensic purposes by increasing the number of PCR cycles during amplification.

In our study, after 48 hours of blood feeding the DNA concentrations were close to zero. However, in samples where a more sensitive amplification kits was used, it was possible to visualize satisfactory genetic profiles after 72 hours. These data demonstrated that even the engorged mosquitoes partially, with long time interval after the blood meal, analysis and obtain DNA profiles is possible. It is still worth noting that to achieve success in amplification factors as maintaining engorged blood, the concentration of the extracted DNA and the time of ingestion of blood are crucial to the success of the analysis (Mukabana et al., 2002a).

3.3 Relationship between time after feeding of mosquitoes and DNA amplification

In the this study, full 16 *loci* (Figures 1 and 2) were visualized in samples obtained from 24h to 72h (Table 1 and 2) after performing the hematophagic process, using the Chelex® 100 Resin and the DNA IQ™ kit. Typing success decreased with increasing digestion period for mosquitoes full profile obtained (all 16 *loci* STR) for DNA IQ™ System kit (Table 2) from 100% mosquitoes after 24h, in 40% after 48h, in 10% after 72h and in 0% after 96h. About Chelex® 100 Resin (Table 2), full profile obtained (all 16 *loci* STR) from 100% mosquitoes after 24h, in 20% after 48h, in 10% after 72h and in 0% after 96h. Thus, we succeeded in obtaining full 16 STR loci (including amelogenin) profile up to 72h, for both methodologies (DNA IQ™ kit or Chelex® 100 Resin), and all DNA profiles matched the donors profiles.

In our paper we observed an inverse relationship between the time of blood feeding and successful PCR amplification, from the time of the interval to time zero intake of blood is less likely to obtain amplification of the 16 STR *loci* that make up the DNA profile quality. Mukabana et al. (2002b) tested the amplification after 0, 8, 16, 24 and 32h after the practice of biting mosquito *Anopheles gambiae* (Diptera: Culicidae) and after 8 hours, the successfully profiled decreased slowly with time after ingestion blood, dropping to below until 50% after 15 hours, and our study although not examining such short intervals of time, demonstrated the validity of this technique until 72 hours after the blood meal.

The Federal Bureau of Investigation (FBI) established 13 loci as the minimum amount ideal for forensic applications (Budowle et al., 1998; Butler, 2005). However, the greater the number of loci studied, the most reliable and conclusive becomes the DNA profile questioned. In this sense, our study provides 16 loci, making DNA profile more reliable. Previous study genotyping human DNA found in mosquitoes from crime scene used only 15 STR loci, using the QIAmp Blood and Tissue kit extraction system (QIAGEN), and a multiplex PCR was performed according to the AmpF*l*STR Identifiler (Applied Biosystem) manual supplied by the manufacturer (Spitaleri et al., 2006). However, our study compared two types of DNA extraction, and perform the measurement with the Quantifiler® Duo DNA Quantification Kit (Applied Biosystems, New Jersey, USA) and amplified using 'Identifiler® Plus" kit (Life Technologies).

The authors Spitaleri et al. (2006) reported a crime against a transsexual prostitute who was found dead and partially hidden on a beach of Sicily (Italy). In the house of the suspect a spot of blood from a partially squashed mosquito on the wall that was collected as a trace was found. In the laboratory, it was found that the blood found in mosquitoes, identified as the species *Culex pipiens*, belonged to the victim becoming the undisputed presence of evidence linking the suspect to the crime.

Thus, this study sought to verify the best methodology and temporal interval until after the completion of the blood meal, the hematophagic mosquito used as forensic biological trace would be able to be collected and to suit as auxiliary as criminal evidence in an attempt to link the suspect the criminal scene.

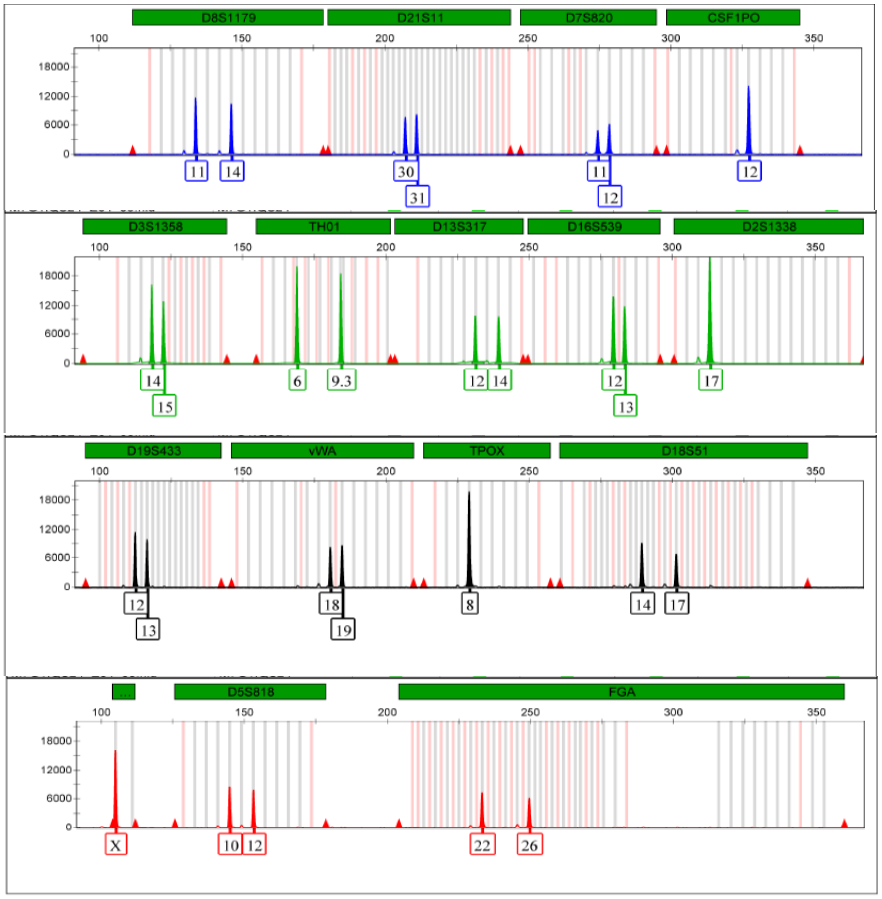

Figure 1 - Profile of human DNA amplified with 15 STR *loci* and amelogenin extracted from hematophagous mosquitoes *Aedes aegypti* using DNA IQ ™ kit 24 hours after blood meal.

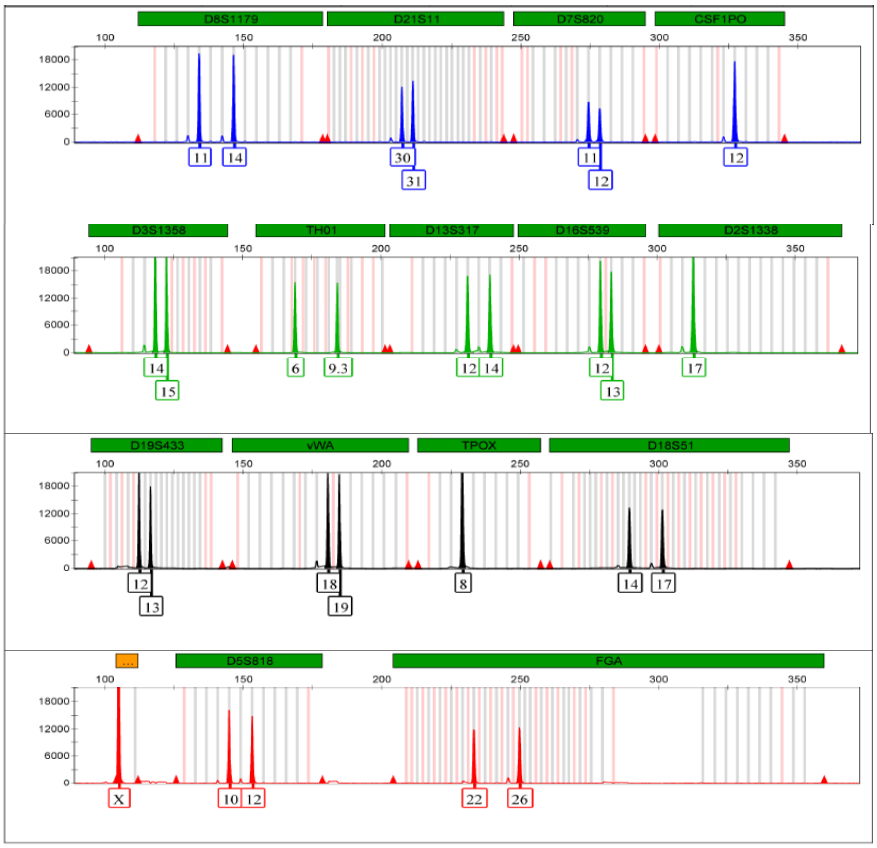

Figure 2 - Profile of human DNA amplified with 15 STR *loci* and amelogenin extracted hematophagous mosquitoes *Aedes aegypti* using Chelex® 100 Resin after 24h of blood meal.

Table 1 - Quality of human DNA classified according to the type of extraction (total n = 150), amplification, and temporal variation profile after the biting.

Type of DNA extraction	**Human DNA**														
	Amplified					**Partially amplified**					**Unamplified**				
	Timeslot (in hours)														
	24	**48**	**72**	**96**	**120**	**24**	**48**	**72**	**96**	**120**	**24**	**48**	**72**	**96**	**120**
DNA IQ Chelex	■	■	■				■	■	■	■		■	■	■	■

Table 2 - Number of amplified alleles in relation to the total of 16 STR *loci* by type of DNA extraction and temporal variation after blood feeding (total n = 10 per type of extraction and time interval).

Type of DNA extraction	Timeslot (in hours)				
	24	48	72	96	120
DNA IQ	16/16 (10)	16/16 (04) 11/16 (02) 00/16 (04)	16/16 (01) 02/16 (02) 05/16 (01) 00/16 (06)	02/16 (01) 03/16 (01) 00/16 (08)	00/16 (10)
Chelex	16/16 (10)	16/16 (02) 06/16 (01) 00/16 (07)	16/16 (01) 02/16 (01) 01/16 (01) 00/16 (07)	02/16 (01) 00/16 (09)	00/16 (10)

4. CONCLUSION

This research demonstrates that the DNA extracted from the digestive tract of blood-sucking mosquitoes can be used as evidential trace and it is a potential tool for forensic analysis aimed at connecting or deleting the suspect of the crime scene. Although there are some studies that prove the effectiveness and efficiency of DNA found in mosquitoes, it is essential to assess possible factors that can affect the quality of this material as forensic evidence since it is a living organism with active metabolism, so that it is not a challenge in some way by the defense of suspects.

The results in this study could be useful for the introduction of DNA extracted from mosquitos in the routine practice of forensic laboratories, as a supplementary trace present at crime scene. This approach could be extremely useful for individual identification when we have to found the author of a crime within a certain number of suspected persons.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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Trace samples of human blood in mosquitoes as a forensic investigation tool

Forensic entomology and genetics

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ABSTRACT

Investigations of any type of crime invariably starts at the crime scene by collecting evidence. Thus, the purpose of this research was to collect and analyze an entomological trace from an environment that is similar to those of indoor crime scenes. Hematophagous mosquitoes were collected from two residential units; saliva of volunteers that were residents in the units was also collected for genetic analysis as reference samples. We examined the allele frequencies of 15 short tandem repeat loci (D8S1179, D21S11, D7S820, CSF1PO, D3S1358, TH01, D13S317, D16S539, D2S1338, D19S433, vWA, TPOX, D18S51, D5S818 and FGA) and amelogenin. A total of 26 female hematophagous mosquitoes were identified as *Aedes aegypti*, *Aedes albopictus*, and *Culex quinquefasciatus*; we were able to obtain 11 forensically valid genetic profiles, with a minimum of 0.028203 ng/μL of human DNA. Thus, the results of this study showed that it was possible to correlate human genetic information from mosquitoes with the volunteer reference samples, which validates the use of this information as forensic evidence. Furthermore, we observed mixed genetic profiles from one mosquito. Therefore, it is clearly important to collect these insects indoors where crimes were committed, because it may be possible to find intact genetic profiles of suspects in the blood found in the digestive tract of hematophagous mosquitoes for later comparison to identify an offender and/or exclude suspects.

Key Words: Mosquitoes blood meal; Forensic DNA; Forensic entomology;

INTRODUCTION

Molecular techniques that profile individuals from a small amount of DNA have facilitated the use of engorged female mosquitoes, which may be crucial for solving some types of crimes. Mosquitoes (Culicidae) are a widely distributed source of human genetic material that can easily be found at crime scenes (Spitarelli, 2006). Genetic profiles obtained from human blood found in the digestive tracts of mosquitoes, for example, can assist in the investigation of crimes committed in a closed environment (such as a room or vehicle; Spitarelli, 2006; Curic, 2014). Comparison of the samples to the suspect's a DNA database of criminals may also indicate whether a crime is a repeated offense (Wallace et al., 2014).

Intrinsic features of the biology and behavior of mosquitoes are potential advantages to the use of these insects as forensic evidence. Different species are active at different times (morning, twilight or night) as well as local feeding and resting (indoors, outdoors, and in forests) (Rozendaal, 1997; Mehlhorn, 2008; Takken and Verhulst, 2012). Additionally, their propensity of eating quickly and remaining at the site (Chadee and Beier, 1996, 1997; Edman et al., 1998; Forattini, 2002) allows us to determine DNA profiles from the blood ingested by female mosquitoes, which facilitates identification of people present at the scene or may indicate whether a victim was at the crime scene.

Furthermore, females only stop feeding when the digestive tract is filled with blood, even if they have to feed on more than one host (Canyon et al., 1998; Forattini, 2002). This characteristic is an additional advantage because it can result in more than one human DNA profile (Clayton et al., 1998; Gill et al., 1998; González-Andrade et al., 2006) by female mosquitoes that may have fed on the victim or criminal. Therefore, the links between the suspect, victim, crime, and/or crime scene can be achieved by analyzing mosquitoes (Curic et al., 2014). In this study, we compared the profile of human DNA saliva with blood from the digestive tract of mosquitoes found in the same environment. We also investigated whether

multiple feedings of a single mosquito, volunteer sex, and mosquito species would affect the genetic profiles of analyzed blood.

MATERIAL AND METHODS

DNA samples obtained from hematophagous mosquitoes

Hematophagous females were collected from student housing in Recife (Pernambuco, Brazil) called residential units (RUs) "A" and "B." RU "A" is composed of the first floor of a building, and RU "B" is a one-story house. Both RUs have several rooms and this structure is analogous to common home. Mosquitoes were mainly collected from common areas such as the TV room, study room, bathrooms, and kitchens. The RUs were chosen because we considered them to be similar environments to those in which indoor crimes are committed, and volunteers/residents represented possible suspects.

All mosquitoes found in RUs "A" and "B" were manually captured and placed in plastic pots and then sacrificed with chloroform to aid identification with a stereoscopic microscope (Olympus) using the Consoli and Oliveira (1994) and Forattini (2002) taxonomic keys. After these procedures, the mosquitoes were crushed in filter paper and left to dry for future genetic analysis, which is the same methodology that was followed by Spitaleri et al. (2006).

Research on the biological reference samples was conducted only after authorization of the volunteers; the research was explained and the volunteer signed the informed consent form (TCLE) that gave permission to collect a buccal mucosa sample by wabbing the oral mucosa for further DNA examination (Sweet et al., 1997; Anzai-Kanto et al., 2005). Genetic analyses were performed at the Laboratório de Perícia e Pesquisa em Genética Forense (Recife, Pernambuco, Brazil).

Reference samples

Genetic analysis was performed by comparative study of the alleles found in the electropherograms of volunteer buccal mucosa samples and human DNA found in the blood from the digestive tract of the hematophagous mosquitoes.

Human DNA extraction

The DNA extraction samples were extracted using the DNA IQ™ System kit (Promega, Madison, WI, USA) as recommended by the manufacturer.

Genetic analysis

DNA quantification was performed after DNA extraction with a Quantifiler® Duo DNA Quantification kit (Applied Biosystems, Foster City, USA) using the Real Time PCR ABI 7500 platform. Later, multiplex amplification was performed by PCR analysis using the AMPF*l*STR Identifiler® Plus® PCR kit (Life Technologies; Carlsbad, CA, USA) following the manufacturer's protocols. Fifteen STR loci were analyzed (D8S1179, D21S11, D7S820, CSF1PO, D3S1358, TH01, D13S317, D16S539, D2S1338, D19S433, vWA, TPOX, D18S51, D5S818, and FGA) in addition to a gender identification marker, amelogenin. PCRs were run for 28 amplification cycles for a total of 2 h, 28 min, and 5 s. Blank controls were used to detect possible contamination.

The amplification products were subjected to capillary electrophoresis with the ABI PRISM 3500HID automated sequencer (Applied Biosystems, Foster City, CA, USA). Data were

analyzed using the GeneMapper® ID –X Software. Non-nucleotide linkers, which are used in primer synthesis for the loci CSF1PO, D13S317, D16S539, D2S1338, and TPOX, allows for simultaneous amplification and efficient separation of the 15 STR loci and amelogenin during automated DNA fragment analysis in accordance with the manufacturer's protocols (Butler, 2005, Grossman et al., 1994, and Baron et al., 1996).

The entire protocol followed in this study is the same used by the Laboratório de Perícia e Pesquisa em Genética Forense (Recife, Pernambuco, Brazil) in the genetic routine analysis is related to traces of research reports of crime scenes.

Ethics approval

This study was approved by the Ethics Committee of the Universidade Federal de Pernambuco (Recife/Brazil) under protocol number 462/11.

Statistical analyses

The likelihood ratio between the questioned sample and the reference was calculated using PATCAN v.1.2 software (Riancho and Zarrabeitia, 2003) using Bayesian analysis to test the hypothesis that blood found in an entomological trace belonged to a reference sample.

The likelihood ratio (LR) and posterior probability are measures of reliability; consequently, trace criminal evidence can be correlated with references, and the probability can be calculated that a genetic profile would be found in other individual in the population.

RESULTS AND DISCUSSION

In the present study, a total of 18 biological samples were collected from the oral mucosa of volunteers/residents from RU "A" (32.5ºC, 66% relative humidity) and nine samples were collected from the oral mucosa of volunteers/residents from RU "B" (34ºC, 56% relative humidity) for a total of 27 samples references. Twenty-six total female hematophagous mosquitoes (Diptera, Culicidae) were captured; 15 were from RU "A" and 11 were from RU "B." Identification revealed that we collected 13 *Aedes aegypti* individuals, 12 *Culex quiquefasciatus* individuals, and one *Aedes albopictus* individual; the mosquitoes collected from each RU included: seven *Aedes aegypti* individuals, seven *Culex quinquefasciatus* individuals, and one *Aedes albopictus* individual were collected from UR A, and six *Aedes aegypti* individuals and five *Culex quinquefasciatus* individuals were collected from UR B. Although it is possible that the DNA will degrade, the consumption of blood by mosquitoes and use of PCR and STR techniques facilitated amplification, enabling interpretation and subsequent validation and confirming the previous results of Alaeddini et al. (2010).

Of the 26 hematophagous mosquitoes, there were 11 samples (Table 1) that would be considered viable trace samples, with amplification of the DNA visualized by the peaks in the electropherograms for profiles of 16 loci (Figure 1). Culicidae mosquitoes, including *Aedes aegypti*, *Culex quinquefaciatus*, and/or *Aedes albopictus*, were used to obtain viable profiles that could be used as a form of evidence for police investigation and judicial proceedings. Even with potentially degraded DNA, it was possible to obtain a number of suitable profiles with electropherogram analysis.

Electropherograms for amelogenin revealed five male and five female genetic profiles. In our study, mosquitoes equally preferred biting males and females; this contradicts the reports of Brouwer (1960), Geier et al. (1996), and Brady (1997), who reported that *Anopheles*

stephensi, *Aedes aegypti*, and *Anopheles gambiae* prefer males because of hormonal differences.

This study revealed that even a mixture of human DNA found in a single mosquito could be quantified. Therefore, when a crime occurs, a mosquito may bite the victim and suspect, because sometimes the victim is immobilized and thus would be easier to bite, and the human DNA profile could be accepted as expert proof.

Human DNA profiles from a single *Aedes aegypti* mosquito revealed a genetic profile mixture of two women (Figure 2). Additionally, individuals of two different mosquito species (*Culex quinquefasciatus* and *Aedes aegypti*) had blood supplied from a single individual (Figure 3).

Using 15 STR loci in addition amelogenin, it was possible to identify the DNA profile of two women in the blood of a female mosquito. The Federal Bureau of Investigation (FBI) recommends a minimum of 13 STR loci for forensic applications (Budowle et al., 1998; Butler, 2005). The authors Haned et al. (2015) reported success in separation and identification of DNA mixture profiles. This type of trace sample has an even greater potential contribution to forensic investigation, because a mosquito could feed on both the victim and offender and both DNA profiles can be analyzed.

DNA extracted from the blood of 11 mosquitoes (Table 1) was amplified and quantified, and the amount of human DNA varied between 0.028203 ng/µL and 0.329996 ng/µL. Mosquitoes 4 and 8 fed on the same woman, and mosquitoes 9 and 10 fed on the same man; however, the quantity of DNA varied among all four mosquitoes (mosquito 4, 0.043155 ng/µL; mosquito 8, 0.304895 ng/µL; mosquito 9, 0.329996 ng/µL; and mosquito 10, 0.133783 ng/µL). Mosquito 3, which had a mixture of genetic profiles, had 0.058803 ng/µL human blood and fed on two separate women.

Curic et al. (2014), described the possibility to quantify human DNA in all mosquitoes analyzed, which is an important factor when analyzing mosquitoes as forensic evidence. Genetic analysis revealed that it is possible to identify individuals at a crime scene based on the amount of DNA that can be found in mosquitoes collected from a crime scene, which can help save time and resources.

The lowest value observed was the LR of mosquito 6, 8.43×10^{18}; this indicates that the likelihood that the genetic profiles of the reference sample obtained from volunteers and the trace sample of human blood found in the digestive tract of hematophagous mosquitoes at the scene of a crime is at least 8.43×10^{18} greater than the chance the DNA came from another person in the population. The posterior probability values were very close to 100% for all samples.

Genetic analysis and interpretation of this kind of criminal evidence is assisted by statistical analysis using the LR (Weir et al. 1997; Hu et al. 2003; González-Andrade et al., 2006; Primorac and Schanfield, 2014), which is the probability of association between possible trace samples (human blood found in the digestive tract of mosquitoes) and references samples. González-Andrade et al. (2006), who reviewed cases of sex offenders and the resulting mixture genetic profiles, the calculation result of LR was one million, whereas the analysis of human blood hematophagous mosquitoes found in the lowest rate was LR approximately eight quintillion on a population; discriminating enough the association between the possible trace and reference samples. Posterior probability, which indicates the percentage of the genetic profile that would be different in any other individual in the population, the values were all close to 100% likelihood of the possible trace and reference samples, taking into

consideration the hypothesis of this paper it is a similar environment to the site of closed crime.

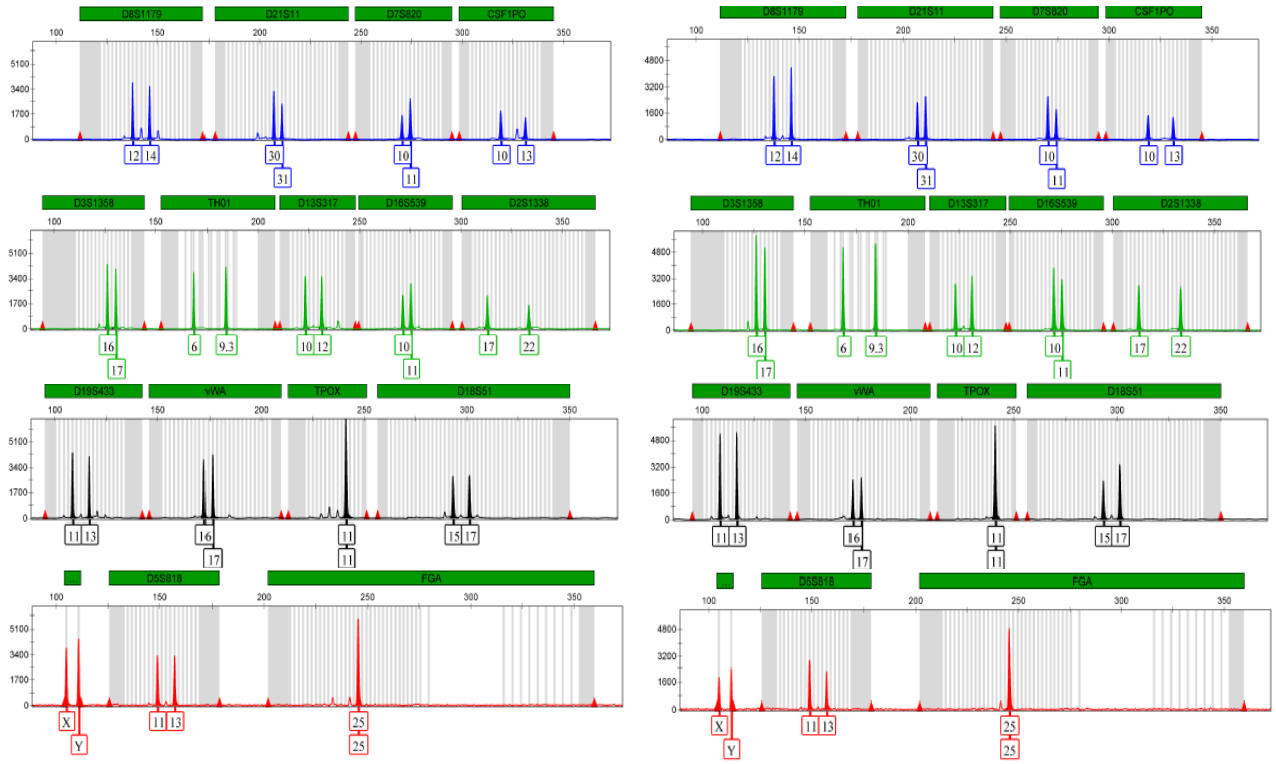

Figure 1. DNA profile of a man. Left, human blood extracted from a *Culex quinquefasciatus* mosquito; right, reference sample from a volunteer's saliva. Fifteen STR loci and amelogenin were amplified.

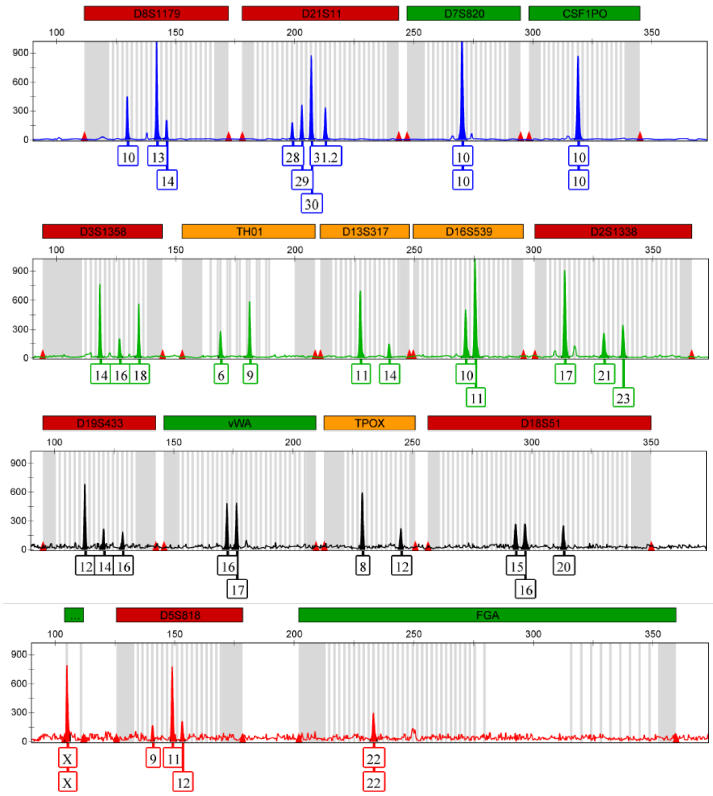

Figure 2. Mixed DNA profiles of two women prepared using human blood extracted from an *Aedes aegypti* mosquito. Fifteen STR loci and amelogenin were amplified.

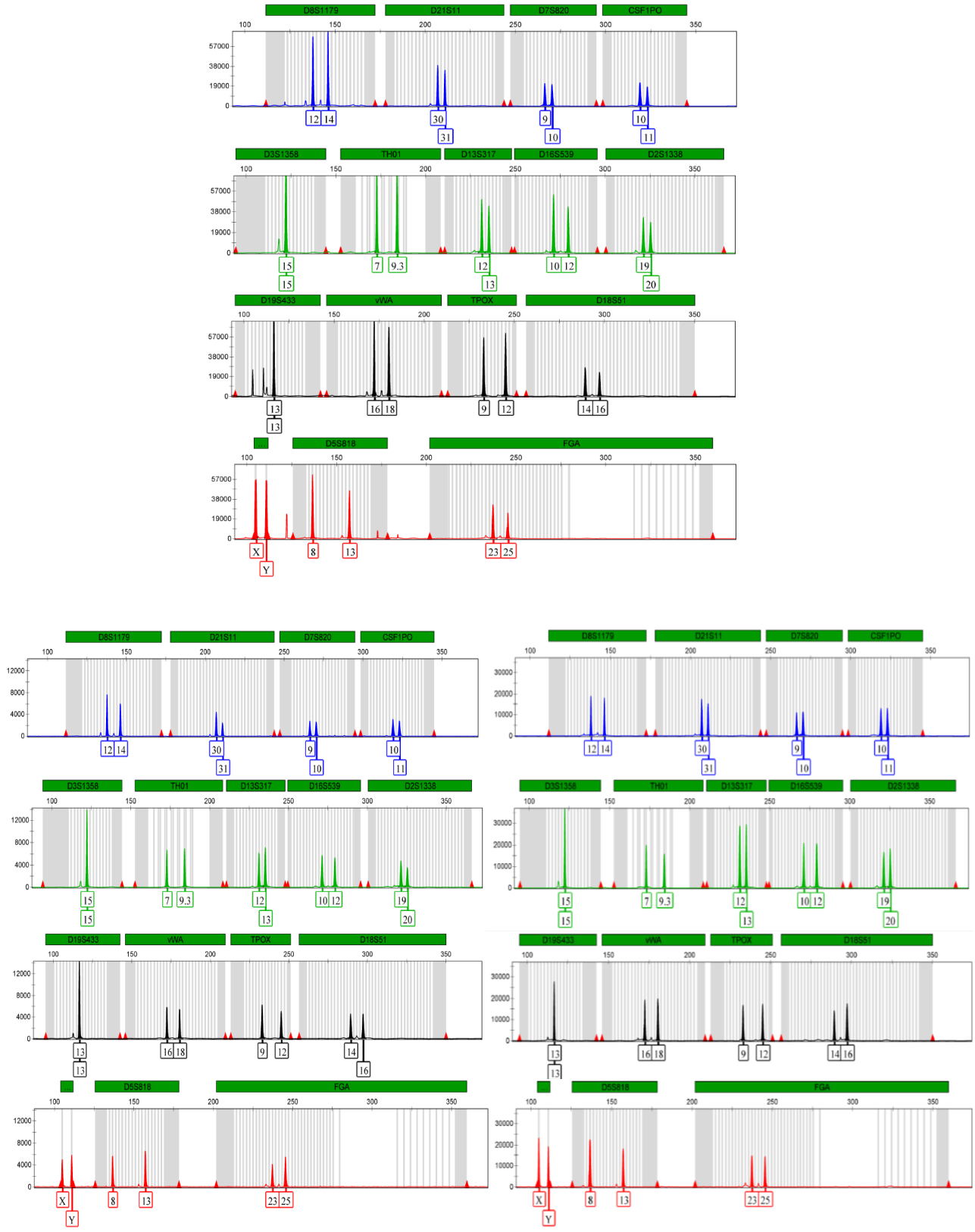

Figure 3. DNA profile of a man whose DNA was detected in blood from two mosquitoes (center, profile of the reference sample; left, profile of human blood extracted from a *Culex quinquefasciatus* mosquito; and right, profile of human blood extracted from an *Aedes aegypti* mosquito). Fifteen STR loci and amelogenin were amplified.

Table 1. Human DNA amplified from blood in the digestive tract of hematophagous mosquitoes.

Sample of human DNA amplified	Sex based on Human DNA profile	Human DNA quantity (ng/µL)	Prior probability (%)	Likelihood ratio (LR)	Posterior probability (%)
mosquito 1	man	0.041996	50	3.67×10^{22}	99.99999999999999999
mosquito 2	man	0.028203	50	1.34×10^{21}	99.9999999999999999
mosquito 3	mixture of two women**	0.058803	50	***	***
mosquito 4*	woman*	0.043155	50	1.73×10^{25}	99.999999999999999999999
mosquito 5	woman	0.109843	50	2.73×10^{19}	99.99999999999999
mosquito 6	woman	0.080513	50	8.43×10^{18}	99.9999999999999
mosquito 7	man	0.042154	50	5.84×10^{26}	99.9999999999999999999999
mosquito 8*	woman*	0.304895	50	1.73×10^{25}	99.999999999999999999999
mosquito 9*	man*	0.329996	50	1.76×10^{20}	99.99999999999999999
mosquito 10*	man*	0.133783	50	1.76×10^{20}	99.99999999999999999
mosquito 11	man	0.138298	50	4.92×10^{19}	99.99999999999999

Bayesian analysis was used for comparisons.

* Distinct mosquitoes fed on the same individual; therefore, LR and posterior probability values are equal in the following analyzed mosquitoes: 4 and 8; 9 and 10.*

** A single mosquito fed on two female individuals.

*** No comparative statistical analysis because of a lack of reference biological material.

CONCLUSIONS

This research confirms that it is possible to analyze human blood found in hematophagous mosquitoes as biological trace evidence and use it as expert evidence. This study revealed that correlation between the reference samples and blood from mosquitoes, regardless of mosquito species analyzed and sex of the individual, can be easily and reliably compared to the obtained laboratory profiles. This facilitates the use mosquitoes as an additional trace for

possible biological evidence in closed environments in which crimes were committed. The results of this study also indicate the possibility of identifying DNA profiles from different individuals in the blood mixture of a single female mosquito.

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The authors declare no conflicts of interest.

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ARTIGO 3

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Detecting multiple DNA human profile from a mosquito bloodmeal

Status: Submetido

Detecting multiple DNA human profile from a mosquito bloodmeal

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ABSTRACT

Traces criminal commonly found at crime scenes may present mixture of two or more individuals. In the crime of location is important the collection of various types of traces in an attempt to find the perpetrator of the crime. Thus, we propose that hematophagous mosquitoes found at crime scenes are collected as a biological trace and from the genetic testing of human blood from the digestive tract of mosquitoes can relate the suspect criminal scene. This study aimed at achieving a single mosquitoe *Aedes aegypti* profiles of mixtures of human DNA until 04 individuals, doing an analogy of the victim and suspect more than one. We also note the multifeed the parameter in time intervals of 24h, 48h and 72h after the blood. 15 STR *loci* were analyzed and amelogenin, the extraction being performed with DNA IQ™ System kit and the amplification using the PowerPlex® HS System. The results demonstrated that only 24 hours after the blood meal was detected mixtures of human DNA profiles from two hematophagous mosquitoes blood with the mixture of three and four individuals, respectively. Thus, we see the possibility of hematophagous mosquitoes are part of the list of biological remains that should be sought and collected the crime scene and can be detected human DNA profile of up to four individuals.

Key Words: Mosquitoes blood meal; DNA mixtures; Forensic DNA; *Aedes aegypti*;

INTRODUCTION

Haematophagy in mosquitoes vectors has various important consequences with impact on characteristics that affect reproductive functions (Attardo et al., 2005), disease transmission (Scott and Takken, 2012), vector control (Harrington et al., 2014) or even forensic entomology (Spitaleri et al., 2006).

Several mosquitoes species such as, *Aedes aegypti* (Scott et al., 1993 a, b; Xue et al. 1995; Scott et al., 2000; Reyes-Villanueva, 2004), *Ae. albopictus* (Delatte et al., 2010), *Anopheles* species (Edman and Downe 1964; Boreham and Garrett-Jone 1973; Burkot et al. 1988; Conway and McBride, 1991) use to supplement their blood meal, as a consequence of physiological traits (anautogeny) (Clements, 1999) or feeding disruption, owing mainly to defensive responses of the host (Conway and McBrid, 1991; Clements, 1999). Such behaviour has many important implications. By having multiple feeding in a gonotrophic cycle, mosquitoes vector can increase the amount of eggs laid (Amerasinghe et al., 1999; Charlwood et al., 2003) increasing density in an area, improving the risk of disease transmission by increasing the frequency of contact with hosts (Garrett-Jones, 1964; Garrett-Jones and Shidrawi, 1969; Dye 1986) and enhance insecticide toxicity, affecting control measures (Barlow and Hadaway, 1956; Halliday and Feyereisen, 1987; Reiter et al., 1990).

In the forense aspect, human blood found in the digestive tract of mosquitoes has been shown to support the investigation of crimes committed in closed environment, i.e, room or vehicle, (Spitaleri et al., 2006; Curic, 2014). Thus, it is plausible suppose that supplementary feeding performed by females in such environment, may be allow genetic material of different subjects in a single mosquito, improving the importance of mosquito as forensic prove. However, such hypothesis has rarely been investigates.

Beside, the wide distribution of mosquitoes, make easily these insects being found at crime scenes, providing an easy source of human genetic material, for forense purpose (Spitaleri et al., 2006). The comparison of the crime suspect's DNA profile database of the Scientific Police, may also indicate whether it is repeated offenses (Wallace et al., 2014) increasing the importance of mosquito as a tool on crime solutions.

In this work we evaluated whether human blood obtaining from mosquitos caught in crime scenes occurred indoors, could show DNA profile from both, victim and the suspects. The effect of temporal intervals of 24, 48 and 72 hours after the blood meal was also analysed.

MATERIAL AND METHODS

Obtaining the mosquitoes

Adult mosquitoes of the *Aedes aegypti* species were obtained from Laboratory of Endemics/LACEN (Recife, Pernambuco – Brazil). Confirmation of species identification was performed with the taxonomic key of Rueda (2004). Adult females were kept in cages, which was offered 10% sucrose solution as food up to 24 hours before the blood supply.

Mixture Human DNA samples obtained from hematophagous mosquitoes

Ten mosquitoes were placed in each cage identified according to the time interval after blood meal (24, 48 and 72 hours) and the number of volunteers to induce blood mixture, totaling nine cages. The volunteers identified by the letters A (woman), B (man), C (woman) e D (woman), exposed their arms in the cages to feed the mosquitoes (Table 01). A control group was also applied with the hematophagy from only one volunteer. Before the females perform the hematophagy, the arms of volunteers were washed with water and mild soap. Furthermore, none of the donors were using perfume to avoid the presence of attractive or repellent substances to mosquitoes.

One arm of each individual was exposed at a time in each cage in order to ensure the blood meal from all volunteers. To avoid any biased data, we established an order of exposure of arms aleatory for the blood meal as shown in Table 1, followed by the stipulated time for removal of the arm, so the meal was not completed with only one of the volunteers' blood. To simulation a hypothetical case: the volunteer A corresponding the victim's and the other volunteers (B, C and D) would be possible suspects.

Table 01 – Order of display of arms of volunteers and blood meal time

Order of display of arms of the volunteers in the cage	Time / each volunteer (in seconds)
A-C	30
A-B-C	20
B-D-A-C	15

After to completion of the blood feeding, the mosquitoes were sacrificed after 24h, 48h and 72h from the blood meal, being crushed on filter paper for subsequent genetic

analysis to be held in the Laboratory of Expertise and Research in Forensic Genetics / Secretary of Social Defense (Recife, Pernambuco, Brazil).

Reference samples

For sample comparisons, 2 mL of each volunteer was pooled altogether into tubes containing EDTA anticoagulant following the same order of the blood meal to serve as a standard analysis for subsequent comparison with the results of genetic profiles obtained from the digestive tract blood of hematophagous mosquitoes. Furthermore, reference samples were collected from oral mucosa of all volunteers.

Human DNA extraction

The DNA samples were extracted using the DNA IQ™ System kit (Promega, Madison, WI, USA) as recommended by the manufacturer.

Genetic analysis

DNA quantification was made by Quantifiler® Duo DNA Quantification kit (Applied Biosystems, Foster City, USA) using the Real Time PCR ABI 7500 platform. Genetic profiling was obtained using PowerPlex® HS System (Promega, Madison, WI, USA), following the manufacturer's protocols, for 15 STR *loci* (D8S1179, D21S11, D7S820, CSF1PO, D3S1358, TH01, D13S317, vWA, TPOX, D18S51, D5S818, D16S539, Penta D, Penta E and FGA) and amelogenin.

Ethics approval

This study was approved by the Ethics Committee of the Universidade Federal de Pernambuco (Recife/Brazil) under protocol number 462/11.

Statistical analyses

The DNA Mix v. 3.2 software was used for statistical analysis for calculate likelihood ratios (Curran et al., 1999) concerning the sample presenting mixture with one more genetic profiles. Used was the ancestry coefficient of 0.01 and minimum frequency of 0.01 following the recommendation of National Secretary of Public Security (SENASP) in a database of allelic frequency to the Brazilian population (Aguiar et al., 2012), adopting a confidence interval of 95%. For each cage, we have the following list of samples for statistical analysis simulating a hypothetical case: mosquito mixture containing human DNA considered as trace (questioned sample); victim's sample corresponding to the volunteer A DNA; and the other volunteers (B, C and D) would be possible suspects.

RESULTS AND DISCUSSION

We obtained the genetic profiles for three and four mixed samples from a single mosquito after 24 hours of the blood meal. However, we did not obtained viable results from mixtures found in mosquitos after 48 and 72 hours from hematophagy. Moreover, we also did not obtained the profiles for two mixed samples after 24 hours, since we could only visualize the profile of one donor. This may be due to a higher time of hematophagy in the first volunteer when only two individuals were involved.

The quantification analysis showed the value of 0.019 ng/µL human DNA, being 0.010 ng/µL of male DNA to the mix with three individuals and the 0.256 ng/µL human DNA (0.007 ng/µL of male DNA) for the mixing of four individuals. The value of statistical

analysis of likelihood ratio (LR), the association that conducts genetic profile found on the trace of the mixture belonging to another individual in the population. The LR value was calculated in relation to trace only the complete markers, so, who had all expressed alleles compared references. Thus, for statistical comparison of traces with two suspects (B e C) the following *loci* were excluded: PENTA E, D16S539, TPOX and FGA; and to calculate with the three possible suspects (B, C and D) they were excluded from the THO1, PENTA E and D5S818. The results LR was 3.2×10^{12} and 128.3×10^{12} for mixtures in question with three or four individuals, respectively. This calculation is widely used in forensic interpretations [Ladd et al., 2001; Gill et al., 2006; Haned et l., 2015).

The reference patterns obtained by in vitro mixture of the blood provided comparable profiles. If one compare the peaks in the Figure 1A and 1B, with the profiles from mosquitos' mixed blood and the reference profile, respectively, only two alleles were missing: allele 8 from THO1 and allele 10 from Penta E. The alleles 7 and 12 had a lower peak probably because of their size in base pairs. It's also important to bear in mind that these positive results are after 24 hour of the hematophagy event, which is the moment that the insect start to metabolize the blood for posterior oviposition (Spitaleri et al., 2006).

For the *loci* D5S818, D13S317, D7S820, D16S539, CSF1PO and Penta D shown in the Figure 2 A-F, we observed that all alleles were obtained when comparing the sample trace profiles with references samples and the balance of the interregions peaks maintained their proportions when they were interrelated.

For the analysis of the markers vWa, D8S1179, TPOX and FGA (Figure 3 A-F), some alleles had their low peaks, and even it is possible to note the disproportionality in amelogenin X donor being greater than the Y logically, since there was three women's donor material against one man. This fact is relevant and explanatory of the disproportionalities of the alleles. For example, we can see this fact in alleles 23 and 25 on the marker FGA that is expressed themselves at low and medium manner. Such marker requires a greater number of pairs of bases, especially because these alleles are only from the man.

In the 15 STR *loci* analyzed, in addition to the sexual marker amelogenin, the values was greater than recommended by FBI for the implementation of Combined DNA Index System (CODIS). This system, composed of 13 STR *loci*, has become a reference for other countries regarding forensic analysis, and the larger the number of *loci* are studied, the greater is the reliability for the comparison between the sample and the reference trace (Budowle et al., 1999; Sun et al., 2003).

The absence of alleles is also a common fact found in the mixture, even when such a mixture is found in inanimate traces at the crime scene, as bottles, and other objects as well as in living organisms, like mosquitoes and necrophagous larvae, for example. Mixed and degraded samples show a forensic challenge, since it may appear imbalanced peaks and limiting expression of one or more alleles, for example (Ladd et al., 2001). Even with the failure to obtain matching alleles between a full-profile of the suspect and evidentiary sample, the profile trace mixing may be essential to include or exclude suspects, helping to solve the case (Butler, 2005).

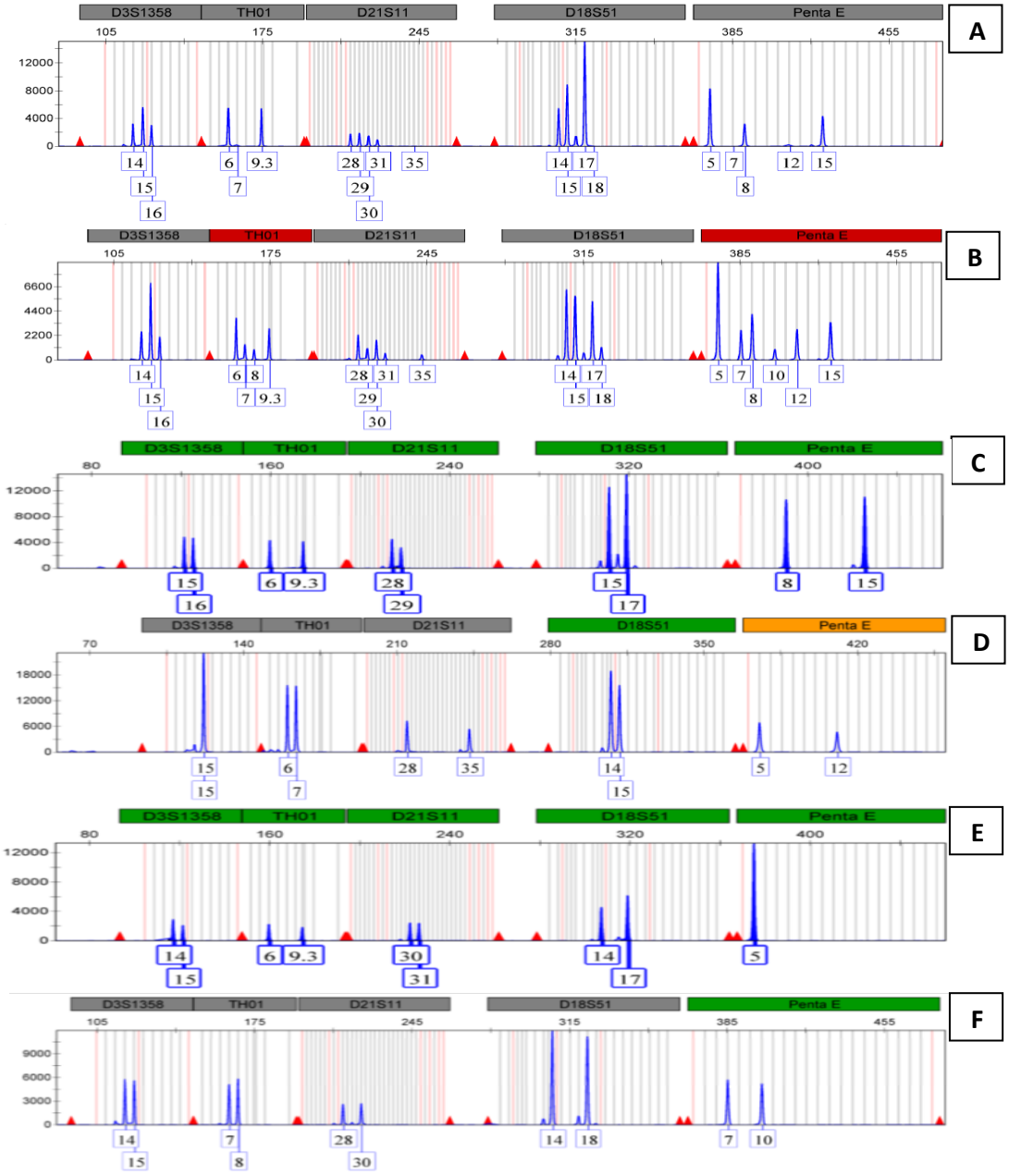

Figure 1 – Comparison of human DNA profiles with *loci* D3S1358, THO1, D21S11, D18S51 e Penta E. A. Mixture profile trace, showing the four volunteers profiles, obtained from the digestive tract of the mosquito *Aedes aegypti* blood after 24 hours prior to the hematophagy; B. Standard mixture profile with the blood of four volunteers; C. Profile simulating the victim (corresponding to the volunteer A); D-F. Profiles of possible suspects (corresponding to the volunteers B, C and D, respectively).

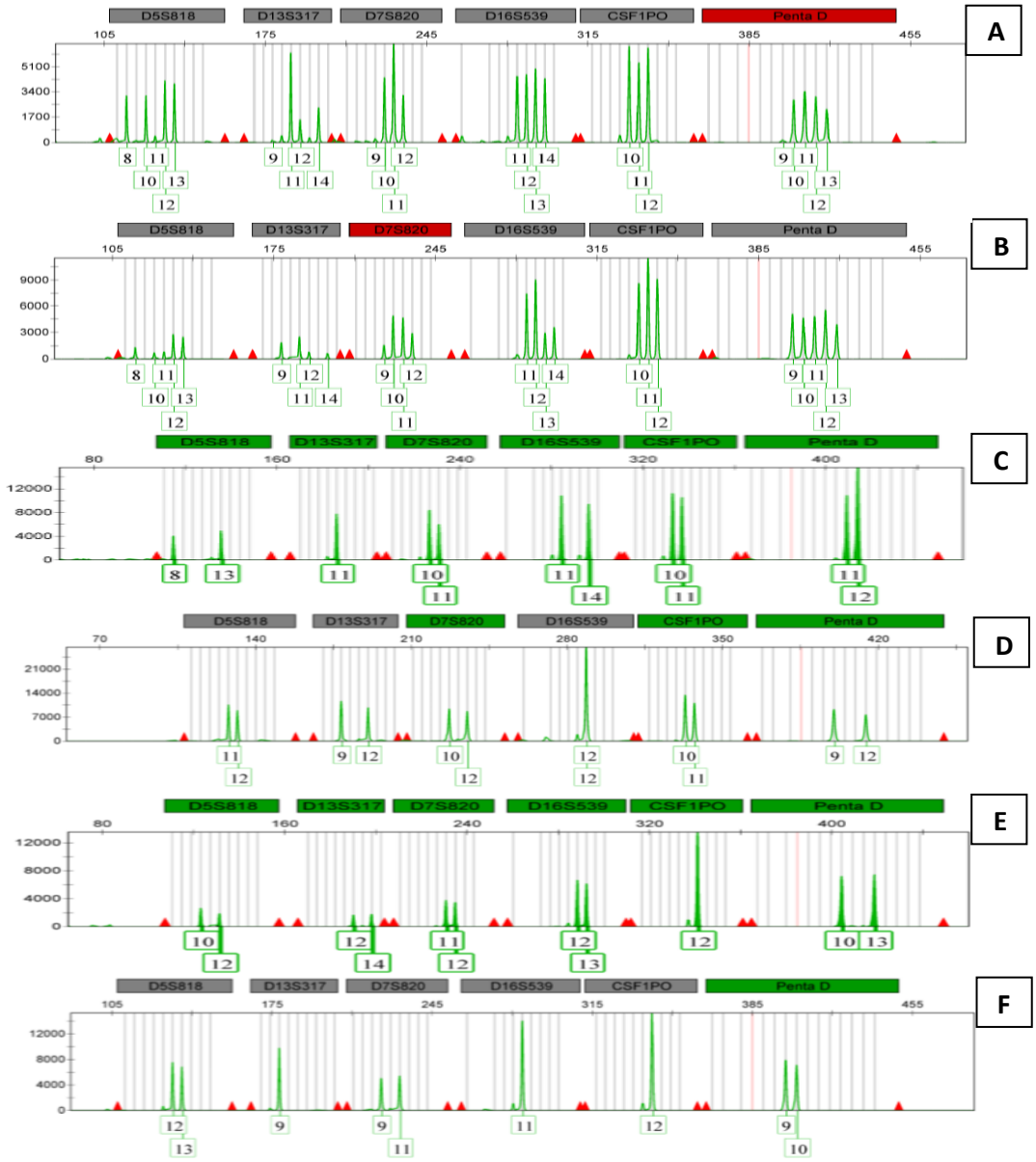

Figure 2 – Comparison of human DNA profiles with *loci* D5S818, D13S317, D7S820, D16S539, CSF1PO e Penta D. A. Mixture profile trace, showing the four volunteers profiles, obtained from the digestive tract of the mosquito *Aedes aegypti* blood after 24 hours prior to the hematophagy; B. Standard mixture profile with the blood of four volunteers; C. Profile simulating the victim (corresponding to the volunteer A); D-F. Profiles of possible suspects (corresponding to the volunteers B, C and D, respectively)

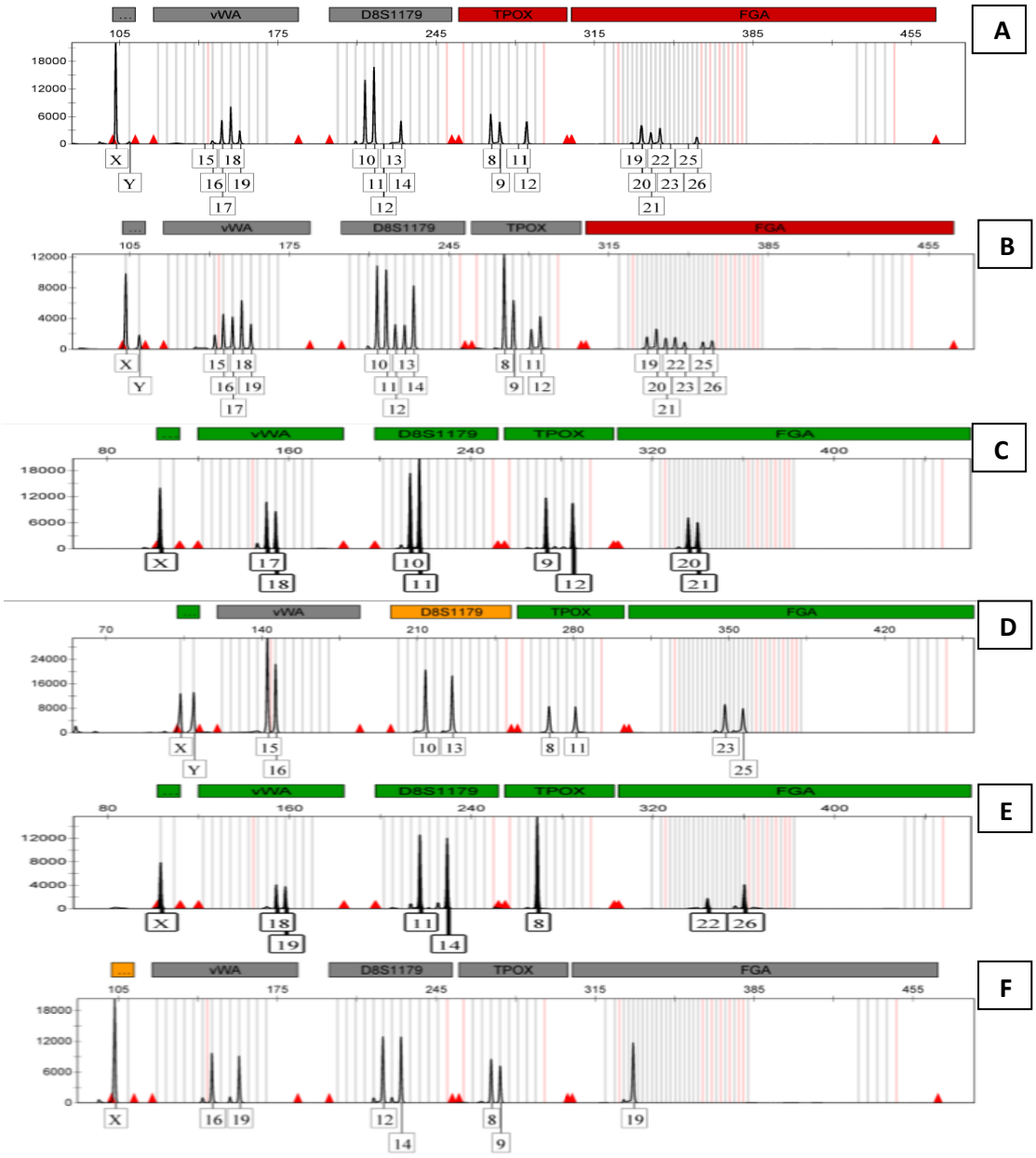

Figure 3 – Comparison of human DNA profiles with *loci* vWA, D8S1179, TPOX, FGA and amelogenin. A. Mixture profile trace, showing the four volunteers profiles, obtained from the digestive tract of the mosquito *Aedes aegypti* blood after 24 hours prior to the hematophagy; B. Standard mixture profile with the blood of four volunteers; C. Profile simulating the victim (corresponding to the volunteer A); D-F. Profiles of possible suspects (corresponding to the volunteers B, C and D, respectively).

CONCLUSIONS

In crime scenes it is common for researchers collect traces that, after analyzed, are identified with a mixture of human DNA. However, when dealing with biological traces that have of mixed genetic profiles, as in hematophagous mosquitoes, the importance to collect

this type of biological material as a potential form of aid the criminal investigation in the inclusion or exclusion of suspects is acknowledged based on the data presented in this research.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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ARTIGO 4

Biochemical and genetic analysis of hematophagous mosquitoes with forensic purpose

Status: a ser submetido

Biochemical and genetic analysis of hematophagous mosquitoes with forensic purpose

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ABSTRACT

Hematophagous mosquitoes can provide additional tools for criminal investigations resulting from indoors. Mosquitoes were collected indoors, in two residential houses, which could be analogous to crime scenes. Material volunteer blood was collected for biochemical analysis of ammonia and lactic acid, using the systems from Roche Diagnostics / Hitachi Cobas C. In addition, analysis was made of the blood typing and measuring the body temperature of the volunteers. Human blood samples found in the abdomen of hematophagous mosquitoes were genetically analyzed, after DNA extraction, performed with DNA IQ ™ System able to identify 15 STR loci and amelogenin. References samples were from the oral mucosa of volunteers. The result of this research showed that it was possible to obtain human genetic material from the mosquito, with no interference of biochemical factors and body temperature in the attractiveness of mosquitoes. Blood type O showed the highest amount of genotyping.

Key words: entomological trace; Forensic DNA; ammonia; lactic acid; blood typing.

INTRODUCTION

Crimes committed in enclosed spaces such as the practice of kidnapping and false imprisonment, showed worrying numbers in the state of Pernambuco / Brazil. Statistics issued by the Social Defense Department of the State of Pernambuco, make mention of quantitative data of these types of crimes: in 2008 were registered 155 cases; in 2009 they were obtained 213 records; in 2010 there was a slight decrease to 168 occurrences in 2011 and increased again to 238 cases recorded in the state.

Mosquitoes found indoors when used in the commission of crimes can serve as a criminal evidence from genetic analysis of ingested blood (Spitaleri et al., 2006). The use of sensitive

and specific techniques such as short tandem repeat and polymerase chain reaction makes it possible to obtain a DNA profile even in degraded samples (Bahlmann et al., 2014).

Hematophagous mosquitoes need blood supply to the oviposition and can to remake the blood meal a few hours after the end of the digestion of the previous meal, and still have the next food source, preferring the same donor (Mukabana, 2002 a,b; Jones and Pilitt, 1973). Therefore, they are extremely useful as traces and contribution in criminal investigation (Spilateri et al., 2006).

Thus, the possibility of identifying persons from the blood meals of hematophagous mosquitoes and subsequent genetic analysis is a viable reality in solving the types of crimes committed in enclosed places (Curic, 2014; Spitarelli, 2006). Corroborating this statement, Rabêlo et al. (2015, in press) obtained human DNA profiles obtained from blood feeding mosquitoes, also being viewed mixtures of genetic profiles in a single mosquito.

The strategy of these insects in search for better blood supply for the realization of their hematophagous meals suffers various stimuli. These include, among others, chemical and physical factors such as ammonia, lactic acid, blood type and skin temperature (Anjomruz et al., 2014, Steib et al., 2001, Geier et al. 1999 Canyon et al., 1998, Grossman; Pappas, 1991).

Thus, this study aims at analyzing human DNA from hematophagous mosquitoes of different species captured indoors and identifying the biochemical factors of ammonia and lactic acid emitted by the human body in the attractiveness of mosquitoes. Also, we verified if body temperature, blood type and sex of the volunteers can influence the hematophagy of mosquitoes and consequently obtaining human DNA.

2. MATERIALS AND METHODS

2.1 The collection of hematophagous mosquitoes

Mosquitoes were collected in two residential houses located in the city of Recife, Pernambuco – Brazil. All mosquitoes found inside the residences were collected by using manual capturing based on the Castro model (Buxton, 1928), placed in pots and then sacrificed with chloroform to aid identification in stereoscopic microscope (Olympus, model SZ2 61, serie 04128), using the taxonomic keys of Forattini (2002) and Rueda (2004).

2.2 Biological samples and body temperature of volunteers

Two kinds of Biological samples were collected from 27 volunteers (Table 1): their blood for biochemical analyzes of ammonia and lactic acid and saliva for genetic analysis. All volunteers signed a written Informed Consent Term approved by the Ethics Committee of the Federal University of Pernambuco (Recife/Brazil) under the protocol number 462/11. The survey was conducted according to the standards of the Declaration of Helsinki, revised in 2008.

Before the collection of biological samples, we administrated a questionnaire at the Consent Term containing questions about smoking habit, alcohol consumption in the last 24 hours, possible diseases (liver, kidneys, heart or other), dehydration, conducting exercises in the last hour before the blood collection, medication use (acetylsalicylic acid or aspirin, barbiturates, biguanides, or any other) blood type, blood transfusion or bone marrow and were sleeping or resting before collection. This questionnaire aims to elucidate possible changes in exams when the practice of some reported item.

We collected 8 mL of peripheral blood. The blood was distributed in tubes containing 4 ml for the analysis of ammonia and 4 mL for the analysis of lactic acid with their specific

anticoagulant indicated by the manufacturer, taking the precaution of blood collection be made into a vein without stasis so that there was no change in biochemical tests. After collecting the samples were stored in a container with thermal isolation and sent to the biochemical laboratory for analysis using systems from Roche Diagnostics / Hitachi Cobas C with the manufacturer's instructions.

The reference values considered for the examination of ammonia were 11-51 µmol/L for women and 16-60 µmol/L for men (COBAS, 2006); and lactate was between 0.5 and 2.2 µmol/L for both genders (COBAS, 2012). Furthermore, the body temperature of each volunteer was measured in °C with the aid of a thermometer.

For the blood typing we used two drops of blood from volunteers arranged on glass slides and subsequently dripped reagents (Prothemo Products haemotherapic Ltda) Anti-A (bright blue) and Anti-B (yellow Tartrazine) for conducting and interpreting the result of agglutination. Some volunteers who did not donate biological material for the realization of blood typing but knew their blood type, was noted only the classification of the ABO system.

2.3 Genetic Analysis

Mosquitoes were individually smashed on filter paper for a later realization of genetic analysis. DNA extraction from both - human blood from the digestive tract of the hematophagous mosquitoes and oral mucosa of the volunteers' samples - was performed using the DNA IQ™ System kit (Promega, Madison, WI, USA) as recommended by the manufacturer. A multiplex PCR was performed according to the AMPF*l*STR Identifiler® Plus® PCR Kit (Life Technologies; Carlsbad, CA, USA) manual supplied by the manufacturer. Were analyzing 15 STR loci (D8S1179, D21S11, D7S820, CSF1PO, D3S1358, TH01, D13S317, D16S539, D2S1338, D19S433, vWA, TPOX, D18S51, D5S818, FGA) and amelogenin. The amplification products were subjected with the ABI PRISM 3500 HID

automated sequencer (Applied Biosystems, Foster City, CA, USA, using 28 PCR cycles for the amplification. Blank controls was used to detect possible contamination.

3. RESULTS

3.1 Human DNA from hematophagous mosquitoes

We captured 26 female mosquitoes (Diptera: Culicidae) identified as *Aedes aegypti* (n = 13), *Aedes albopictus* (n = 1) and *Culex quinquefasciatus* (n = 12) with 11 samples containing human DNA (Table 1) (unpublished observations, Rabêlo et al., 2015 in press). We also detected genetic mixed profiles from a single mosquito (unpublished observations, Rabêlo et al., 2015 in press).

3.2 Biochemical analyzes, blood type and body temperature correlated with the amplified human DNA

In the mosquitoes that had human blood with the amplified DNA and the sample correlated reference, it was observed that within the six matches (Table 1), two volunteers have not provided blood materials making it impossible for quantitative examination of ammonia and lactic acid. Moreover, a mosquito of the *Aedes aegypti* species showed human DNA mixture without correlation of the samples of volunteers and two mosquitoes of the *Culex quinquefasciatus* species showed genetic profile, with no correlation with references samples. From the analysis of the results of genetic profiles we obtained six male DNA human profiles and six related females.

Biochemical analyzes (Table 1) showed that human DNA profiles (volunteers 4, 14, 16 e 18) were obtained from mosquitoes that fed on subjects who had normal to take lactic acid. Besides, it was observed that all the volunteers who took the test for lactic acid was

detected done normal except for the volunteer number 21 which we had no mosquitoes with the corresponding human DNA.

For biochemical examinations of ammonia (Table 1), DNA was obtained in human volunteers profile of 4, 14, 16 and 18, tests have shown that being with altered to higher values than in normal individuals 4 and 18, with the other having normal reference values for this test. Thus, it was observed that both individuals with normal values for this biochemical examination as altered values for more were attractive to hematophagous mosquitoes.

Volunteers represented by numbers 8, 9, 13 and 20 had altered values to take ammonia, but without obtaining genetic profile correlation. The other positive results for the genetic analysis identified as individuals 15 and 27 not sanguine donated materials preventing the correlation analysis biochemistry and genetics. Moreover, the volunteers 18 and 27 were shown to be attractive to mosquitoes hematophagous since two distinct blood repast different mosquitoes made of the same volunteer.

Of the volunteers who knew and have donated blood material for analysis of blood typing, 52.17% had blood type O, and 34.78% were classified as blood type A, and 13.4% for type B blood and no blood type AB it was observed.

The body temperature variation of the volunteers was 1 ºC, and the slightest recorded temperature of 36 °C and the greater was 37 ºC with a genetic profile presentation in all amplitudes of the temperatures recorded. Thus, not observed a body temperature value be preferable to demonstrate the attractiveness of mosquitoes to the blood supply.

Table 1 – Relationship between biochemical analyzes, blood type, body temperature and amplification of human DNA found in hematophagous mosquitoes.

volunteer	volunteer		biochemical analyzes /ABO			body temperature	amplification DNA
	sex	age	ammonia (µmol/L)	lactic acid (µmol /L)	Volunteer blood typing	(ºC)	(specie of mosquitoes)
1	M	20	50	1.3	O	36.4º	--
2	M	19	48	1.8	O	36.7º	--
3	M	23	36	1.8	O	37.0º	--
4	M	20	75	1.7	O	36.4º	profile amplified (*Aedes aegypti)*
5	M	21	27	1.3	--[b]	36.7º	--
6	W	64	--[a]	--[a]	B	36.6º	--
7	W	49	13	0.7	A	36.0º	--
8	W	22	8	2.0	A	36.7º	--
9	W	20	8	0.8	O	36.6º	--
10	M	19	41	1.1	O	36.0º	--
11	W	27	--[a]	--[a]	A	36.8º	--
12	W	23	--[a]	--[a]	A	36.5º	--
13	M	21	5	1.0	A	36.4º	--
14	M	22	52	1.3	A	36.0º	profile amplified (*Culex quinquefasciatus)*
15	M	26	--[a]	--[a]	O	36.4º	profile amplified (*Aedes aegypti)*
16	W	18	28	0.7	B	36.6º	profile amplified (*Culex quinquefasciatus)*
17	W	24	13	0.8	O	36.0º	--
18	W	38	59	1.1	--[b]	36.5º	profile amplified (two mosquitoes of specie *Aedes aegypti* fed on this same volunteer)

19	M	35	36	1.2	--[b]	36.7º	--
20	W	23	276	1.4	B	36.4º	--
21	M	20	44	4.2	A	36.5º	--
22	W	24	--[a]	--[a]	O	36.7º	--
23	W	20	--[a]	--[a]	A	36.5º	--
24	W	20	--[a]	--[a]	O	36.5º	--
25	M	20	--[a]	--[a]	O	36.0º	--
26	M	33	--[a]	--[a]	--[b]	36.7º	--
27	M	20	--[a]	--[a]	O	37.0º	profile amplified (two mosquitoes of species *Aedes aegypti and Culex quinquefasciatus* fed on this same volunteer)

a - it not donated blood for biochemical analysis.
b - it not donated blood for typing blood and/or did not know what blood type .
a mosquito of the *Aedes aegypti* species showed human DNA mixture without correlation of the samples of volunteers of two women. Two mosquitoes of the *Culex quinquefasciatus* species showed genetic profile of man and woman, with no correlation with reference samples.

4. DISCUSSION

The possibility of obtaining human DNA profiles using the blood originating from haemathophagous meals is already a viable reality (Curic, 2014). Criminal evidence was found successfully in investigations using this trace in the work Spitaleri et al. (2006) reporting that a murder case was solved with a mosquito blood the *Culex pipiens* specimen found at the residence of the suspect. However, this work did not analyze variables that could affect the results, now presented in our study.

We observed human DNA profiles obtained with hematophagous mosquitoes of the species *Aedes aegypti* and *Culex quinquefasciatus*. Even with the human DNA possibly being degraded by the consumption of human blood, it was possible to obtain human DNA profiles (Rabêlo et al., 2015, in press). The work of Alaeddini et al. (2010) reported that viable results for subsequent interpretation and validation are possible, in spite of DNA degradation, using the same STR approach employed in this study.

Mixture of DNA profiles from a single hematophagous mosquitoe was also identified. El-Alfy and El-Hafez (2012), using 15 STR *loci* (plus amelogenin), reported the successful separation and identification of profiles mixture of human DNA. Thus, this type of trace even has a greater contribution because, on the site of the mosquito crime could perform the haematophagic feed of both the victim and the offender, without invalidating or one and not another human DNA profile. When there is a reference sample, these types of cases the separation of these genetic profiles is possible to point the perpetrator through the multiplex system (Gill et al 1998, González-Andrade et al., 2006).

Considering the behavior of the mosquitoes no preference among female and male was observed in relation to the choice of blood feeding subject. This finding is contradictory with respect to other papers, such as Brouwer (1960), Geier et al. (1996) and Brady (1997) reporting the preference of hematophagous mosquitoes towards men, explaining this fact due to hormonal differences.

We did not detect that biochemical factors could be related to the attractiveness of the haematophagy, but this result may be due the limited number of volunteers of biological material donors for such observation, even though the volunteers who showed abnormal values with amount higher than normal for ammonia were obtained viable genetic profiles. This is also in contrast with the studies of Geier (1999) et al and Steib (2001) reporting that the biochemical components expelled by the human body, such as lactic acid and ammonia are attractive components for hematophagous mosquitoes.

Regarding the laboratory tests for lactic acid almost all were normal, except for the voluntary 21, which showed almost double the limit value for lactate. However, when we observed the Consent Term of the questionnaire it reported that it had done exercise at the last minute before the collection of blood material, a fact that was probably responsible for changes in such examination.

The volunteers 4 and 18 that showed higher values than normal for ammonia when verified the questionnaire only differed in their responses to the negative item was were sleeping and / or resting before collection, which the number of individual 18 answered so, differently from the number 4. However this fact does not seem to be very relevant in the justification of the amendment of such examination, since the voluntary four became negative the question that would have done physical exercise in the last hour prior to blood collection.

Individuals 8,9,13 and 20 that showed a decrease of biochemical values of ammonia, when consulted responses was observed that the individual 8 is a smoker and was using Rivotril two weeks ago; the voluntary 9 negatively answered the questions; the voluntary 13 replied that he was doing the Busonid medication administration and the individual 20 said it was making use of contraceptive and resting before collection.

The variation in registered body temperature of the volunteers was 1 ° C difference between the minimum and maximum temperature recorded which was obtained amplification of genetic profiles, and thus we can not infer whether there was influence of this variable, only report that more was registered blood meals with nearer to 37 ° C temperatures. Pappas and Grossman (1991) studied the variation in the body temperature between 29 C and 36.2ºC in nine individuals reporting that the behavior of the mosquitoes and the amount of blood intake did not differ, however engorgement time decreases as the increase of body temperature.

The analysis of blood group indicated a greater preference in the practice of haematophagy by blood type O as opposed to blood type AB in which no individual who was not registered been fueled by the species under study. Anjomruz et al. (2014) reported the preference of *Anopheles stephensi* by AB group and the least preferred as belonging to the group O, as opposed to the species collected and identification DNA profiles in this study who were *Aedes aegypti* and *Culex quinquefasciatus*.

Thus, although reporting work influence of biochemical factors as attractive to haematophagy, this fact was not explicit in the results of this work. With obtaining viable genetic profiles for judicial review, even with some altered values in biochemical analyzes indicate that external factors, such as reported in the questionnaire, do not prevent blood feeding and consequent attainment of human DNA, thus valuing the mosquito as a hematophagous important forensic trace.

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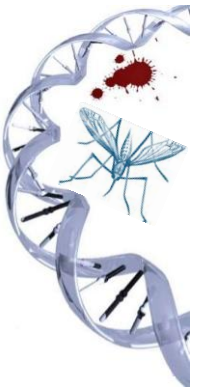
CONCLUSÕES



5. CONCLUSÕES

1. O melhor método de extração de DNA foi com a utilização do DNA IQ;
2. Foi possível a obtenção de perfis de DNA humano em até 72h após a realização da hematofagia;
3. A partir de 0,01 ng/µL foi obtido perfil de DNA humano proveniente do trato digestório de mosquitos hematófagos;
4. Foi possível corresponder o perfil de DNA humano encontrado nos mosquitos hematófagos e as amostras referências de voluntários;
5. Fatores como amônia, ácido lático e temperatura não afetam a atratividade dos mosquitos. Além disso, foi registrada a preferência na prática da hematofagia pelo sangue tipo O;
6. A obtenção de mistura de perfis genéticos foi obtida com até quatro indivíduos e em até 24h após a refeição sanguínea.

PERSPECTIVAS

6. PERSPECTIVAS

Os resultados desse trabalho aumentam o escasso número de estudos que comprovam a viabilidade do uso de mosquitos hematófagos como prova forense. Tal ferramenta, é principalmente importante em locais de crime fechados, e quando o criminoso procura limpar a cena de crime, na tentativa de eliminar os demais vestígios de seu ato criminoso. Além disso, temos a perspectivas de que, com a divulgação destes tipos de pesquisas, e consequente aumento de estudos aplicados na área da entomogenética forense, as universidades brasileiras passem a dar mais ênfase a esse foco de pesquisa como forma de ampliarmos as possibilidades de aplicabilidade dos insetos, com o advento da genética forense, na resolução de casos criminais, auxiliando a polícia técnico-científica na resolução de casos criminais difíceis.

APÊNDICE

TERMO DE CONSETIMENTO LIVRE E ESCLARECIDO - TCLE

Eu_____,RG_____, CPF_____, data de nascimento ____/____/_____, com _____ anos de idade*, residente na _____, n°____, aptº_____, bairro _____, do município de _____ estou ciente e concordo em participar da pesquisa intitulada I**SOLAMENTO, AMPLIFICAÇÃO E SEQUENCIAMENTO DE DNA HUMANO ENCONTRADO EM TRATO DIGESTÓRIO DE CULICÍDEOS HEMATÓFAGOS PARA FINS FORENSES**

***os voluntários desta pesquisa precisam ter mais de 18 anos de idade.**

A JUSTIFICATIVA, OS OBJETIVOS E OS PROCEDIMENTOS: O motivo que nos leva a estudar este problema é de importância social para conseguir identificar quem é o autor de crimes cometidos em locais fechados como sequestro/cárcere privado, ou seja, manter a pessoa presa sem sua autorização, a partir da análise genética dos mosquitos hematófagos encontrados no local. O objetivo do projeto é verificar se o sangue encontrado no trato digestivo de mosquitos hematófagos pode ser comparado aos das pessoas que moram nas casas onde os mosquitos foram coletados, fazendo-se uma analogia a este tipo de crime. O procedimento da coleta de material será da seguinte forma: serão coletados os mosquitos encontrados no interior das residências, sangue (8 mL) e saliva de moradores voluntários. O material bucal será coletado com swab estéril (instrumento parecido com cotonete) para servir de material biológico de referência. A seringa e o swab serão abertos na frente do voluntário para verificação do material estéril. A coleta de sangue será realizada por profissional capacitado, garantindo a saúde do voluntário.

DESCONFORTO, RISCOS E BENEFÍCIOS: Será coletada somente a quantidade de sangue suficiente para a pesquisa sem causar dano ou dor sem necessidade. Será solicitado também ao voluntário uma amostra de saliva, no qual não representa nenhum prejuízo ou dor para o voluntário. Em resumo, o desconforto da participação deste projeto para o voluntário, está caracterizado num possível constrangimento ao responder o questionário, e os riscos normais e habituais de uma coleta de sangue. O benefício para a sociedade será que a partir deste trabalho poderá ficar provado que o suspeito de praticar o crime de sequestro ou cárcere privado poderá ser identificado a partir do sangue retirado dos mosquitos encontrados no local do crime. Já o benefício individual, será que a partir das respostas apresentadas no questionário e consequentemente informações de seu hábito diário em relação a sua saúde, o voluntário terá orientações de cuidado em relação à mudança de hábito que deva adquirir para a obtenção de uma melhor saúde e consequente qualidade de vida, além de esclarecimentos de como prevenir a presença de mosquitos na residência.

GARANTIA DE ESCLARECIMENTO, LIBERDADE DE RECUSA E GARANTIA DE SIGILO: o voluntário será esclarecido(a) sobre a pesquisa em qualquer aspecto que desejar, sendo livre para recusar-se a participar ou retirar seu consentimento a qualquer momento. A sua participação é voluntária e a recusa em participar não irá acarretar qualquer penalidade ou perda de quaisquer benefícios. A pesquisadora irá tratar a sua identidade com padrões profissionais de sigilo. Os resultados dos exames da pesquisa permanecerão confidenciais. O voluntário não será identificado individualmente em nenhuma publicação que possa resultar deste estudo. Uma cópia reprográfica deste consentimento será arquivada pela pesquisadora deste projeto e uma outra cópia será fornecida ao voluntário.

CUSTOS DA PARTICIPAÇÃO, RESSARCIMENTO E INDENIZAÇÃO POR EVENTUAIS DANOS: A participação no estudo não acarretará custos para o voluntário e não será disponível nenhuma compensação financeira adicional.

CONTATO COM A PESQUISADORA DO PROJETO: Kaynara Cecília Nery Rabêlo, Av. Prof. Rego, s/n, cidade universitária, Recife-PE. CEP 50.670-901. Tel.: 2101 2655 / 8718 4984. Email: kaynaracecilia@yahoo.com.br

CONTATO COM O COMITÊ DE ÉTICA EM PESQUISA: Av. da Engenharia, s/n, 1º andar, Cidade Universitária, Recife-PE. CEP 50. 740-060. Tel: 2126 8588.

QUESTIONÁRIO (verificação de alguns fatores para realização dos exames bioquímicos):

Fumante: ( ) SIM ( ) NÃO Se afirmativo, fumou o último cigarro a quanto tempo?
Ingeriu etanol (álcool) nas últimas 24 horas: ( ) SIM ( ) NÃO
Apresenta doença hepática (fígado): ( ) SIM ( ) NÃO
Apresenta insuficiência renal (rins): ( ) SIM ( ) NÃO
Apresenta desidratação: ( ) SIM ( ) NÃO
Fez na última hora exercício : ( ) SIM ( ) NÃO
Fez uso de medicamentos, como salicílicos (ASS ou Aspirina), barbitúricos (sedativos ou calmantes, ex diazepam, gardenal) ou biguanidas (contido em alguns remédios para diabetes): ( )SIM ( ) NÃO
QUAL(IS):_____
É diabético: ( ) SIM ( ) NÃO
Apresenta ou apresentou infecção urinária no último mês: ( ) SIM ( ) NÃO
Faz terapia de hiperalimentação: ( ) SIM ( ) NÃO
Tipo sanguíneo/fator Rh:
Fez transfusão sanguínea e/ou de medula óssea? ( ) SIM ( ) NÃO Se afirmativo, quando?
Está tomando alguma medicação?
Possui insuficiência cardíaca e toma remédios?
Possui animal em sua residência? quais?
Estava dormindo e/ou descansando antes da coleta? ( ) SIM ( ) NÃO
Temperatura voluntário:
Temperatura e umidade da residência :
OBS.:

Recife, _____ de _____ de _____

_____

Assinatura do voluntário (a) e nº do telefone

_____

Assinatura da pesquisadora responsável pelo projeto

_____

Assinatura de testemunha (Vânia Tavares – LABEND/LACEN)

_____

Assinatura da bióloga responsável pela coleta de sangue (Natália Oliveira – LIKA/UFPE)