Title: Impact of sample degradation and inhibition on field-based DNA identification of human remains

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Abstract

The advent of DNA technologies for field-based application promises to provide rapid intelligence to aid investigations. Their validation and adoption by enforcement groups have demonstrated utility in sample screening and prioritisation, but field application in some areas of forensic science, such as human remains identification, is little evidenced. Assessing the ability of such approaches to provide meaningful data is critical as decomposition is likely to complicate analysis and limit the effective use of such field-based DNA interventions. This research assessed the ability to collect viable DNA data in the field using the ParaDNA Field Instrument and Intelligence Test chemistry. Different sample collection methods were assessed; direct from skin surface; direct from exposed tissue; indirect from muscle swab transferred to FTA card; and from larvae on the donors. Samples were collected and processed on-site at the Anthropology Research Facility, University of Tennessee. The data show that the muscle tissue provided the most effective sample template and, using this approach, it was possible to generate STR profiles from human remains in under two hours from the time of sample collection. STR profile data were collected up to four days from donor placement (114 Accumulated Degree Days). After this time there was a rapid decrease in the quality of the profiles collected due to the onset of decomposition. The data also show that effective sample recovery was not possible from the surface of the skin, exposed tissue or from carrion larvae. Inhibition studies in the laboratory suggest that by-products of the decomposition process are the primary mode of failure. Together these data suggests a possible application for screening and prioritisation in criminal casework but highlights issues that may affect the success of the approach.

Key words: Field based PCR; DNA; degradation; inhibition; direct PCR; human remains

Introduction.

The advent of non-laboratory-based DNA analysis promises to increase the speed in which an STR profile is generated and searched against a database, compared to reference samples and used for sample prioritisation [1]. In doing so, rapid investigative intelligence is provided to law enforcement agencies which may allow better casework management [2]. These devices have been validated for use by crime scene investigators and police officers and are undergoing adoption by many global enforcement groups [3-5]. The speed and ease-of-use in which these systems provide an identification result is of obvious benefit to criminal investigations. It is also important to recognise that rapid identification in a civil investigation (e.g. a natural disaster) or military investigation (e.g. remains collection and repatriation) may also be useful. Questions about the utility (accuracy, reproducibility likelihood of database match) of each system have been answered using mock forensic evidence items, such as blood, semen, and saliva swabs [6], but there is little evidence supporting field application on decomposing human remains.

When discovered, human remains have been traditionally categorised as falling into one of the following five decomposition stages [7]; fresh (beginning at the point of death); bloat (beginning when the corpse begins to inflate due to gasses created by anaerobic bacteria in the abdomen); active decay (starting when the carcass deflates due to invertebrate feeding with liquefaction beginning); advanced decay (when most of the flesh has been removed); and dry remains (mainly bones remaining). The propose of such categorisation is to help estimate Post Mortem Interval (PMI) and the rate of change between stages is dependent on a number of factors which can vary considerably between environments such as the ambient temperature [8], body mass, humidity [9], insect activity [10], scavenging [11, 12] and the presence of micro-organisms [13]. Another method used to estimate PMI is to calculate the Total Body Score (TBS) [14]. This method divides decomposition into four broad categories: fresh, early decay, late decay, and skeletal, with each category sub-divided into point-valued stages. Remains are assessed and those with a higher TBS are more decomposed. The TBS can be converted to Accumulated Degree Days (ADD), a measure of heat-energy that represent the accumulation of thermal energy in a system, which represents chronological time and temperature combined [15]. Research has

shown that ADD contributes to nearly 80% of the variation observed in the decomposition process [14] and can be used to normalise between different environments and experimental observations.

Further variables associated with successful human remains identification include the biological sample type available for analysis. Samples typically collected for human remains identification include blood or buccal swabs (non-invasive approaches), but can also include deep muscle/organ tissue, bone and teeth (invasive approaches) when decomposition is advanced [16, 17]. Invasive sampling techniques often provide the most DNA for analysis and are typically collected once the remains have been recovered and removed from the site. However, non-invasive collection is important as, wherever possible, evidence should be preserved in the state it is found. Handling or physical manipulation of the sample should be minimised to prevent further sample destruction and contamination. Finally, a non-invasive sampling approach minimises exposure to possible bio-hazards which would safeguard the crime scene, enforcement officers and other practitioners on the ground.

Assessing the merits of common sample recovery approaches in conjunction with field-based DNA profiling is the first step in understanding whether such methods can support investigators working with human remains. This research project looks to assess the utility of the ParaDNA Field Unit and Intelligence Test assay for generating rapid, usable data collected from human remains in the field, at different stages of decomposition represented by ADD scores. Different sample recovery approaches were tested and the impact of each approach on the data quality were assessed.

Methods.

Donor Information and Field Site set-up

Recovery of cellular material from human remains occurred at the Anthropology Research Facility operated by the Forensic Anthropology Center (FAC) at the University of Tennessee over two, week-long, periods – once in spring 2017 and again in summer 2017. Local weather conditions were recorded daily across both field sessions using data collected by the Federal Aviation Administration from McGhee Tyson airport, TN (Latitude: 35.811, Longitude: -83.994) [18]. Accumulated Degree Day

(ADD) scores were obtained by summing the average daily temperate (Celsius) using 0°C as the baseline to provide a single ADD score for the day of collection (Table 1). This allowed for some normalisation between datasets collected at different time points and in different geographical locations. Donors received by the FAC were collected following approved ethical guidelines and this study was ethically approved by both the University of Tennessee and also Liverpool John Moores University. In total, 10 donors were included in the research (eight in spring and two in summer) and were selected based on availability at the time of each study.

The collection of biological samples followed by on-site DNA analysis followed a methodology designed to minimise possible contamination to human remains. Items needed for collecting tissue included swabs, sample collectors, indicating FTA cards, forceps, scalpels, sharps-bin, plastic storage box and marker pen. Personal Protective Equipment (PPE) included safety glasses, facemasks and overshoes. The test chemistry was removed from a laboratory freezer each morning and held in an insulated cool-bag containing freezer packs for the day, approximately 6 hours of sampling time. All equipment (Figure 1a) was stored in a rucksack that was carried from donor to donor. Once enough samples were collected for analysis they were taken to a centralised rainproof shelter. This acted as the traditional Post-PCR environment and contained the DNA instrument, disinfectant wipes, laboratory gloves, small zip-lock bags and large waste bag (Figure 1b). Waste, including assay plates, gloves, and wipes, was removed from the field site at the end of each day. After samples were loaded into the ParaDNA Field instrument, gloves were changed, and sampling resumed. The shelter remained in place for the five-day study period. The DNA instrument was taken to the shelter at the start of each day having been re-charged off-site overnight.

Table 1. *Sample data generated at University of Tennessee detailing four collection methods.*

Donor ID	Placement Date	Sampling Method	Area of collection	Sampling Date	ADD for day of sampling	DNA Score (%)	Alleles recovered (%)
HR1	25.4.17	Indirect from swab	gums	1.5.17	147	0	0
		Direct from donor	gums			0	0
		Direct from donor	skin surface (calf)	4.5.17	202	0	0
		Direct from donor	skin surface (calf)	5.5.17	213	5	0
HR2	28.4.17	Indirect from swab	skin surface (flank)	1.5.17	89	0	0
		Direct from donor	skin surface (flank)			0	0
		Direct from donor	skin surface (ankle)	4.5.17	144	18	0
HR3	28.4.17	Indirect from swab	skin surface (armpit)	1.5.17	89	1	0
		Direct from donor	skin surface (armpit)			8	0
		Direct from donor	exposed tissue (skull)	4.5.17	144	2	0
HR4	28.4.17	Indirect from swab	exposed tissue (calf)	1.5.17	89	0	0
		Direct from donor	exposed tissue (calf)			0	0
		Direct from donor	exposed tissue (lower leg)	4.5.17	144	0	0
		Direct from donor	exposed tissue (forearm)	5.5.17	155	5	0
HR5	26.4.17	Direct from donor	skin surface (calf)	1.5.17	130	2	0
				2.5.17	148	2	0
				5.5.17	196	2	0
HR6	26.4.16	Direct from donor	skin surface (shoulder)	1.5.17	130	87	92
				2.5.17	148	53	75
				5.5.17	196	4	0
HR7	26.4.17	Direct from donor	exposed tissue (skull)	1.5.17	130	0	0
				2.5.17	148	0	0
				5.5.17	196	0	0
HR8	26.4.17	Direct from donor	exposed tissue (forearm)	1.5.17	130	1	0
				2.5.17	148	4	0
				5.5.17	196	0	0
HR9	23.6.17	Indirect from larvae on FTA	Upper torso	26.6.17	93	0	0
						4	0
						0	0
				28.6.17	135	0	0
						0	0
						0	0
HR10	23.6.17	Indirect from muscle swab on FTA card	R-Upper Thigh	26.6.17	93	67	83
						73	75
						69	92
			L-Upper Thigh	27.6.17	114	76	83
						70	92
						66	92
			R-Calf	28.6.17	135	24	0
						16	0
						20	58
			L-Calf	29.6.17	159	0	0
						0	0
						0	0
			L-Calf	30.6.17	183	0	0
						0	0
						0	0
HR11	26.6.17	Indirect from muscle swab on FTA card	L-Upper Thigh	26.6.17	19	57	92
						38	67
						56	92
			R-Upper Thigh	27.6.17	40	54	75
						57	83
						65	75
			R-Calf	28.6.17	61	50	75
						68	100
						56	92
			R-Hand	29.6.17	85	72	92
						60	100
						65	83
			R-Foot	30.6.17	109	1	0
						0	0
						7	0

ADD values calculated from date of placement. Single results (n=1) are provided for DNA Score (calculated following [19]) and the allele recovery rate (determined as a percent of the maximum 12 alleles).

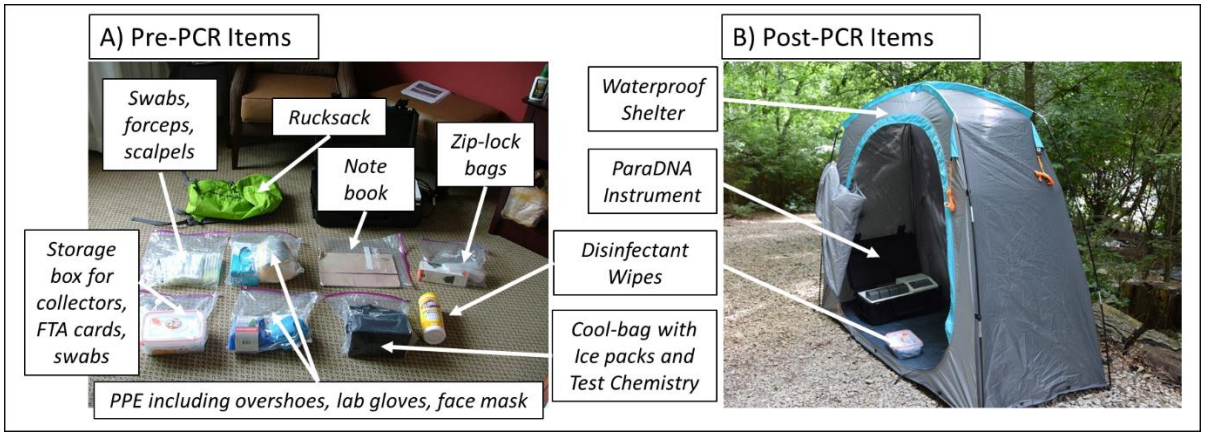

Figure 1. *Equipment required for field-based DNA analysis. Items identified as Pre-PCR (A) were taken between samples. Anything being transferred to the Post-PCR Shelter (B) was not removed until the end of the sampling period.*

Optimisation and Assessment of Collection Methods

Two non-invasive methods were initially assessed on four donor samples (HR1-4) in spring (Table 1). An indirect collection method was trialled where an area of the donor was swabbed using a wetted rayon swab (Thermo Scientific, Sterilin, F155CA) (Figure 2a) before recovering any material from the moist swab using the ParaDNA Sample Collector for approximately 30 seconds (n=4). This closely follows the process of collection outlined by the developers of the approach [19]. A direct collection method was also trialled where the same area of the donor was targeted but using the Sample Collector directly (Figure 2b) for 30 seconds (n=4). Given the rapidity of the data generation, assessment of the samples on-site, and the manufacturer's advice, the direct sampling approach was considered more likely to be effective at collecting biological material and two direct treatments were then compared; direct from skin surface (n=9) and direct from tissue exposed by scavengers (n=9). Sampling using these treatments was performed across five days. After consultation with practitioner groups, two additional collection methods were trialled in the summer; indirect invasive muscle swab (Figure 2c) and indirect non-invasive larvae collection (Figure 2d). For muscle collection, a 5-10 cm incision was made using a sterile disposable scalpel. New incision sites were selected daily in tissue showing the least visible decomposition. The muscle tissue was cross hatched with the scalpel and swabbed with a dry cotton swab for 30 seconds. The swab was then firmly pressed onto an indicating FTA card (GE Healthcare, Whatman, WB120412) and left to dry in a small paper bag containing silica gel desiccant for 30 minutes

(n=30). Larvae samples were collected directly from the donor's remains and placed in a screw top 50 ml plastic collection tube. The samples were frozen and transferred to a 1.5ml micro-centrifuge tube containing 75 µl of PCR grade water before being pulped using micro-pestles (Eppendorf, 0030120973). The solution was then transferred to an FTA card and left to dry in a paper bag containing silica gel for 1 hour before sampling (n=6). When recovering material from FTA cards, the ParaDNA Sample Collector was scratched against the sample deposition site on the FTA card for 30 seconds to recover FTA fibres before being transferred to the PCR assay.

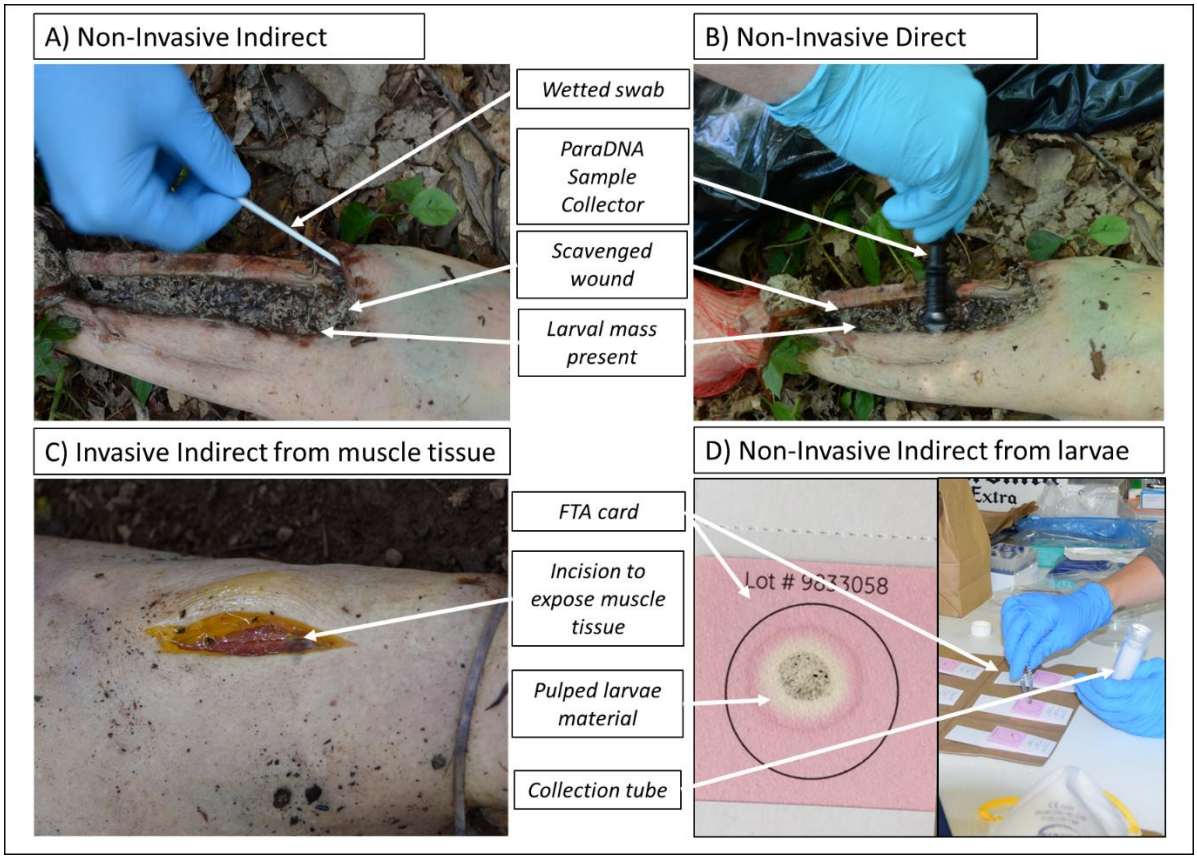

Figure 2. *Non-invasive indirect methods based on subsampling from a swab (a) and non-invasive direct methods based on sampling from the donor using the Sample Collector (b) were initially attempted. The latter was further divided into direct from skin surface and direct from scavenged wound. Further sampling included invasive indirect from muscle swab transferred to FTA card (c) and pulped larvae transferred to FTA card (d).*

Field-based DNA Analysis

Biological material collected from each donor was analysed using ParaDNA® Intelligence Test Chemistry (LGC, PARA-070) [6] with 8-16 independent samples run each day (Table 1). The test provided a rapid presumptive identification of an individual based on five Short Tandem Repeat (STR)

markers (D3S1358, D8S119, D16S539, D18S1358, TH01) and a male/female identification result based on the Amelogenin gene. All samples underwent amplification on a ParaDNA Field Portable Instrument (LGC, PARA-020). Automatic software analysis (Intelligence Version 1.1) was performed which provided allele calls for the five STR markers, the sex identification and a DNA 'Detection Score' representing a relative measure of DNA quantity and quality detailed in [19].

Laboratory-based assessment of DNA amplification success and inhibition

The impact of sample inhibition on DNA amplification was assessed by first spiking purified human DNA into the Intelligence Test chemistry (final assay concentration at 2ng per well) effectively making a positive control plate. Inhibition may be in the form of nucleases, proteases and lipids released during decomposition, from biological inhibitors associated with the larvae and also any chemical reagents on the FTA cards. Decomposition inhibitors were re-created in the field in the UK by leaving pork belly to decompose outdoors to similar ADDs to those measured in Tennessee. Material was then recovered from the skin surface using the Sample Collector (simulating the direct skin collection), or from the flank of the pork belly (simulating the exposed tissue collection). The use of a non-human proxy was necessary to prevent co-amplification with the spiked purified DNA in the human specific ParaDNA test. Inhibition from larvae was tested by processing larvae collected from the pork tissue in the same manner as described above. Inhibition from FTA cards was tested by processing blank FTA cards. Six replicates were analysed for each inhibition treatment.

Results and Discussion

The period of sampling in both spring and summer saw the donors undergo rapid decomposition with most samples collected at the 'fresh' and 'early decomposition' stages, up to about 213 ADD [20]. Some of the donors were entering advanced decomposition during the sampling process, typified by moist decomposition with bone exposure which can occur between 234 and 546 ADD. No samples were collected when skeletonized which can occur between 657 and 5500 ADD [20]. Comparison of approaches clearly shows that the muscle incision approach provided the highest median DNA Detection Score of 55% (Figure 3) suggesting that this approach yields the highest amount and/or best

quality of DNA. The collection of forensic samples using FTA cards is common in casework applications and it has been shown to work with direct PCR approaches [21].

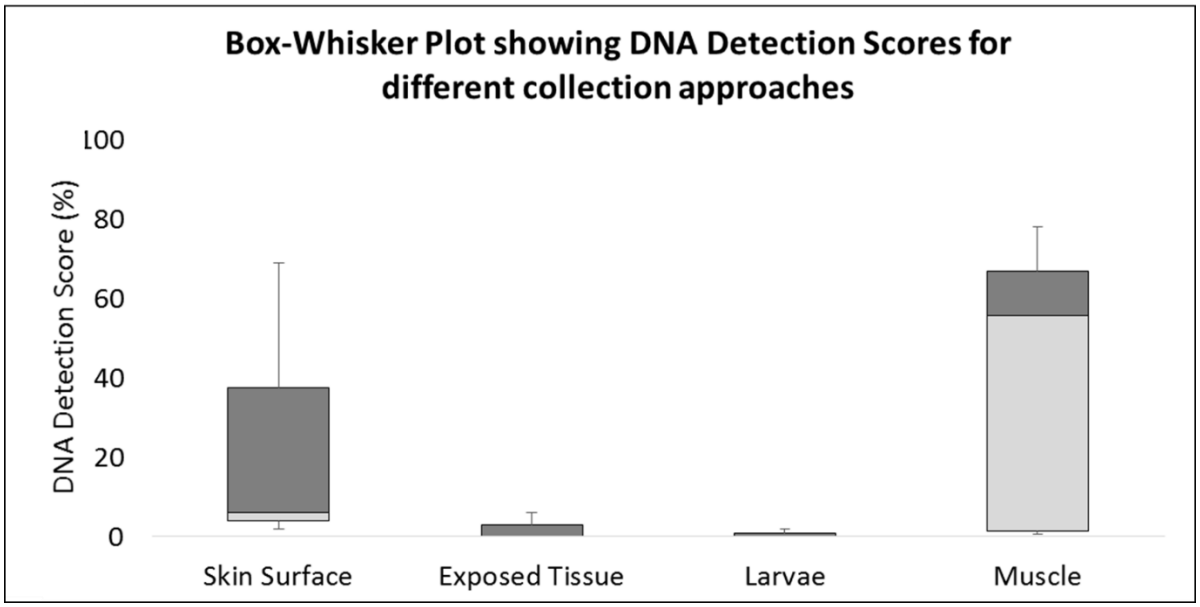

Figure 3. *Box whisker plot showing DNA Detection Scores (%) generated by ParaDNA Intelligence Test. Each plot shows the second quartile (light grey) and third quartile (dark grey) with the first and fourth interquartile ranges provided by the error bars. The median value is the line separating the second and third quartiles.*

DNA amplification was also shown to be possible when collecting direct from the skin surface giving a median DNA score of 4% (Figure 3). This is considered largely due to a single sample (HR6) amplifying in two instances (Table 1). When collecting samples from this donor it was noted that the donor exhibited some blistering on the skin surface which appeared to contain blood. As such, the sampling action caused the blisters to rupture meaning that the blood-based material was collected as well as skin (author's personal observation). If these two samples are removed from the data in Figure 3 then both skin surface and exposed tissue data are broadly equal. The data also show reduced amplification in the exposed tissue samples with a median DNA score of 0% (Figure 3) which is unexpected given that muscle tissue and suspected blood were visually observed at the time of collection. Collection of human material from carrion fly larvae also failed to yield results in the field (median DNA Score of 0%), although amplification was observed in one single sample (Figure 3). Possible explanations for the poor results observed in the skin, exposed tissue and larvae treatments include low template recovery using the Sample Collector, DNA degradation and/or PCR inhibition.

Previous research using the plastic sample collector has been shown to be successful at collecting material from a variety of different surfaces [19] and images of the sample collector taken immediately after the sampling clearly shows biological material is being recovered (authors personal observation). Also, sample collection direct from fish tissue (raw and cooked) has been shown to work [22] which would suggest that the plastic collection device is capable of lifting biological material. DNA fragmentation resulting from the release of cellular nucleases [23] is considered a more likely explanation. The destruction of high molecular weight DNA as ADD increases will create a smaller pool of potential DNA targets for amplification during PCR, and can be seen in the data collected from the muscle tissue which shows a reduction in the amount of DNA amplified as ADD increases (Figure 4). This can be combatted by using PCR primers designed to amplify short DNA fragments, resulting in greater PCR efficiency and increased success when profiling degraded and inhibited samples [24, 25]. The size of the PCR amplicons generated using the ParaDNA Intelligence Test cannot be ascertained as the system uses a melt curve detection approach to differentiate between alleles and the primer sequences are not in the public domain. As such it is not known to what extent amplicon length may be affecting the observed results.

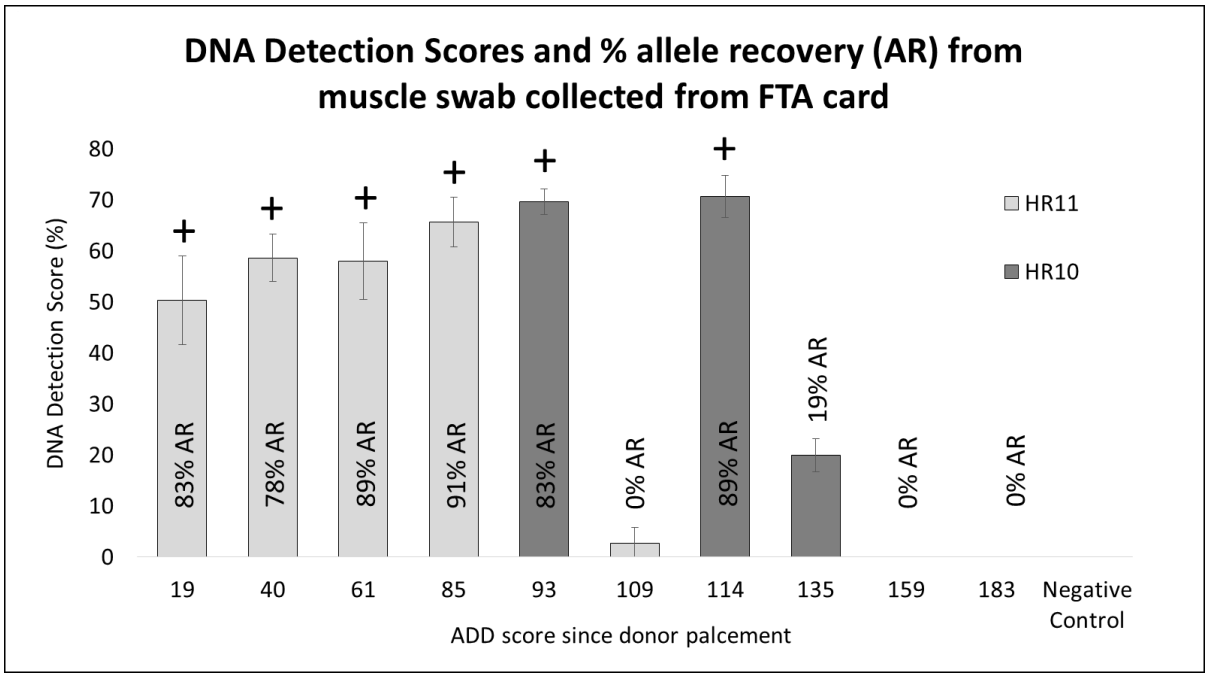

Figure 4. *Data showing decreasing DNA Detection Scores as a function of increasing ADD. The number of alleles recovered (AR) is provided as a % of the maximum 12 alleles observed. No amplification was observed in negative FTA cards. Error bars denote 1 Standard Deviation. + Denotes instances when a consensus DNA profile based on the three replicates was generated*

Muscle data also show that when DNA is collected and amplified, the Allele Recovery (AR) rate is ~80% up until ~100 ADD, five days post placement (Figure 4). When STR alleles were not positively identified, the sample was given a reduced confidence score and the putative allele identity was 'masked' by the software. Using this approach, it was necessary to build up a consensus DNA profile across the three replicate samples that were collected from each donor. In such instances the full five STR profile was generated together with Amelogenin. Across the sampling period there were no observed instances of sample contamination, allele drop-in or discordant profiles between individuals. There were instances in which the muscle collection approach yielded 0% DNA detection scores, although these were towards the end of the sampling period (Figure 4). Together, these data suggest that DNA is recoverable and a complete mini STR profile is obtainable from muscle when using the ParaDNA Field Unit and Intelligence Test Chemistry.

The same pattern was not observed for the other sampling methods, possibly due to variation in the sampling periods between the methods (Figure 5). Samples from HR11 were collected on the day the donor was placed and sampling from HR10 began two day after placement. Together these samples represent both fresh and early decomposition and range from 19-183 ADD. Collection from the skin, exposed tissue and larvae did not start until ~90 ADD and no meaningful DNA Detection Scores or STR profile results were observed. The data reveal a potentially critical period, between 80 and 150 ADD, where DNA amplification stopped in all samples. This raises the possibility that amplification may be possible from the skin and exposed tissue samples if performed earlier.

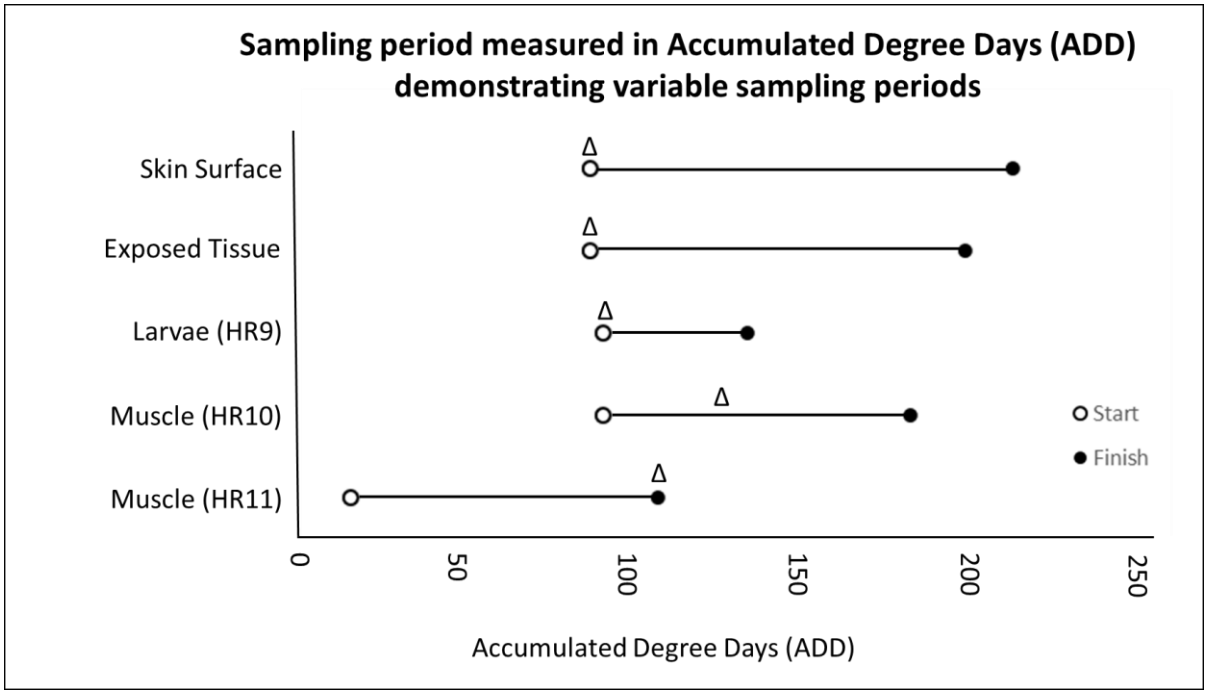

Figure 5. *Accumulated Degree Day (ADD) and range over which sample collection occurred for each treatment. Delta (Δ) indicates the first point in which a consensus DNA profile was not generated for each sampling method.*

Another explanation for the poor amplification observed in the skin and exposed tissue samples is that co-recovery of biological and environmental PCR inhibitors caused the observed failures. The ParaDNA Intelligence Test uses a direct PCR approach dispensing the need for DNA extraction and purification but potentially makes it more susceptible to PCR inhibition. The impact of degrading samples on the ParaDNA Intelligence Test amplification was investigated by using test plates spiked with DNA as positive controls with inhibitors then added. The data clearly show there was a negative effect on the amplification of control DNA and the pattern mirrors the results observed when working in the field (Figure 6). The largest amount of inhibition observed is in the decomposing pig flank tissue, which mimics the poor performance observed in exposed human tissue. Decomposing skin tissue also had an inhibitory effect although to a lesser extent. Even 'fresh' tissue had a negative impact although given the pork tissue was sourced from a local supermarket it is likely to have been butchered days before and kept chilled. The observed inhibition is consistent with other research that has shown that during decomposition, the purging of putrefactive fluids occurs which can have a significant effect on DNA typing results [26].

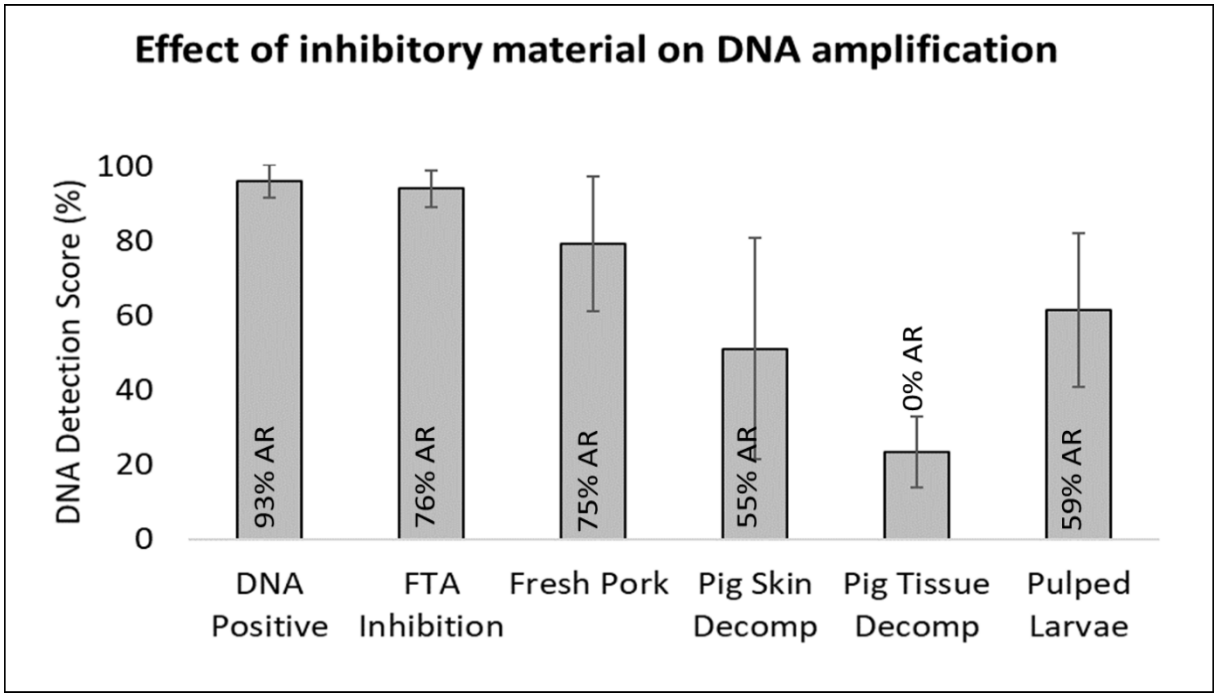

Figure 6. *Data showing that DNA Detection Scores and allele recovery rates (AR) vary depending on what samples are collected. The pattern observed is similar to that observed when collecting human data. Error bars denote 1 Standard Deviation.*

Blow flies are commonly encountered within the proximity of decomposing cadavers and are considered of high importance within forensic entomology. Research has shown that partial STR profiles are obtainable from the gut of fly larvae [27] and the ability to collect human DNA from flies using a field-based DNA profiling system offers some exciting possibilities in terms of human remains detection. The inability to recover and/or amplify any human DNA from larvae may be due to the presence of lipids and fats, accumulated by the larvae during development to fuel subsequent metamorphosis [28, 29], which contribute to PCR inhibition. This suggestion is supported by the inhibition study which shows a large decrease in DNA detection score and recovered alleles from the larvae (Figure 6). As amplification success is heavily dependent on both DNA quantity and the absence of inhibitors, it seems likely that improved results may be observed across all sample types if there was additional purification of the material to remove any PCR inhibitors. Interestingly, when blank FTA cards were tested using the spiked DNA plates there was a reduction in the number of alleles recovered from the positive control, although not in the overall DNA detection score. This suggests again that there may be a slight inhibitory effect on the PCR reaction due to the addition of fibres from the FTA card. The proprietary chemicals on the FTA cards are designed to lyse cells thereby releasing the DNA

which is then fixed onto the fibres. Typical application of FTA cards include a wash step to remove the storage chemicals from the punch allowing direct PCR [30]. Given the cards were unwashed at the time of processing it is likely that there was transfer of these chemicals to the DNA plate which may be the cause of the reduced amplification. Inhibition from FTA cards has been previously noted but is considered small and unlikely to impact modern STR kits [31].

Summary

The ability to recover biological samples in the field from human remains is required in many casework scenarios. The advent of field-based DNA processes offers many different approaches to allow recovery to be performed outside the laboratory by field-specialists. The results generated using the ParaDNA Field Instrument suggest that across the four sampling methods tested, the only approach that demonstrated any utility was the muscle incision approach where the FTA storage card was used to recover a small amount of the material. These observations support the idea that rapid processing in the field can be used to triage human remains although further optimisation of the collection process is required. A limitation to the approach described here is that the ParaDNA Intelligence Test assay only amplifies five core STR loci which will likely limit the utility of the test when trying to differentiate between close relatives. The observation that certain biological components of decomposition inhibit PCR also suggest that improving assay robustness will facilitate data collection allowing the generation of an STR profile from a single sample rather than using a consensus profile approach as done here. The correlation between DNA Detection Score and downstream profiling success has been previously established using mock case type samples [19]. However, it is possible that the combination of sample degradation and presence of PCR inhibitors associated with decomposition may weaken this correlation and further work is needed to assess whether the system can be used to screen samples of this nature. Finally, the authors observe that while this proof of concept research shows promise, further research is required prior to implementation; both to confirm STR allele concordance between the field results and those generated from traditional CE based approaches and also to assess the contamination risk from collection via FTA cards.

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