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Jason G. Linville,[1] *Ph.D.; Jack Hayes,*[2] *Ph.D.; and Jeffrey D. Wells,*[3] *Ph.D.*

Mitochondrial DNA and STR Analyses of Maggot Crop Contents: Effect of Specimen Preservation Technique*

ABSTRACT: DNA analysis of maggot crop contents can be used to identify a missing body or aid entomologists with interpreting evidence used for PMI estimations. Entomological evidence is often collected and preserved to keep identifiable external features intact. The preservation methods currently in use may not be suitable for preserving DNA in the maggot crop for later analysis. In this study, carrion maggots raised on human tissue were preserved under the following 8 preservation conditions: no fluid at −70°C, no fluid at 4°C, no fluid at 24°C, 70% ethanol at 4°C, 70% ethanol at 24°C, 95% ethanol at 24°C, Kahle's solution at 24°C and formaldehyde at 24°C. Maggots were dissected following 2 weeks, 8 weeks and 6 months of preservation. The maggot crops were extracted, human DNA was quantitated, and an attempt was made at amplifying mitochondrial DNA (mtDNA) and short tandem repeat (STR) loci. Both mtDNA and STRs were successfully amplified from maggots stored in ethanol or without any preservation fluid. Formalin-containing preservation solutions reduced the recovery of DNA. The best results were observed from maggots stored without any preservation fluid at −70°C.

KEYWORDS: forensic science, forensic entomology, postmortem interval, death investigation, Calliphoridae, *Calliphora vicina*, human DNA analysis

Insects and other arthropods are often collected from a corpse during a criminal investigation. This entomological evidence can be used to estimate the amount of time the victim has been dead, also known as the postmortem interval (PMI) (1,2). Since eggs are almost never deposited on a corpse before death, determining the age of immature insect specimens recovered from a corpse can be an accurate way to estimate the minimal PMI. The specimen's age is determined by comparing its size or state of development to published growth data from insects of the same species. This PMI estimation process relies heavily on the experience of the entomologist, and may only require a low-tech microscopic examination of the specimen. Therefore, investigators are encouraged to choose a preservation method that will keep the quality of identifiable features intact until the entomologist can examine the data (3,4). Specimens are often preserved at room temperature in alcohol or formalin-based solutions.

Recently, analysis of entomological evidence has moved beyond microscopic examination. Because identification of immature specimens can be difficult through visual examination, DNA analysis has been recruited to accomplish this task (5–7). Also, DNA analysis of maggot crop contents can be used to identify what a maggot has been feeding on (8–10). Crop content analysis may be useful in helping to identify a missing corpse that has been removed from a crime scene, or answering questions about whether a maggot has fed on multiple food sources (8).

[1] Department of Biology, University of Alabama at Birmingham, Birmingham, AL.
[2] Posthumous, formerly University of Texas at San Antonio, School of Public Health.
[3] Department of Biology, West Virginia University, Morgantown, WV.
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Preservation of entomological evidence becomes even more important if DNA analysis of the maggot crop is to be attempted, because some preservation methods suitable for storing maggots for microscopic examination may not be suitable for keeping DNA intact. As with other DNA-containing biological evidence, steps should be taken to prevent bacterial growth and the enzymatic degradation of DNA. Typical DNA evidence is stored under dry conditions at a low temperature (11). Storage of crop contents presents a unique situation because it is not practical for investigators to dissect, remove, and dry out the maggot's crop at the crime scene. Therefore, we investigated how some preservation methods currently used for entomological evidence affect the ability to recover DNA from maggot crops.

In this study, maggots fed on human tissue were preserved under eight separate combinations in various fluids at different temperatures. Maggots were dissected and crops were removed and extracted after being preserved for time periods of two, eight, and six months. Sequencing of mitochondrial DNA (mtDNA) and analysis of nuclear short tandem repeat (STR) loci were attempted on the crop extractions.

Materials and Methods

Maggot and Tissue Samples

All eggs were collected from a colony of *Calliphora vicina* Robineau-Desvoidy (Calliphoridae) flies on beef liver obtained from commercial source. Immediately following collection, eggs were transferred to a piece of human spleen in a rearing jar. The use of human tissue in this study was approved under UAB IRB Protocol X991104002. Maggots developed on the spleen at room temperature under a 24 h light source. The density of maggots was approximately 200 maggots per 70 g of tissue.

Maggots were collected after four days of development. At the time of collection, the maggots' crops were visible from the

exterior and near a maximum size. Five maggots were immediately killed and dissected (see below) to serve as a control. The remaining live maggots were divided into 24 groups of three maggots and each group was placed in a 1.5 mL tube. Each group was preserved under one of the following eight preservation conditions, chosen because either the published literature or our conversations with investigators indicated that they were the most common currently used (three groups per condition): no fluid at −70°C, at 4°C, at 24°C, 70% ethanol at 4°C, 70% ethanol at 24°C, 95% ethanol at 24°C, Kahle's solution (30 mL 95% ethanol, 12 mL formaldehyde, 4 mL glacial acetic acid and 60 mL water) (12) at 24°C and formaldehyde at 24°C. One group (three maggots) from each condition was removed for dissection after two weeks, eight weeks and six months of preservation. A second experimental block (an additional 72 larvae) was created by repeating the entire procedure beginning on a different date. The creation of additional treatments or replications was not possible because of the limited availability of human tissue.

Maggot Dissection and Crop Extraction

Before dissection, maggots were washed to remove potential external contaminants (9). Each maggot was individually soaked for 2 h in a 1.5 mL tube containing 1 mL of 20% bleach. The bleach was removed and each maggot was rinsed twice with 1 mL of distilled water.

Each clean maggot was dissected using the method described in Linville and Wells (9). Briefly, posterior segments were cut with iris scissors, then a ventral incision was made from the posterior to anterior end of the maggot. If possible, the crop was removed with forceps. Due to physical changes caused by prolonged exposure to preservation fluids and room temperature, some crops were difficult to remove (see discussion). Therefore, in these circumstances, either the entire anterior inside of the maggot was removed or the entire maggot was extracted.

A tissue sample (human spleen) and all maggot crops were extracted using Qiagen's Dneasy Tissue Kit (Valencia, CA) following the manufacturer's protocol for animal tissues. The crop extractions from block one maggots (three maggots per group) were quantitated using Applied Biosystems' Quantiblot Human DNA Quantitation Kit (Foster City, CA). Quantiblot data from block two maggots were not included since, unlike the block one samples, there were discrepancies among repeated quantitations from these maggots.

PCR and Sequencing

A segment of human hypervariable region II (HVII) of mitochondrial DNA was amplified using the polymerase chain reaction (PCR). The primer set used was F34 (5′-CACCCTATTAACCACTCACG-3′) and R370 (5′-CTGGTTAGGCTGGTGTTAGG-3′) (13). Amplifications were performed using Promega PCR Master Mix (Madison, WI) and the protocol for a 25 μL reaction volume. Each reaction included 1 μL of each primer (5 pmol/μL) and 5 μL of DNA extract. For the amplifications, the PCR program consisted of an initial denaturation cycle of 95°C for 3 min, 45°C for 1 min and 72°C for 1 min 30 s, then continued with 33 cycles of 94°C for 1 min, 45°C for 1 min and 72°C for 1 min 30 s, with a final extension at 72°C for 3 min 30 s. The success of PCR reactions was determined using an agarose yield gel stained with ethidium bromide.

Some amplifications were repeated if amplified DNA was present, but there was failure to obtain a sequence. These failures were associated with either a low amount of PCR product (indicated by a weak band in the ethidium bromide gel) or interference from non-specific amplified products resulting in a high background. These reactions were repeated using a different PCR program consisting of an initial denaturation step of 95°C for 2 min, then continued with 5 cycles of 94°C for 1 min, 62°C for 1 min and 72°C for 1 min 30 s, 10 cycles of 94°C for 1 min, 56°C for 1 min and 72°C for 1 min 30 s, and 24 cycles of 94°C for 1 min, 45°C for 1 min and 72°C for 1 min 30 s, with a final extension at 72°C for 3 min 30 s.

PCR product was cleaned using Qiagen's PCR Purification Kit (Valencia, CA). Cycle sequencing was performed with the F34 primer using Applied Biosystems' BigDye Terminator v3.0 Cycle Sequencing Ready Reaction kit and detected using an Applied Biosystems' 310 Genetic Analyzer (Foster City, CA). Sequences were aligned and edited using Sequence Navigator software (Applied Biosystems).

STR Analysis

STR analysis was attempted on all crop extractions that successfully produced a mtDNA sequence. Crop extractions were amplified using Promega's *GenePrint* PowerPlex 1.2 System (Madison, WI) according to the manufacturer's protocol. Amplified fragments were separated using an Applied Biosystems' 310 Genetic Analyzer (Foster City, CA). Data was analyzed using Genotyper Software (Applied Biosystems).

Results

Each control maggot produced a complete HVII haplotype and STR profile. Both the mtDNA haplotype and STR genotype matched those of the maggot's food source (human spleen).

Quantitation of crop extractions showed the amount of DNA recovered decreased over time for most preservation methods (Table 1). The Quantiblot results did provide an indication of whether subsequent analysis would be successful for individual maggots. In crop extractions that fell above the detection limit of 0.06 ng/μL, all samples produced the correct mtDNA haplotype and all but one sample produced the correct STR genotype. However, a DNA quantity below the detection limit did not necessarily mean the analyses would fail as mtDNA sequencing was successful in many, and STR analysis was successful in some of the crop extractions falling below the detection limit. Therefore, larvae from both blocks were included in the genotyping results.

Overall, the most frequent genotyping success resulted from maggots stored without preservation fluid at −70°C (Table 2). At 24°C

TABLE 1—*Quantiblot results for human DNA recovered from the maggot crops as a function of specimen preservation method and duration.*

Preservation Method		Quantiblot Results ng/μL (SD) $n = 3$		
Fluid	Temperature	2 Weeks	8 Weeks	6 Months
70% ethanol	24°C	0 ···	0.02 (0.03)	0.04 (0.08)
95% ethanol	24°C	0.5 (0.5)	0.2 (0.1)	0.2 (0.03)
None	24°C	0.2 (0.1)	0.02 (0.03)	0 ···
Kahle's	24°C	0.1 (0.05)	0 ···	0 ···
Formaldehyde	24°C	0 ···	0 ···	0 ···
None	−70°C	1.3 (0.3)	0.3 (0.3)	0.3 (0.1)
None	4°C	0.9 (0.2)	0.2 (0.1)	0 ···
70% ethanol	4°C	0.1 (0.04)	0.1 (0.1)	0.1 (0.1)

Control maggots = 1.4 ng/μL (0.6) $n = 5$.

TABLE 2—*Frequency of mtDNA sequencing and STR genotyping success from maggot crops as a function of specimen preservation method and duration.*

Preservation Method		Successful Results (# of maggots out of 6)					
		2 Weeks		8 Weeks		6 Months	
Fluid	Temperature	mtDNA	STR	mtDNA	STR	mtDNA	STR
70% Ethanol	24°C	6	2	6	3	6	2
95% Ethanol	24°C	6	2	6	3	6	3
None	24°C	6	6	6	4	4	0
Kahle's	24°C	6	4	0	0	0	0
Formaldehyde	24°C	1	0	1	0	0	0
None	−70°C	6	5	6	6	6	6
None	4°C	6	5	6	5	3	1
70% Ethanol	4°C	6	3	6	4	6	4

and 4°C, maggots stored in ethanol resulted in more frequent success over time than maggots stored in formalin solutions or without preservation fluid. Within each group of maggots, a mtDNA sequence was successfully obtained more often than a STR profile. This is expected as mtDNA results are typically easier to achieve due to the high copy number of mtDNA and protection by the organelle (14).

For maggots preserved at room temperature, ethanol solutions best preserved DNA for analysis using these protocols, while formaldehyde preserved DNA the worst (Table 2). All crops from maggots preserved in 95% and 70% ethanol were successfully sequenced at each time period, including the 6-month maximum. STR analysis was also successful in 42% of the maggots preserved in ethanol at room temperature. The duration of time stored in ethanol did not seem to greatly affect DNA recovery from the crops, as seen in both the Quantiblot and genotyping results. Maggots stored without any preservation fluid at room temperature became degraded over time. Although correct mtDNA haplotypes and some STR genotypes were obtained from these maggots after eight weeks of preservation, at six months sequencing was only partially successful and all STR analyses failed.

Preservation in formaldehyde and Kahle's solution inhibited DNA analysis. Storage in Kahle's allowed for mtDNA sequencing after two weeks, but prevented any STR analysis at this time. At eight weeks, the amount of DNA fell below the detection limit and both mtDNA and STR analyses were not possible. Formaldehyde reduced the DNA recovery to below the detection limit in all samples although some mtDNA amplification was still possible.

Storing maggots at 4°C as opposed to room temperature only slightly improved the preservation of DNA and success of subsequent analyses (Tables 1 and 2). However, for maggots preserved without any fluid at the extreme low of −70°C, almost all mtDNA sequencing and STR analyses were successful even after six months. Storage without preservation fluid at 4°C was similar to storage at 24°C with both having some loss of STR analysis at eight weeks and some loss of mtDNA sequencing at six months. Varying the temperature had similar results for maggots stored in 70% ethanol. Storage at 4°C in ethanol showed only slight improvement over storage at 24°C in ethanol.

Discussion

Several texts on the subject of collecting entomological evidence recommend preserving larvae in Kahle's solution (3,4). The formaldehyde-containing Kahle's solution is an excellent preservative and killing solution in that it stops the maggot's growth and prevents visible decomposition of the maggot. The preservative properties of both Kahle's and formaldehyde held true in this study as the external physical condition of preserved maggots was excellent, even after six months of storage. The internal structures of these maggots, including the crop, were also well preserved. Although the crop would occasionally break during removal, it remained solid, and the pieces were easily transferred to the extraction tube. However, DNA degradation has been reported in tissues preserved in formaldehyde (15). Formalin–fixation can also reduce the efficiency of DNA extraction from a tissue sample (16). These problems were apparent in formaldehyde and Kahle's preserved maggots as DNA recovery was reduced and amplification of DNA was less successful than in maggots stored without fluid.

Ethanol is often recommended as a preservation solution because of its ability to denature nucleases and dehydrate specimens (17). Although there was an initial decline in the quantity of DNA recovered when compared with control maggots, there appeared to be little further decline during the 6-month period. A similar observation was reported for ethanol-preserved samples of bear feces (18). In this study, even though DNA amplification was moderately successful throughout, the physical task of dissection and crop removal was more difficult in maggots preserved in ethanol. The crops were fragile upon removal and, in many cases, would appear to leak as the crop was removed. In some cases, it was necessary to scoop the entire crop out of the maggot using the iris scissors, with the crop found to have the consistency of a thick liquid. However, the slight variations in the dissection processes did not appear to have a direct effect on the recovery of DNA. Although some broken crops provided less DNA than other maggots in the same group, other broken crops provided an amount of DNA that was comparable to others in the same group (data not shown). This fact also held true for fragile crops from maggots preserved without any fluid.

Colder temperatures are often used as a preservation strategy as they help to reduce or eliminate bacterial growth and enzymatic activity. In maggots held at various temperatures, a cooler environment helped physical preservation as well as DNA preservation. Although there was not much improvement in maggots stored at 4°C compared with room temperature, maggots stored without preservation fluid at −70°C most closely resembled control maggots that were dissected immediately following their collection from the food source. Even after six months of preservation, the physical condition of these maggots was excellent and both mtDNA sequencing and STR analyses were successful.

Although formalin-based solutions may adequately preserve entomological evidence for visual examination, these solutions may permit the degradation of DNA inside the maggot crop over time and prevent the complete recovery of DNA from the specimen. Ethanol solutions may initially reduce the recovery of DNA from maggot crops, but there is not a further decline at longer storage times. Keeping maggots frozen at a temperature below refrigeration (4°C) will greatly improve the preservation of DNA in the maggot crop. However, if freezing is not possible, storage in ethanol is preferred over formalin-based solutions.

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Additional information and reprint requests:
Jeffrey D. Wells, Ph.D.
Department of Biology
West Virginia University
Morgantown, WV 26506
E-mail: jdwells@mail.wuv.edu