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Extraction and Detection of Domestic Pig DNA from *Sarcophaga carnaria* Linnaeus and *Chrysomya albiceps* Wiedemann Larval Crops Fed on Deceased Pig Remains

Mostafa I. Hassan[1]; Ahmed S. Bream[1]; Ahmed M. Galal El-Din[2], Mohammad R. K. Abdel-Samad[1]; and Eslam S. Metwaly[2]

1- Zoology and Entomology Department, Faculty of Science, Al-Azhar University, Nasr City, Cairo, Egypt,

2- Forensic Investigation Laboratory, Ministry of Interior, Abbassia, Cairo, Egypt

E-mail: mostafa012@gmail.com

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ABSTRACT

In order to investigate the possibility of detecting host DNA isolated from larval crops and the effect of time elapse during digestion, the present study was carried out. Domestic pig cadaver was used as a case study model instead of real human corpse tissue by collecting of 3rd instar larvae post-feeding and starving during24, 48, 72, and 96 hours. The identification of DNA extracted from pig tissues and from *Sarcophaga carnaria* Linnaeus and *Chrysomya albiceps* Wiedemann larval crops fed on deceased pig remains were carried out using PCR. The detection of DNA bands in the larvae of the two dipteran species was carried out by using Gel electrophoresis. Results showed that the extracted DNA from larval crops of the two fly species was identical to that extracted directly from domestic pig decaying tissues. In addition, the PCR specifically amplified the domestic pig mtDNA *cyt b* gene with 203 bp amplicons. Results also showed that DNA could be clearly detected within approximately 72h after the removal of larvae from pig corpse, suggesting reliable consecration of insects as valuable evidence in crime scenes. These results are useful for further consideration of the estimation of postmortem intervals (PMI) by the aid of forensic entomology.

INTRODUCTION

Forensic entomology, or the application of arthropod science to legal matters, has significantly increased owing to importance within the forensic sciences (Amendt *et al*., 2010; Byrd and Castner, 2010; and Brundage and Byrd, 2016). It is most commonly associated with the use of death investigations, although the scope of this science is broadly allowed for more than just its definition. Recently, more researches are carried out different researches in order to know the associated insect and arthropod evidences that can assist in the estimation of the postmortem interval (PMI) (Amendt *et al*., 2007; Byrd and Castner, 2010; Bugelli *et al*., 2015; and Brundage and Byrd, 2016). Insect fauna can also assist the detection and recognition of wounds, serve as indicators of perimortem and postmortem treatment of remains, and demonstrated neglect in both humans and animals (Anderson, 2004). The application of forensic entomology in cases involving animals is relatively

straightforward since the bulk of forensic entomological research has been carried out on animal models. This yields a large amount of data that can be used when associating insect species and their development time with animal remains. It is possible to answer questions about the circumstances of an animal's death by looking at decomposition and insect succession on the same animal species. This leads to a direct application of forensic entomological research to this particular discipline. The evaluation of entomological evidence at a scene has the potential to give investigators valuable information about the circumstances of animal death or neglect at different conditions. Insect evidence can be found in orifices, sores and wounds hence helping investigators in determining the events that may have taken place before death (location of injuries and wounds) (Sharma *et al*., 2015; and Njau *et al*., 2016). In addition, insect larvae found on human or animal corpses are considered as an important indicator for estimating corpse relocation due to human activity, especially steps taken to hide a cadaver (Byrd and Castner, 2009; Bala, 2015; and Charabidze *et al*., 2017). Under these circumstances, DNA detection in human or animal material ingested by the insect larvae could yield a genetic profile suitable for comparison to that of a corpse. So, for allocation of larvae to a specific corpse, the examination of the crop content with typing methods commonly used in DNA analysis such as STR typing or evaluation of a Hypervariable Region (HVR) within the mitochondrial d-loop sequence is reasonable. Because of the higher copy number of mtDNA in cells, it can be effectively isolated and employed from aged and degraded tissues (Monthatong and Thongchaitriwat, 2016). Human DNA could be successfully amplified and analyzed from several insect sources, according to the fact that, within the Dipteran larvae crops, the food is temporarily stored in the anterior portion into which proteolytic enzymes are not secreted. Under this situation, human mitochondrial DNA (mtDNA) analysis is preferably chosen. Therefore, the current study aimed at studying the possibility of obtaining DNA profiling for the animal through the examination of the crop content of founded insects through time-pass intervals.

MATERIALS AND METHODS

Larvae Samples Collection:

Samples of larval stages found on/in the inner tissues at different anatomical districts, around, and beneath both human and pig cadavers in decaying stage were collected. Dead samples (if any) were also removed and maintained in a domestic freezer (-20 °C). All collected specimens were then thoroughly cleaned with sterile water and/or 20% bleach solution before final preservation to assure the elimination of contaminant DNA on the external surface (Linville and wells, 2002). Taxonomic determination was carried out using current keys (Dillon and Dıllon, 1972; Hall and Townsend, 1977; White, 1985; Pape, 1996; Riberio and Carvello, 1998; Háva, 2004; Whitworth *et al*., 2007; Carvalho and Mello-Patiu, 2008; Almeida and Mise, 2009; and Dekeirsschieter *et al*., 2011). All taxonomic identification processes were carried out at the Museum of Entomology Department, Faculty of Science, Ain Shams University.

Pig Cadavers:

The present study was carried out at a garden of the Animal House, Zoology and Entomology Department, Faculty of Science, Al-Azhar University, Cairo, Egypt. A quarter of a domestic pig was purchased from a piggery farm at Manshiat Naser, Cairo, Egypt and weighed about 8 kg which was a suitable model for estimating decomposition stages (Catts and Goff, 1992).

The carcass was packed immediately after being killed in a heavy-duty polythene trash bag, and transported to the study site, and divided into two equal pieces. The first piece was transported into a cage and placed outdoor and wire mesh of 20 mm mesh size was used in the form of wire cage which secured with blocks around the carrion and

removed during larval collection. The Wire mesh was placed over the pig carcass to protect it from large vertebrate scavengers (Slone *et al*., 2005). The wire mesh used in the form of a wire cage was secured with blocks around the carrion. These were removed and set aside at every sampling time. The recorded temperature during the experiment was ranged from 29-33° C and R.H. of 60-65%.

The second piece was placed inside an insectary and subjected to *Chrysomya albiceps* Wiedemann and *Sarcophaga carnaria* Linnaeus larvae infestation at a temperature of 25 ± 2° C and R.H. of 65± 10%. The time of deposition was noted daily.

Pig DNA Recovery:

1. Sample Preparation:

Larval sample preparation was carried out following the recommendations by Kondakci *et al*. (2009). Immature larvae were removed from the substrate and placed in 70% alcohol for 10 sec. to remove impurities that may be on the surface of the individual. Subsequently, the dissection was carried out on an exemplary glass slide by extracting the cephalic part and the entire cephalopharyngeal skeleton using histological scalpel and tweezers. The remainder of the larva was squeezed and extracted viscera were placed individually into 1.5-ml microtubes containing one ml distilled water. After dissection, the samples were stored at 4°C. Each larva corresponds to a single sample being tested.

2. DNA Extraction:

Total DNA was extracted by the Phenol-Chloroform method, followed by ethanol precipitation (Russell and Sambrook, 2001). Each preserved larva was washed with sterilized water, cut into small pieces, and ground in a plastic grinder in a 1.5 ml microcentrifuge tube containing 300 µl extraction buffer (10 mM Tris, 0.5 M NaCl, 10 mM EDTA, 2 % SDS, and 200 µg/ml Proteinase). Then, the lysate was incubated in a water bath at 37 °C overnight. A volume of 20 µL of 100 mg/ml RNaseA was added, mixed well, and incubated at 65 °C for 45 min. One volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added to the sample, and vortexed thoroughly, before being centrifugated at 13,500 rpm for 15 min. The aqueous layer was removed to a new microcentrifuge tube, and DNA was precipitated with 500 µl of cold absolute ethanol and washed with 70 % (v/v) ethanol. The DNA pellet was air-dried and resuspended in 50 µl of TE buffer. Extracted DNA was quantified using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, Delaware, USA).

3. Amplification of Pig Partial mtDNA cyt b Gene by PCR:

The nucleotide sequence of the domestic pig *Sus scrofa domesticus cytochrome b* (*cyt b*) gene (Accession number: AP003428) was retrieved from GenBank and used for primer design using Primer3 (Rozen and Skaletsky, 2000). The PCR amplifications were conducted in a 50 µL volume containing 5 µL of 10x reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM from primer (**Table 1**), 1.5U *Taq* DNA polymerase (Fermentas, Germany) and approximately 100 ng mtDNA. The reaction was cycled for 1 min at 94°C, 1 min at an optimized annealing temperature that was determined for primer and 2 min at 72°C for 35 cycles. The PCR products were electrophoresed on 2% agarose gel stained with ethidium bromide to test the amplification success. PCR products were visualized by ethidium bromide-stained 1.5 % agarose gel electrophoresis. The PCR products were purified and sequenced by Macrogen Incorporation (Seoul, Korea).

Table (1). Sequences of primers used and amplification conditions

Gene	Primer sequences5´————————3´	PCR condition	Size of amplified Fragments
PG2	5'TCGCAGCCGTACATCTCATA3' 5'TGCTGGGGTGTAGTTGTCTG3'	95°C 1 min 57°C 1 min 72°C 1 min	203bp

Effect of Time Elapse on Recovered DNA Concentration:

To assess the effect of maggot post-feeding period on the probability of DNA detection by PCR, 100 well-fed larvae were collected randomly from human cadavers. From pig meat pieces, 100 well-fed larvae of both *Sarcophaga carnaria* Linnaeus and *Chrysomya albiceps* Wiedemann (from outdoor and indoor, respectively) were collected randomly. Of the collected larvae, 20 larvae were killed immediately and dissected for further DNA analysis (zero-time starvation).

The remained larvae were then divided into four groups; each with 20 larvae. Larvae were starved successively for 24, 48, 72, and 96 hours. Larvae were then removed for dissection, killed in near-boiling water and preserved in 70% ethanol prior to analysis. Previously mentioned steps for DNA analysis for both human and pig were then applied.

RESULTS AND DISCUSSION

Pig DNA Recovery from Larval Crops at Outdoor And Indoor:

1.DNA Quantification:

Quantification of DNA extracts from the larval crop showed that the recovered amount of DNA decreased over time compared to control. The highest DNA amount was obtained from samples obtained directly from the pig remains, but it was reduced due to nuclease activity. The ideal DNA concentration was obtained from maggot crop at zero-day starvation till the 2nd day starvation and they were used for generating STR profiles. The quantity of DNA reduced by time elapse due to the digestion process in maggot but the DNA still can be amplified. After 4-days starvation the DNA was barely detected as the amount was too low and can't be amplified (Table 2 and Fig. 2).In a previous study, the periods in which it is feasible to detect DNA from the gut contents of *Aldrichina grahami* blowfly larvae ranged from a maximum of 24 h at 32 °C to 42 h at 16 °C (Li *et al*., 2007). Furthermore, how a corpse decomposes, and the factors that may alter the rate of decay, is extremely important for host DNA quality and quantity (Monthatong and Thongchaitriwat, 2016).

Table (2): DNA concentration extracted from maggots' crops collected from decaying pig remains from both indoor and outdoor

Samples	**DNA concentration (ng/µl)**	
	S. carnaria	***C. albiceps***
From pig remains	150	150
From insect remains	233	235
Zero-day starvation	67.9	69.7
24h-Starvation	22.5	25.7
48h-Starvation	6.09	10.9
72h-Starvation	0.11	8.7
96h-Starvation	0.0001	0.0002

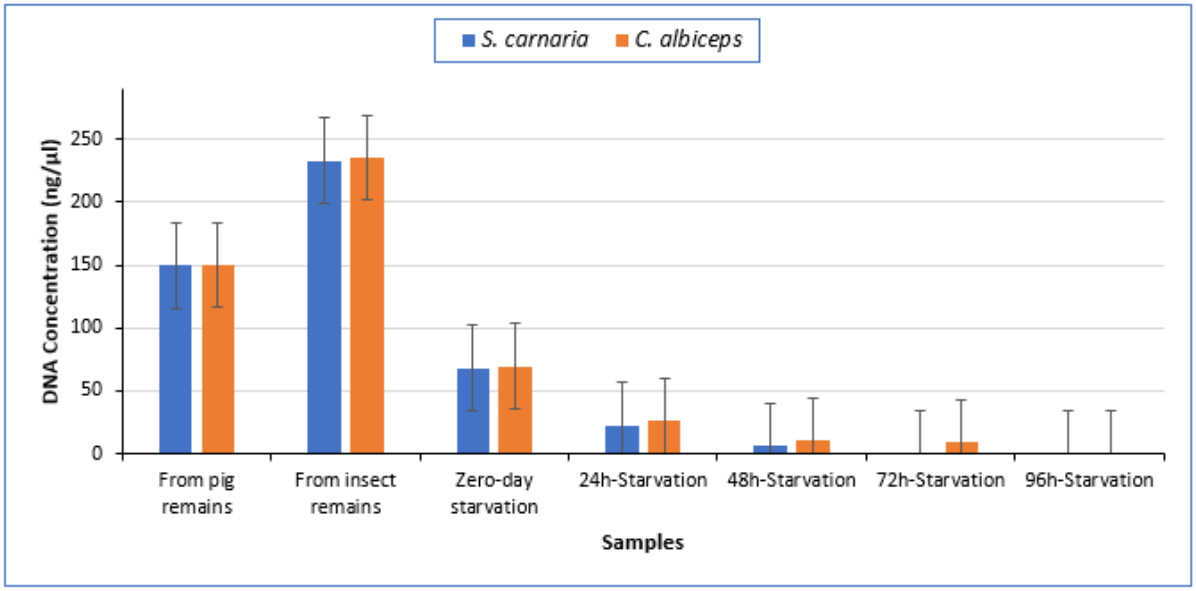

Fig. (2): Mean Pig DNA quantity from larval crops content at successive days interval

2. DNA Isolation and Detection:

The total DNA concentrations obtained from the pig meat (as positive control) and insects (as negative control) were 150 and 235 ng/ µL, respectively. The resultant fragment of the amplification of fresh and decaying pig meat was constantly observed at 203 bp. Because of the higher copy number of mtDNA in cells, it can be effectively isolated and employed from aged and degraded tissues (Foran, 2006). Human DNA could be successfully amplified and analyzed from several insect sources, according to the fact that, within the dipteran larvae crops, the food is temporarily stored in the anterior portion into which proteolytic enzymes are not secreted (Zehner *et al*., 2004). Under this situation, human mitochondrial DNA (mtDNA) analysis is preferably chosen. Determination of vertebrate and human usually relies on mitochondrial D-loop in the hypervariable region; however, *cytochrome b* (*cyt b*) and *cytochrome oxidase* subunits *I* and *II* (*COI* and *COII*) are also loci of choices for vertebrate and insect identifications, respectively (Campobasso *et al*., 2005).

The detection of pig *cyt b* fragments by PCR from the DNA obtained from *S. carnaria* larvae grown on decayed pork meats varied greatly, depending on the time elapsed after meat-feeding. The agarose gel electrophoresis photography showed that as the time elapsed the concentration of recovered DNA from insect crops decreased (Fig. 3). The highest DNA concentration was obtained at zero-day starvation, followed by 24h and 72h. Results also showed that after 72h starvation DNA concentration was greatly decreased and at 96h post starvation DNA fragments is barely detected.

In addition, the detection of pig *cyt b* fragments by PCR from the DNA obtained from *C. albiceps* larvae grown on decayed pig tissues varied greatly, depending on the time elapsed after tissues-feeding. The agarose gel electrophoresis photography showed that as the time elapsed the concentration of recovered DNA from insect crops decreased (Fig. 4). The highest DNA concentration was obtained at zero-day starvation, followed by 24h, 48h and 72h. Results also showed that after 48h and 72h starvation DNA concentration was greatly decreased and at 96h post starvation DNA fragments is barely detected.

Since the age of fly larvae is relevant to PMI, this estimation is based on the age of the larvae development and feeding on the victim (Linville *et al*., 2004). This is useful in cases where larvae are directly collected from or near a corpse which shows any signs

of decomposition. However, in certain circumstances, such as in the presence of live larvae at a crime scene without a dead body, the only alternative evidence which can be used for proving the identity of the victim is the food source left in the larvae crops (Wells *et al*., 2001 and Monthatong and Thongchaitriwat, 2016).

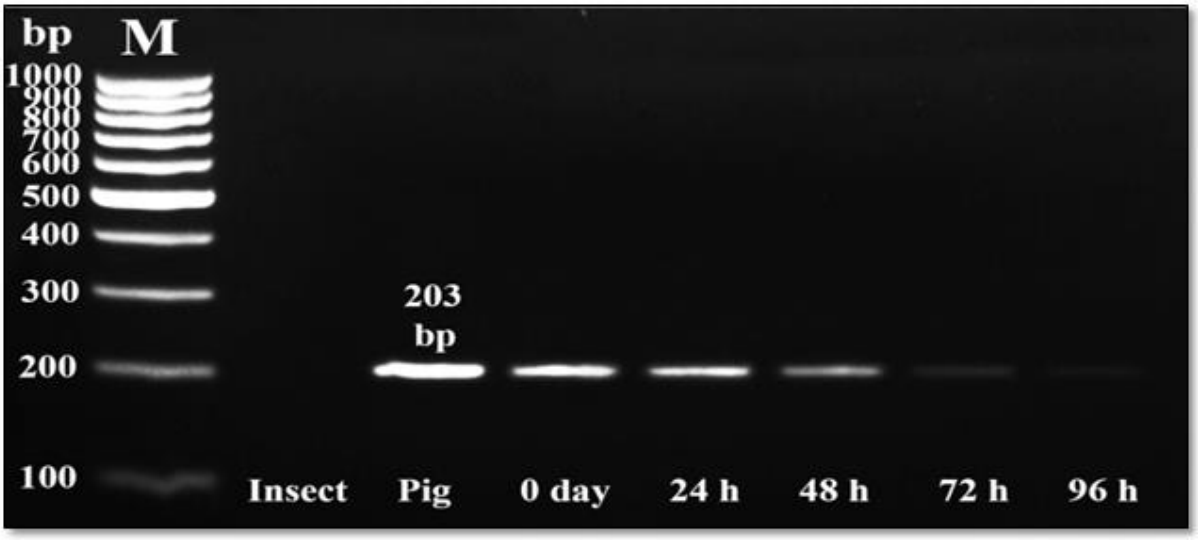

Fig. (3): Agarose gel electrophoresis showing pig-specific partial *cyt b* gene PCR products amplified from *S. carnaria* larval crops after being fed on pig meat at varying post-feeding periods (0-96 h). M= 1000 bp DNA ladder, Insect = negative control, and Pig = positive control.

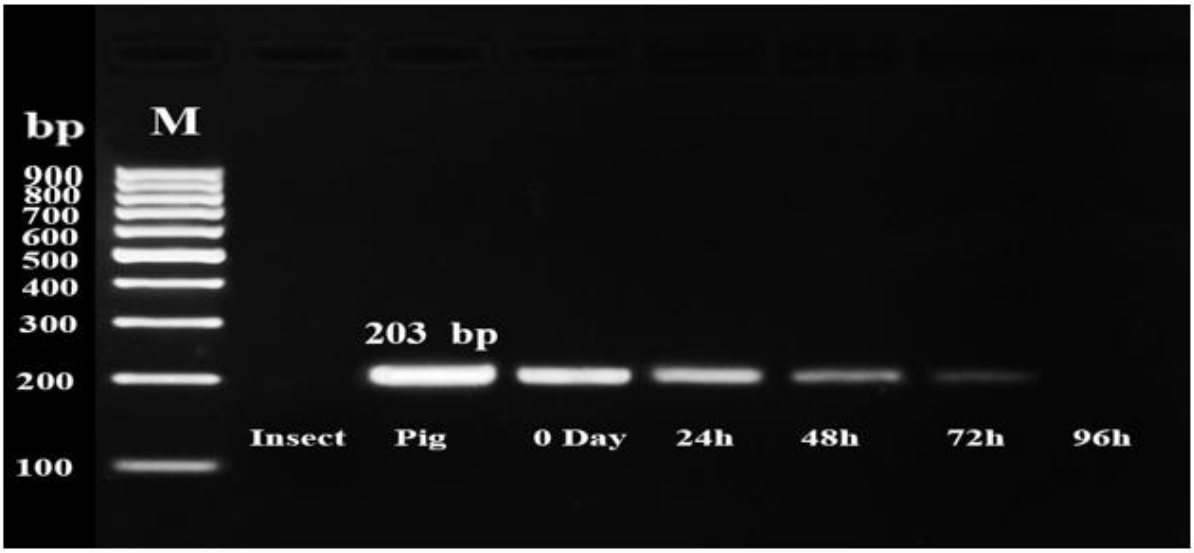

Fig. (4): Agarose gel electrophoresis showing swine-specific partial cyt b gene PCR products amplified from *C. albiceps* larval crops after being fed on pig meat at varying post-feeding periods (0-96 h). M= 1000 bp DNA ladder, Insect = negative control, and Pig = positive control.

Conclusion:

According to the obtained results in the current study, the DNA analysis from maggots represents an excellent alternate in the identification of corpse from which maggots have been feeding on. Starved maggots represent ideal evidence in the identification of decomposing bodies and are only useful in body identification up to the third day if they are removed from the food

source. In addition, results of the current study showed that the recovered DNA from maggot crops decreased with time due to further digestion process occurs in maggot crop. These results are useful for further consideration of the estimation of PMI by forensic entomology. So, it is recommended to analyze the recovered DNA in a maximum of three days after collecting maggots either from the decomposed body or from the crime scene.

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