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TECHNICAL NOTE

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Surface Sterilization of a Maggot Using Bleach Does Not Interfere with Mitochondrial DNA Analysis of Crop Contents*

ABSTRACT: Mitochondrial DNA analysis of a maggot crop can identify the corpse a maggot has been feeding on. Analysis of the crop could be useful in a criminal investigation if maggots are found at a suspected crime scene in the absence of a body, or if there is a question of whether a maggot used in postmortem interval estimations moved onto the corpse from another food source. Such analysis can also resolve a chain of custody dispute if it has been suggested that larval samples have been switched. When recovering DNA from a maggot crop, the analyst must be careful to avoid external contamination. We investigated the effects of three simple wash methods developed to reduce external contamination. Maggots raised on pig liver and intentionally contaminated with cow blood were washed using water, 20% bleach, or a solution containing the enzyme DNase. Only washing the maggots in 20% bleach reduced the amount of vertebrate DNA amplified from the maggots' exteriors. No wash method affected the ability to recover DNA from the maggot crops.

KEYWORDS: forensic science, mitochondrial DNA, maggot crop

Recently Wells et al. identified several situations when analysis of a carrion-feeding maggot's crop would be useful in a forensic investigation (1). In carrion-feeding maggots, the crop is a food storage organ located at the anterior end of the gut. Mitochondrial DNA (mtDNA) recovered from the crop can be sequenced to determine who or what the maggot had been feeding on. This information could help investigators identify a missing victim if maggots are discovered at a suspected crime scene in the absence of a corpse. Maggot crop analysis also could provide a forensic entomologist with another way to associate a maggot with a victim when making a postmortem interval (PMI) estimation. If maggot age is to be used for PMI estimation, it is assumed that the insect specimen's entire development took place on the victim. Maggot crop analysis could reveal that the maggot had moved onto the victim from a different nearby food source. We are also aware of one case in which it was suggested that a forensic entomologist had returned the wrong sample to the police. The detection of the victim's DNA within a maggot's gut would resolve such a chain of custody dispute.

Vertebrate DNA has been successfully amplified and analyzed from several insect sources. DNA recovered from insect blood meals has been used to identify the species or individual identity of the host (2,3). The potential forensic utility of using DNA recovered from crab louse blood meals and excreta to identify the host has also been explored (4,5). Wells et al. were successful in sequencing human mtDNA from the crops of maggots raised on human tissue (1).

Just as with other biological samples such as hair or bone, when amplifying mtDNA from a maggot crop the forensic analyst must consider the risk of contamination (6). In maggot crop analysis, the analyst must be certain the extracted DNA is from the crop and not contaminant DNA from the maggot's exterior. In the case of identifying a missing victim, exterior contamination of human origin could lead to incorrect assumptions about the identity of the missing person. Exterior contamination would also interfere with making correct inferences about whether a maggot had been feeding on multiple food sources. We advocate DNA extraction of the crop rather than the entire maggot because careful removal of an internal structure preserves taxonomically important cuticular surface structures and because the crop contents are relatively undigested compared to those of the stomach or intestine.

Care taken during the dissection of the crop, including frequent sterilization of instruments, is clearly called for. However, given the relative impermeability of the maggot cuticle, we hoped that further precautions might be taken by destroying DNA on the external surface prior to dissection without degrading the crop contents.

Simple washing of the maggot's exterior could be sufficient to significantly reduce the risk of external contamination interfering with the crop analysis. The goal of finding an appropriate wash method is to identify one that will eliminate exterior DNA contam-

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ination without compromising the ability to recover DNA from the crop. In this study, we investigate three wash methods designed to reduce the amount of external contamination through physical, chemical, or enzymatic means. The exteriors of maggots raised on pig (*Sus scrofa)* liver were intentionally contaminated by soaking the maggots in cow (*Bos Taurus*) blood. The maggots were then washed by soaking them in water, 20% bleach, or a DNase solution. Before maggot dissection, the maggots were rinsed with water in order to detect any external contaminant that remained following the wash process. These exterior rinses were extracted and analyzed. The maggot crops were removed, extracted, and analyzed.

Materials and Methods

Maggot and Tissue Samples

Eggs were collected from a laboratory colony of *Cynomyopis* (= *Cynomya*) *cadaverina* (Calliphoridae) flies on a piece of pig liver obtained from a commercial source (Freemont Beef Co.). After four days of development at room temperature under a 24-h light source, a total of 27 maggots were collected. All collected maggots were approximately the same lengths (13 to 17 mm). At the time of collection, the maggots' crops were visible and near their maximum size (4 to 7 mm). Maggots were stored at −70°C until needed.

A small piece of the pig liver used as the food source was collected at the time of the maggot collection. Cow blood was collected from a commercial source (Freemont Beef Co.) to be used as the external contaminant.

Wash Method

The 27 maggots were divided into nine groups of three maggots. Five of these groups were intentionally contaminated by soaking the maggots in cow blood for 3 h. The remaining four groups of maggots were not intentionally contaminated and used as control groups. The treatment of the nine groups is summarized in Table 1.

Following the contamination period, all of the maggots were individually separated into sterile 1.5 mL microcentrifuge tubes. Two of the nine groups (one contaminated, one not contaminated) were treated with a DNase enzyme, two groups were soaked in 1 mL of 20% bleach, and two groups were soaked in 1 mL of distilled water. The remaining three groups were not washed. The maggots in water or 20% bleach solution were vortexed briefly, then left in their corresponding wash fluid overnight (19 h). The maggots that were treated with DNase solution were incubated for 30 min at 37°C in a solution of 450 μL H_2O, 50 μL DNase buffer, and 50 μL RQ1 RNase-Free DNase (Promega Corporation, Madison WI). Following this 30 min incubation period, 50 μL of stop solution were added to the Dnase-treated maggots, which were then incubated at 65°C for 10 min.

Following the wash period, most of the maggots were individually transferred into separate tubes containing 500 μL of distilled water and briefly vortexed. This exterior rinse of the maggot was performed to collect DNA still remaining on the outside of the maggot following the initial wash step. Only one group of intentionally contaminated maggots was not rinsed in order to represent untreated maggots during the dissection process.

Maggot Dissection

The maggot crops were removed in the following manner. The two or three most posterior segments of the maggot were cut off with iris scissors. This was followed with a ventral incision made from the posterior to the anterior end of the maggot. During this incision, the scissors must be kept just under the cuticle to prevent damaging the crop. Following the incision, the crop was exposed and removed with forceps (Fig. 1). After each maggot dissection, the instruments were wiped clean with distilled water, then flame sterilized. Each dissection was performed in a separate sterile Petri dish.

After the crop removal, each crop was placed into a separate 1.5 mL tube. All crops were approximately 4 to 7 mm in length. The remainder of each maggot was discarded. If the maggot was removed from a tube containing 500 μL water (rinse), the tube was recapped and saved for later extraction.

Crop Extractions and Exterior Rinse Extractions

All crops were extracted using Qiagen's DNeasy Tissue Kit (Valencia, CA) following the manufacturer's protocol for animal tissues. All of the 500 μL exterior rinses were first concentrated using Microcon YM-100 Centrifugal Filter Device (Millipore Corporation, Bedford, MA) and then extracted using Qiagen's DNeasy Tissue Kit and protocol for animal tissues.

The pig liver used as the food source and the cow blood used as the external contaminant were also extracted using the Qiagen kit.

PCR and Sequencing

A segment of cytochrome b was amplified from the food source, the blood contaminant, all crop extractions, and all exterior rinse extractions using a previously published and newly designed PCR primer. One of the primers, designed by Kocher et al. (7), is a highly conserved primer and is commonly used for amplifying cytochrome

TABLE 1—*Summary of experimental treatment and detection of vertebrate cytochrome b haplotypes for the maggots used in this study.*

Group*	Food Source	Crop Haplotype	External Contaminant	Wash Method	External DNA Haplotype
1	Pig Liver	Pig	Cow Blood	None	Not attempted
2	Pig Liver	Pig	None	None	None Detected
3	Pig Liver	Pig	Cow Blood	None	Cow
4	Pig Liver	Pig	None	Water	None Detected
5	Pig Liver	Pig	Cow Blood	Water	Cow
6	Pig Liver	Pig	None	20% bleach	None Detected
7	Pig Liver	Pig	Cow Blood	20% bleach	None Detected
8	Pig Liver	Pig	None	DNase	None Detected
9	Pig Liver	Pig	Cow Blood	DNase	Cow

* Each group consisted of three maggots.

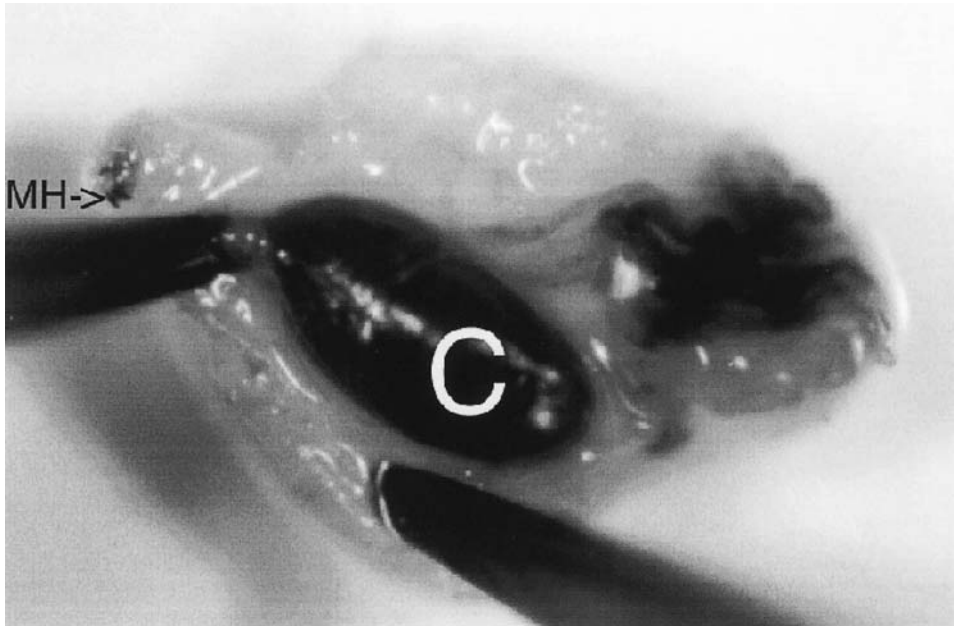

FIG. 1—*Crop of a* Cynomyopsis cadaverina *maggot prior to removal for DNA analysis. The larva has been cut along its longitudinal axis on the ventral side. The cuticle has been folded back exposing the viscera. Forceps are shown grasping the anterior end of the crop (C) where it connects to the remainder of the maggot gut. The anterior end of the maggot is in the upper left corner as indicated by the mouth hooks (MH).*

b from a variety of species including flies (5′-CCCTCAGAATGATATTTGTCCTCA-3′). The other primer was newly designed for this study and will amplify most vertebrate DNA but will not amplify fly DNA (5′-GACTAATGATATGAAAAACCAYCGTTGT-3′). Amplification reactions were performed using Promega (Madison, WI) PCR Master Mix and the protocol for a 25 μL reaction volume. For the amplifications, the PCR program consisted of an initial denaturation cycle of 95°C for 3 min, 60°C for 1 min, and 72°C for 1 min 30 s, then continued with 33 cycles of 94°C for 1 min, 60°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 from an agarose yield gel stained with ethidium bromide.

Successful PCR reactions were cleaned using Qiagen's PCR Purification Kit (Valencia, CA). Cycle sequencing was performed with the newly designed primer using the BigDye Terminator sequencing kit and detected using an Applied Biosystems (Foster City, CA) 310 Genetic Analyzer. Sequences were aligned and edited using Sequence Navigator software (Applied Biosystems). Confirmation of the cytochrome b gene followed translation to the amino acid sequence using Sequencher 3.1.1 software (Gene Codes Corporation, Ann Arbor, MI). Both the newly designed primer and the Kocher et al. primer were used to sequence contaminated, unwashed maggot crops in order to confirm a lack of a DNA mixture.

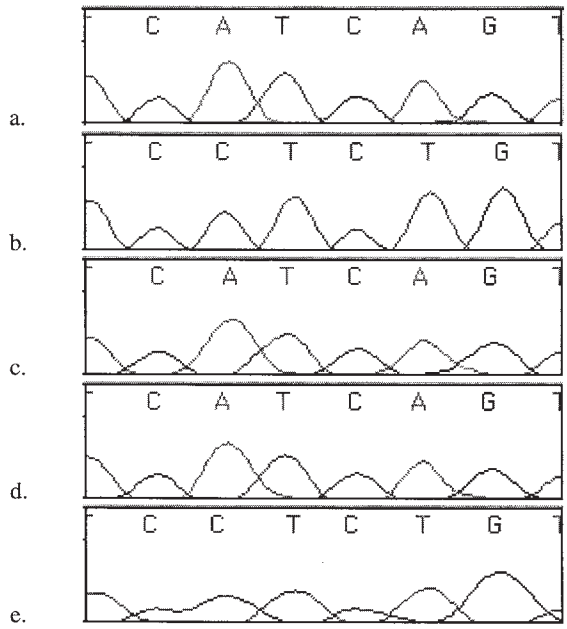

FIG. 2—*Electropherograms representing nucleotide positions 14704–14709 of cow* (Bos taurus) *mitochondrial DNA as numbered in Anderson et al. (8)* (a) *pig* (Sus scrofa) *liver used as the maggots food source in this study;* (b) *cow blood used as the external contaminant;* (c) *crop extraction of intentionally contaminated maggot that was not washed;* (d) *crop extraction of intentionally contaminated maggot that was washed in 20% bleach;* (e) *exterior rinse extraction of intentionally contaminated maggot that was not washed.*

Results

Crop Extractions

A comparison of the pig liver used as the food source and the cow blood used as the external contaminant revealed the samples had 71 base pair (bp) differences in the 423 bp analyzed region.

This cytochrome b fragment was successfully amplified from all of the maggot crop extractions. The type of wash method did not appear to have any effect on the quantity of the PCR product as all of the bands on the yield gel were of the same intensity. DNA sequencing of the crop extractions produced the genotype from the food source (pig liver) in all 27 of the samples (Table 1). A sample of the resulting electropherograms is shown in Fig. 2. There was no evidence of a mixture of DNA in any of the intentionally contaminated samples. Reverse sequencing performed on the crop extrac-

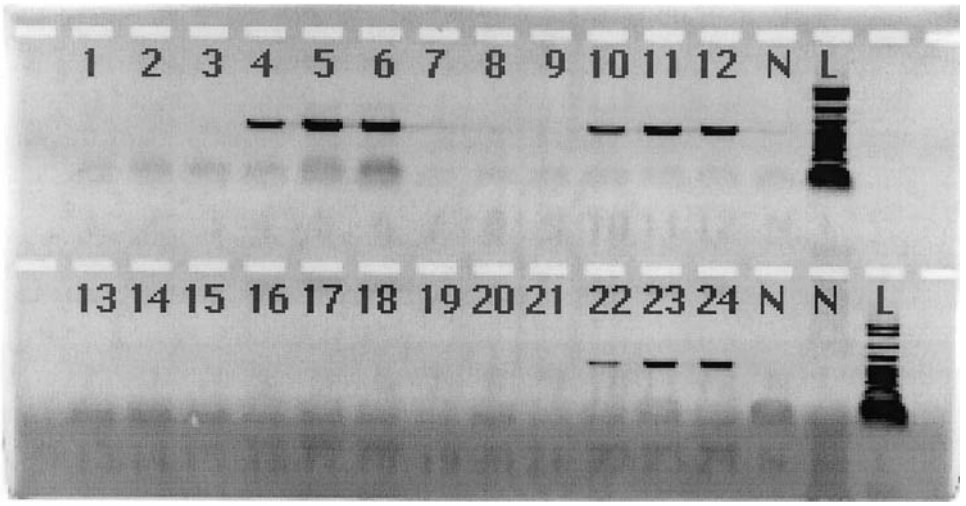

FIG. 3—*Yield gel of PCR reactions used to detect DNA rinsed from external surface of maggot following the wash procedures in this study. Lanes are from maggots that were: not contaminated, not washed to remove DNA prior to extraction (1–3); contaminated, not washed (4–6); not contaminated, washed in water (7–9); contaminated, washed in water (10–12); not contaminated, washed in bleach (13–15); contaminated, washed in bleach (16–18); not contaminated, treated with DNase (19–21); contaminated, treated with DNase (22–24). Gel includes negative controls (N) and ladders (L).*

tions of maggots that were not washed and not rinsed confirmed the lack of any DNA mixture in the crop extractions.

Exterior Rinse Extractions

The yield gel of PCR products from the exterior rinse extractions is shown in Fig. 3. External DNA was successfully amplified from the intentionally contaminated maggots that were not washed, washed only in water, or treated with DNase solution (Fig. 3, Lanes 4–6, 10–12, 22–24). Amplification of these exterior wash extractions produced strong bands in the yield gel. The DNA sequence of these amplified fragments matched the genotype of the cow blood used as the external contaminant (Table 1, Fig. 2).

DNA was not successfully amplified from the outside of intentionally contaminated maggots that had been washed with 20% bleach (Fig. 3, Lanes 16–18).

DNA was not successfully amplified from the outside of any maggots that had not been intentionally contaminated (Fig. 3).

Discussion

The intentional exterior contamination of the maggots did not interfere with the DNA analysis of the crop extractions. DNA sequencing revealed the DNA recovered from the crop was from one source, the food source, and not from the external contaminant. Even in heavily contaminated unwashed maggots, there was not a detectable mixture of DNA. Initially, this seems to suggest that there is not a need for washing the maggots. After all, if heavy external contamination doesn't interfere with the crop analysis of unwashed maggots, why bother washing them? There are two reasons why the analyst should consider a wash method. First of all, there can be variation in the dissection process itself. Even though the external contaminant didn't interfere in this experiment, this doesn't mean that it couldn't happen. Second, in this experiment we were dealing with "ideal" maggots. The maggots were collected when their crops were largest, and immediately preserved in a −70°C freezer. In real casework, the samples are likely to be less than ideal. We are currently evaluating our method with maggots that have smaller crops and that were preserved using other methods. In casework, when there may only be trace amounts of DNA in the maggot crop, the investigator should take steps to reduce the presence of external contamination.

A 20% bleach solution appears to be an effective method for sterilizing the external surface of a blow fly maggot without interfering with the analysis of mtDNA from the crop contents.

Since many forensic analysts are not familiar with the anatomy of a maggot, we have included a brief description of the dissection process. Depending on the steadiness of the analystís hand, this process is easy when the crop is large, but can become more difficult as the crop size decreases.

In this experiment, we used a newly developed primer specifically designed to amplify most vertebrate DNA, but not insect DNA. Boakye et al. successfully used universal cytochrome b primers to amplify vertebrate DNA from blood meals in the presence of insect DNA (2). However, when using universal primers, there is chance that a moderate amount of insect DNA in the extract may prevent the amplification of small amounts of vertebrate DNA. We felt it would be best to avoid this problem by developing a new primer set incapable of amplifying insect DNA. Our primer set utilizes one universal primer and another specific primer that anneals in the tRNA-glu region located next to cytochrome b gene in many vertebrates, but not in insects.

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