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In vitro recovery and identification of Y-STR DNA from *Chrysomya albiceps* (Diptera, Calliphoridae) larvae fed a decomposing mixture of human semen and ground beef

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ABSTRACT. Worldwide, several women become victims of rape every day. Many of those women are also murdered, with their bodies sometimes being found in an advanced state of decomposition, resulting in loss of evidence important to criminal investigations. Diptera is one of the main orders associated with human body decomposition. Fly species that belong to the family Calliphoridae are usually scavengers and are frequently found on

decomposing bodies, thereby playing an important role in forensic entomology. The recovery and genotyping of human Y-STR DNA from the gastrointestinal contents of the calliphorid *Chrysomya albiceps* larvae has promising applications in the investigation of sexual crimes, such as rape, and in cases of murder and abandonment of the victim's body, which may be found in a state of decomposition. We studied this species of fly with the aim of supporting such investigations. After establishment of a colony, larvae were fed with decomposing human semen mixed in ground bovine meat (1 mL per 200 g beef). Larvae (10–15) were collected every 24 h and kept in 70% ethanol, to give a total of 96 larvae obtained after eight days of decomposition. The digestive system of each larva was resected. Molecular typing was conducted, which comprised sample extraction, quantification, amplification, and capillary electrophoresis with 16 STR loci from the Y chromosome. We succeeded in establishing a Y-STR DNA profile, with amplification of up to 11 loci, from individual samples, or up to 15 loci, when a combination of samples corresponding to the time-points 48, 72, 120, 144, and 192 h was used.

Key words: Sexual crimes; Homicide; Forensics; Forensic entomology; Forensic genetics

INTRODUCTION

Advances in the recovery, isolation, and identification of human DNA ingested by larvae of forensically important flies can contribute to the materialization of forensic evidence. Thus genetic analyses of insect gut contents may prove to be crucial evidence to link a suspect to the victim or the scene (Campobasso et al., 2005), for example, in cases of rape followed by homicide, when the victim is found in an advanced state of decomposition (Clery, 2001). A study performed in 56 countries showed that one in every 14 women has been, at least once during her lifetime, a victim of sexual abuse. The same study indicated that there is underreporting of this type of crime, since most women are afraid of being blamed and have no support from family, friends, and public services. The world average for women aged 15 or older who have been sexually assaulted by someone beside their partners is 7.2%. The aforementioned study was performed in collaboration with the World Health Organization (WHO) and was based on research published between 1998 and 2011 (Abrahams et al., 2014).

Violence against women in Brazil is alarming. A study performed in 2013 by the Institute of Economical and Applied Research (IPEA) estimated that between 2009 and 2011, the country recorded nearly 17,000 female deaths caused by gender conflicts, or feminicide, a crime that occurs simply because the victim is a woman. This is equivalent to saying that, on average, 5,664 women are violently murdered per year or one every 90 min (Garcia et al., 2013). Official data show that in 2012, 51,101 reports of rape occurred in Brazil or 26.3 for every 10,000 people (Lima & Bueno, 2013). However, in 2013, based on a questionnaire about victimization, in the context of the Social Perception Indicator System

(SPIS), it was estimated that, every year, 527,000 people suffer sexual violence, with only 10% of the cases being reported to the police, indicating considerable underreporting of this type of crime. The same study indicated that, according to sources from the Brazilian Health Ministry, 88.5% of rape victims are women and 96.7% of the aggressors are men, that is, carriers of the Y chromosome (Cerqueira & Coelho, 2014).

In comparison to the national average number of rape cases, the numbers in the state of Rio de Janeiro are even worse. In 2012, there were 6,029 reports, that is, 36.9 for every 100,000 people, representing an increase of 23.8% in rape cases, compared to the previous year. These numbers indicate that 17 cases of rape occur every day in Rio de Janeiro, and 82.8% of these victims are women (Teixeira et al. 2013).

The bodies of many of these victims had been dumped in deserted and difficult-to-reach places, causing them to be discovered in an advanced state of decomposition, several days after the rape and murder. The longer the interval that has elapsed after the crime, the scarcer will be the available evidence. Forensic expertise shows that there is great difficulty in collecting technical evidence in cases of sexual violence when ejaculation of the perpetrator is followed by the death of the victim, because as body decomposition advances, there is no longer sufficient semen available for collection and genetic identification.

Forensic evidence becomes even more important for criminal prosecution when we consider the fragility of evidence obtained from eyewitnesses. Lindsay et al. (2004) found that there is a real possibility that details may be introduced between distinct events; that are a witness could confuse episodes experienced at different time points. Clarck and Godfrey (2009) showed that eyewitness identification as criminal evidence in cases of sexual violence results in greater chances of unfair convictions, because of memory limitations, especially under high stress conditions, changes in compensatory mental criteria, caused by memory limitations, and biased police procedures, encouraging the identification of innocent individuals as culprits.

Insects that colonize human bodies under different states of decomposition are an important source of information and can be used in forensic studies. One of the main orders associated with decomposing bodies is the order Diptera, which constitutes one of the largest groups of insects. The Calliphoridae family comprises several species of scavenging flies that play an important role in forensic entomology (Carvalho & Mello-Patiu, 2008). Maggots are commonly used during a death investigation to estimate the *postmortem* interval (Oliveira-Costa & Mello-Patiu, 2004; Oliveira & Vasconcellos, 2010).

Chrysomya albiceps is a calliphorid species that has been found abundantly in cadavers and carcasses at all stages of decomposition, especially in regions such as Southern Europe, the Afrotropics, Eastern Asia (From India to China), and the Neotropics (Grassberger et al., 2003); it has tremendous potential for forensic applicability in these regions. Many studies have been developed based on genetic and DNA applications of forensic entomology, which can help to solve various types of crime (Wells & Stevens, 2008; Reibe et al., 2009; Boehme et al., 2010).

Forensic molecular entomology has proven to be a promising scientific tool; Szalanski et al. (2006) performed the first isolation and amplification of human DNA from the bedbugh *Cimer lectularus* (Hemiptera, Cimicidae) by utilization of the forensic STR marker D18S51. Similarly, human DNA was recovered from fly larvae that fed on a human corpse, obtaining a complete autosomic and Y-STR profile from various samples (Di Luise

et al., 2008). Prior to that, Zehner et al. (2004) used fly larvae found in 13 human corpses to obtain complete STR profiles in most of the samples.

Curic et al. (2014) were able to recover human DNA from the mosquito family Culicidae, 72h after females fed from human blood. More recently, researchers were successful in the amplification of DNA from several surfaces using samples defecated and regurgitated from adult flies fed with swine blood and biological fluids (Kulsein et al., 2015). Turkish studies with third instar larvae from *Lucilia sericata* species that fed from diabetic human wounds were able to obtain complete human DNA STR and SNP profiles (Kondakci et al., 2009).

We examined the possibility of *in vitro* recovery and identification of human Y-STR DNA from *Chrysomya albiceps* larvae, with the purpose of producing material evidence that would be difficult to contest, and that could contribute to the acquittal of innocent suspects.

MATERIAL AND METHODS

The study was conducted in two phases: first, "Larvae Feeding and Dissection," and next, "DNA Extraction and STR Amplification." A few of the main mapped and cataloged short tandem repeats (STRs) on the human Y chromosome (Y-STR) were used.

Larvae Feeding and Dissection

This phase was performed in the Diptera Laboratories of the Museu Nacionnal da Universidade Federal do Rio de Janeiro (MN/UFRJ) and in the Forensic Entomology Lab of Instituto de Criminalística Carlos Éboli, da Polícia Civil do Rio de Janeiro. Adult Calliphoridae Diptera were attracted by the decomposing carcasses of domestic pigs (*Sus scrofa domesticus*) and captured by daily harvesting, starting on the second day after slaughter until the stage of animal skeletonization. The harvested insects were anesthetized for up to 90 s at -20°C (Estrada et al., 2009) and identified using a stereoscopic microscope and the identification key developed by Carvalho and Mello-Patiu (2008). After isolation and identification, the *C. albiceps* flies were transferred to cages with dimensions 45 cm × 45 cm × 45 cm, covered with nylon fabric, fitted with an entrance protected by a cotton cloth, closed by an elastic thread, in order to allow the handling of the specimens without their being able to escape, where they received water ad libitum and honey until further testing (Queiroz & Milward-de-Azevedo, 1991); these were offered in containers closed by a string, in absorbent fabric, in order to allow the solution to rise by capillarity (Byrd & Tomberlin, 2010).

Females of this fly species should be fed on putrefying meat to complete reproductive development and stimulate oviposition. Studies have shown that the volume of semen released in human ejaculation ranges between 2.0 and 7.0 mL, with an average of 4.5 mL (Guzick et al., 2001). Therefore, in we opted to use a volume of semen that was less than the average normally present in human ejaculation. For the tests, 3.0 mL of semen (from volunteer and anonymous donors) was added to and thoroughly mixed with 600 g of ground beef, which was equally divided into three parts (200 grams of beef and 1.0 mL semen each) and placed into three transparent plastic boxes with covered sides and a lid

(with a 4.0 cm^2 opening). The boxes were kept under reduced light conditions to simulate the natural environmental conditions of oviposition (Grassberger et al., 2003).

Subsequently, the boxes containing the eggs of *C. albiceps* were placed in an empty entomological cage, as described above (Queiroz & Milward-de-Azevedo, 1991) followed by harvesting 10 to 15 immature insects (larvae and/or pupae), taken from the boxes (3–5 individuals from each box) every 24 h. Throughout the duration of the experiment specimens were kept at 27 ºC +/- 3 ºC in a 12/12 Light/Dark cycle.

The harvesting of immature *C. albiceps* began when the first larvae appeared (first instar larvae) and continued until the eighth day of decomposition, that is, 192 h after oviposition. These specimens were preserved in flasks with 70% ethanol and identified by date and decomposition day. Decomposition beginning was the moment of oviposition in the meat boxes and was used as a baseline for harvesting every 24 h.

The collected maggots were surface washed with 70% ethanol and distilled water to remove any trace of semen that might have been adsorbed onto the external surface. Subsequently, the larvae were dissected for resection of internal structures, including the proximal portion of the intestine and the surrounding tissues. The dissection was performed consistently on the ventral region of all samples, in a posteroanterior direction, using a metal scalpel (Figure 1) using a metal forceps, a glass Petri dish, and a stereoscopic microscope. The harvested contents were preferentially that found in the first two thirds of the immature insect's body.

Figure 1. Dissection of one larvae of *C. albiceps*.

The visceral contents were placed in 2.0-mL microtubes containing 70% ethanol and stored until subsequent use in the molecular analyses. Between dissections, scalpels, forceps, and Petri dishes were washed sequentially with tap water, 70% ethanol, and distilled water.

DNA Extraction and STR Amplification

This phase of the investigation was carried out in the Institute for Research and Examination in Forensic Genetics of the Rio de Janeiro Civil Police. The reference sample, obtained from a semen donor, was subjected to extraction using Chelex 100® resin (Walsh et al., 1991). Samples from the gastrointestinal contents of immature insects, obtained from the entomological step of the investigation, were subjected to organic DNA extraction,

using the phenol-chloroform method (Baechtel, 1989) according to a standard protocol for extraction from soft tissues.

For quantification, we used 2 µL of the original sample resulting from final resuspension of the extraction (kept at -20°C), as well as 48 µL of ultrapure MilliQ water, which yielded a 1:25 dilution (one part of sample to 25 parts of water). Possible contamination with protein, phenol, or RNA was assessed using a spectrophotometer through the 260/280 and 260/230 ratios.

To determine DNA concentration, 50 µL of the aforementioned dilution was analyzed in a Gene Quant Spectrophotometer (GE Healthcare Life Sciences, USA). Dilutions were prepared until DNA concentrations reached values between 0.5 and 1.0 ng per µL of the sample. Y-STR DNA amplifications were performed using PCR technique, in a GeneAmp PCR System 9700® thermocycler (Life Technologies). Sixteen loci on the Y chromosome were tested: DYS456; DYS389 I; DYS390; DYS389 II, DYS458; DYS19; DYS385; DYS393; DYS391; DYS439; DYS635; DYS392; GATA H4; DYS437; DYS438; and DYS448.

The amplification products were analyzed by capillary electrophoresis, in the molecular analysis system ABI PRISM® 3100 Avant Genetic Analyzer (Life Technologies), using the ABI Prism 3100® Data Collection and GeneMapper v3.2 (Life Technologies) software, to detect human Y-STR polymorphisms. A haplotype investigation was performed, with the purpose of verifying the Y haplotype, using the Y-STR Haplotype Reference Database (YHRD) (http://www.yhrd.org), with a haplotype containing 16 loci as the reference.

The samples were collected and anonymized in accordance with the methods approved by the Ethics in Research Committee of Clementino Fraga Filho Hospital/UFRJ (CEP N° 536/10).

RESULTS

During the eight days of decomposition, ambient temperature varied between 23.5 and 34.8°C. The relative air humidity during this period varied between 56% and 86%. The harvesting of immature *C. albiceps* started after 48 h of decomposition (coinciding with the emergence of the first instar larvae) and continued until 192 h (or eight days) of decomposition. Ninety-six immature insects were harvested, consisting of 81 larvae and 15 pupae. The lowest number of immature insects harvested was 12, and the highest, 15 (Table 1).

Table 1. Number of larvae and pupae of *Chrysoma albiceps* harvested during the fly sample collection

DECOMPOSITION TIME (h)	NUMBER OF IMMATURES	
	Larvae	Pupae
48	15	0
72	14	0
96	14	0
120	15	0
144	13	0
168	8	4
192	2	11

The electropherograms demonstrated that there was amplification of Human Y-STR DNA at almost all stages of decomposition, varying between 5 and 11 amplified loci when

the samples were individually analyzed. The results from the combined samples show amplification of 15 of the 16 tested loci. The electropherograms of the samples from 96 h (fourth day of decomposition) and 168 h (seventh day of decomposition) showed no amplification.

An evident variation in amplification sensitivity of the various genetic loci was observed. The following loci were more successfully amplified: DYS393, DYS437, DYS389 I, DYS458, and DYS391. On the other hand, the less amplified loci included DYS389 II, DYS390, DYS385, DYS439, DYS635, and DYS392.

Table 2 shows the results of amplification of individual samples and the combined results compared to the reference haplotype. The amplified loci presented complete allelic compatibility with the haplotype of the reference sample donor, with DYS19 being the only locus that was not amplified in any of the samples.

Table 2. Y haplotype profiles amplified in the immature fly gut samples after 48 72, 120, 144, and 192 h of decomposition of the semen/ground beef samples.

GENETIC LOCI	SAMPLE ALLELES					
	DECOMPOSITION TIME				Investigated Haplotype	Reference Haplotype
	72h (Day 3)	120h (Day 5)	144h (Day 6)	192h (Day 8)		
DYS456	16	16	16	16	16	16
DYS389 I	14		14	14	14	14
DYS390	23			23	23	23
DYS389 II			30		30	30
DYS458	15		15	15	15	15
DYS385	15			15	15	15
DYS393	14	14	14	14	14	14
DYS391	10		10	10	10	10
DYS439		12			12	12
DYS635		22	22		22	22
DYS392	12			12	12	12
GATA H4	11		11	11	11	11
DYS437	15	15	15	15	15	15
DYS438		8	8	8	8	8
DYS448	20	20		20	20	20

DISCUSSION

In the *in vitro* analyses of immature (larvae and pupae) *C. albiceps*, almost all samples were successfully amplified. The exceptions might have resulted from the interference of contaminants and/or from uneven distribution of semen in the meat similar to Campobasso et al. (2005), who found good results from older larvae (third instar). Some results were obtained from samples in an advanced state of decomposition, which could be explained by the exposure of these larvae to larger amounts of semen, as they remained in contact with the oviposition substrate for a longer period.

The results of the spectrophotometric analyses using the 260/280 nm and 260/230 nm ratios show that only a few samples presented values higher than the purity limit, between 1.8 and 2.0, confirming the interference of proteins and phenol after a second washing with ethanol. The results of all the samples that were washed in ethanol only once demonstrated, without exception, considerable interference by proteins and phenol. In addition, the extracted DNA may display PCR inhibitors, similar to what was observed by Netto et al. (2015). Furthermore, a DNA amount much greater than the ideal was observed in the quantification tests, affirming the need for serial dilutions to achieve ideal values of around 0.5 and 1.0 ng DNA/µL before PCR amplification.

With the aim of improving ways to recover material evidence for forensic investigative purposes, the successful amplification of DNA from samples even in an advanced stage of decomposition validates this technique for criminal investigations, since, as the time after the criminal act progresses, less evidence becomes available for forensic experts and medical examiners. Forensic experience shows that there is a clear negative correlation between time elapsed after the crime and the amount of available evidence; that is, the longer the time after the homicide, the smaller the chances of finding any traces of evidence crucial to solving the crime that are specifically linked to the perpetrator. Therefore, recovery of human DNA from immature flies present on decomposing bodies may be a useful tool for criminal experts and medical examiners.

In a study performed over a decade ago on the stability of the prostate specific antigen (PSA) and genotyping of human Y-STR from immature *Lucilia sericata* (Diptera: Calliphoridae), Clery (2001) tested the amplifications of four Y-STR *loci* (DYS19, DYS389 I, DYS389 II, and DYS390) using a proportion of semen 4–12 times higher than that used in our study. Temperature was kept constant, which is unlikely to happen in a violent crime scene, and only two time-points were used to harvest the insects (48 h and 145 h). Positive results were observed only in the samples of material recovered at a very early stage of decomposition.

In contrast to Clery's (2001) study and with non-controlled temperature and humidity (a general rule for sexual crimes), 16 Y-STR loci were tested in our study, using a proportion of semen way below the average amount present in a normal human ejaculation, to test the possibility of detection of genetic material even in small amounts of semen. We highlight the fact that seven different decomposition time points (48, 72, 96, 120, 144, 168, and 192 h) were used, with successful amplification of up to 15 of the 16 tested loci.

Clearly, one of the main reasons for these satisfactory results, in comparison to those of Clery (2001), is the fact that the techniques and reagents have improved in recent years. For example, the amplification capacity of the kits currently used in laboratories for forensic DNA has improved and the capillary electrophoresis equipment has become more efficient. In addition, there are differences between the extractions techniques used in each of the studies Clery (2001) made an extraction only with Chelex.

The elaboration of a hypothetical haplotype profile using a combination of partial profiles is possible and is very likely in routine forensics. The reason for this is that mixed profiles were not detected in any of the experiments performed, and from a criminalistic point of view, the forensic expert present at the crime scene would harvest highly heterogeneous samples, with immature insects at different developmental stages, resulting in a mixture of samples. The same would hold true for the harvest of samples performed in the Departments and Institutes of Legal Medicine. In our study, samples were independently analyzed, and harvested at every 24 h of decomposition; however, in an actual criminal case, the victim may be found in a single state of decomposition, there would probably be only one harvest soon after discovery of the body, and the analyses would consequently be made using several immature insects at different developmental stages.

Even if the results are considered independently, the study is still relevant, since the individual results achieved for three samples (120, 144, and 192 h) showed amplification for 11 genetic loci each. It would already be possible to use these results as the minimal haplotype according to the most widely used international parameters, the European Minimal Haplotype (nine loci) and the Scientific Working Group on DNA Analysis

Methods (SWGDAM), which establishes the minimal haplotype as 11 loci (the sum of the previous 9 loci plus DYS438and DYS439) (Mulero et al., 2006, Thompson et al., 2013). These loci can also be used with the genetic databases that use CODIS (Combined DNA Index System), which are established in more than 60 countries, with more than 36 million catalogued genetic profiles, with the latest statistical data from July 2013 by the INTERPOL Global DNA Profiling Survey. Nowadays, of 190 member countries, 135 utilize DNA profiles in criminal investigations, including Brazil (Interpol, 2012).

According to some researchers, the amplification of Y-STR DNA originating from samples of rape victims, decomposed or not, should be a routine procedure in forensic laboratories, since it can detect male profiles present in smaller samples than those required for the amplification of autosomal STRs (Roewer, 2009, Sibille et al., 2012). Recent studies using the PowerPlex®Y23 kit (Promega) show high sensitivity for the amplification threshold of Y-STR loci. These studies managed to obtain complete Y-STR DNA profiles from samples containing 62.5 pg of male DNA mixed with 400 ng of female DNA. The same occurred when 125.0 pg male DNA was mixed with 3000 ng of female DNA (Thompson et al., 2013).

The Y chromosome is usually used in forensics to indicate the patrilineal lineage of an individual or the suspect of a crime, and it helps to guide the investigation and reduce the chance of errors, apart from the fact that it facilitates the release of innocent individuals. However, a few studies have suggested that, contrary to popular belief, significant differences may exist in some loci on the Y chromosome, namely rapidly mutating (RM) Y-STR, between men of the same patrilineal lineage. Among the discovered RM Y-STR regions, 13 correspond to loci that are already routinely used in forensic analyses of the human Y chromosome (Goedbloed et al., 2009).

With further development of studies using RM Y-STR, there could be a major change in the use of the Y chromosome, which could shift from an indicator of patrilineal lineage to a tool for individual identification of suspects of the same lineage (Ballantyne et al., 2010). In later studies, Ballantyne et al. (2010) corroborated the positive prospects of using these RM regions (which present high mutation rates on the Y chromosome), in forensic analyses, to aid the investigation of sexual crimes and identify the perpetrators, particularly in those more complex cases, in which two or more men who belong to the same patrilineal lineage may be suspects (Ballantyne et al., 2012).

The fact that only the DYS19 locus was not amplified in any of the samples corroborates the conclusions of a few studies that had been performed with the purpose of testing the sensitivity of amplification of genetic loci achieved with kits that are routinely used worldwide in forensic analyses. In those studies, it was observed that DYS19 was one of four loci with a higher rate of amplification failure (Maiquilla et al., 2011). According to these authors, success in the amplification of a particular locus is not significantly associated with the size of the tested amplicons. Ben Zakour et al. (2004) showed that one of the main factors responsible for differences in the amplification rate between genetic loci seems to be due to differences in the rate of prime hybridization of each locus.

The differences in the sensitivity of amplification between the loci tested in our study are consistent with the results of previous studies. In a study carried out in the Philippines with 154 female children victims of rape, it was observed that the DYS391, DYS393, and DYS437 loci were those with the highest amplification success, whereas the

DYS385, DYS392, and DYS428 loci were less amplified (Maiquilla et al., 2011), which is consistent with the results of our study.

CONCLUSIONS

The recovery and identification of human Y-STR DNA using immature Diptera is quite possible. The results of the analyses showed that Y-STR present in human genetic material could be amplified from the digestive system of immature (larvae and pupae) *C. albiceps*, under conditions similar to those of a sexual crime scene, with the victim in an early, moderate, or advanced state of decomposition.

The results indicate that under the experimental conditions of the present study, recovery of human Y-STR DNA samples from immature *C. albiceps* was possible up to eight days (192 h) of decomposition. The greatest number of loci amplified in a single harvesting time-point was 11, and when the samples were combined, 15 loci were amplified.

It is necessary to conduct further studies associated with this technique, with the aim of improving the quality of the final extraction product. In addition, *in situ* assays using animal models (for decomposition) should be performed to establish standard operational procedures for forensic investigations.

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