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

3

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29 **Abstract**

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

49

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

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54 **Introduction.**

55 The advent of non-laboratory-based DNA analysis promises to increase the speed in which an STR
56 profile is generated and searched against a database, compared to reference samples and used for sample
57 prioritisation [1]. In doing so, rapid investigative intelligence is provided to law enforcement agencies
58 which may allow better casework management [2]. These devices have been validated for use by crime
59 scene investigators and police officers and are undergoing adoption by many global enforcement groups
60 [3-5]. The speed and ease-of-use in which these systems provide an identification result is of obvious
61 benefit to criminal investigations. It is also important to recognise that rapid identification in a civil
62 investigation (e.g. a natural disaster) or military investigation (e.g. remains collection and repatriation)
63 may also be useful. Questions about the utility (accuracy, reproducibility likelihood of database match)
64 of each system have been answered using mock forensic evidence items, such as blood, semen, and
65 saliva swabs [6], but there is little evidence supporting field application on decomposing human remains.

66

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

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

83

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

94

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

101

102 **Methods.**

103 *Donor Information and Field Site set-up*

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

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

116

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

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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
HR2	28.4.17	Direct from donor	skin surface (calf)	5.5.17	213	5	0
		Indirect from swab	skin surface (flank)	1.5.17	89	0	0
		Direct from donor	skin surface (flank)			0	0
HR3	28.4.17	Direct from donor	skin surface (ankle)	4.5.17	144	18	0
		Indirect from swab	skin surface (armpit)	1.5.17	89	1	0
		Direct from donor	skin surface (armpit)			8	0
HR4	28.4.17	Direct from donor	exposed tissue (skull)	4.5.17	144	2	0
		Indirect from swab	exposed tissue (calf)	1.5.17	89	0	0
		Direct from donor	exposed tissue (calf)			0	0
HR5	26.4.17	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
		Direct from donor	skin surface (calf)	1.5.17	130	2	0
HR6	26.4.16	Direct from donor	skin surface (shoulder)	2.5.17	148	2	0
				5.5.17	196	2	0
				1.5.17	130	87	92
HR7	26.4.17	Direct from donor	exposed tissue (skull)	2.5.17	148	53	75
				5.5.17	196	4	0
				1.5.17	130	0	0
HR8	26.4.17	Direct from donor	exposed tissue (forearm)	2.5.17	148	0	0
				5.5.17	196	0	0
				1.5.17	130	1	0
HR9	23.6.17	Indirect from larvae on FTA	Upper torso	26.6.17	93	4	0
				28.6.17	135	0	0
						0	0
HR10	23.6.17	Indirect from muscle swab on FTA card	R-Upper Thigh			67	83
						73	75
			L-Upper Thigh			69	92
						76	83
			R-Calf			70	92
						66	92
			L-Calf			24	0
						16	0
			L-Calf			20	58
						0	0
HR11	26.6.17	Indirect from muscle swab on FTA card	L-Upper Thigh			0	0
						0	0
			R-Upper Thigh			0	0
						0	0
			R-Calf			0	0
						0	0
			R-Hand			0	0
						0	0
			R-Foot			0	0
						0	0

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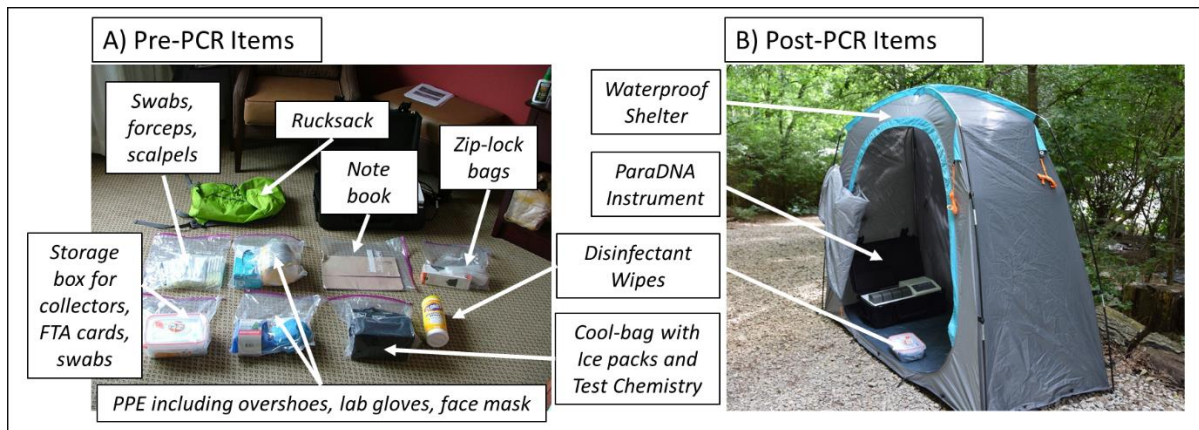
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ADD values calculated from date of placement. Single results (n=1) are provided for DNA Score (calculated following [19]) and the allele recovery rate (determined as a percent of the maximum 12 alleles).



143

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

147

148 *Optimisation and Assessment of Collection Methods*

149 Two non-invasive methods were initially assessed on four donor samples (HR1-4) in spring (Table 1).

150 An indirect collection method was trialled where an area of the donor was swabbed using a wetted rayon

151 swab (Thermo Scientific, Sterilin, F155CA) (Figure 2a) before recovering any material from the moist

152 swab using the ParaDNA Sample Collector for approximately 30 seconds (n=4). This closely follows

153 the process of collection outlined by the developers of the approach [19]. A direct collection method

154 was also trialled where the same area of the donor was targeted but using the Sample Collector directly

155 (Figure 2b) for 30 seconds (n=4). Given the rapidity of the data generation, assessment of the samples

156 on-site, and the manufacturer's advice, the direct sampling approach was considered more likely to be

157 effective at collecting biological material and two direct treatments were then compared; direct from

158 skin surface (n=9) and direct from tissue exposed by scavengers (n=9). Sampling using these treatments

159 was performed across five days. After consultation with practitioner groups, two additional collection

160 methods were trialled in the summer; indirect invasive muscle swab (Figure 2c) and indirect non-

161 invasive larvae collection (Figure 2d). For muscle collection, a 5-10 cm incision was made using a

162 sterile disposable scalpel. New incision sites were selected daily in tissue showing the least visible

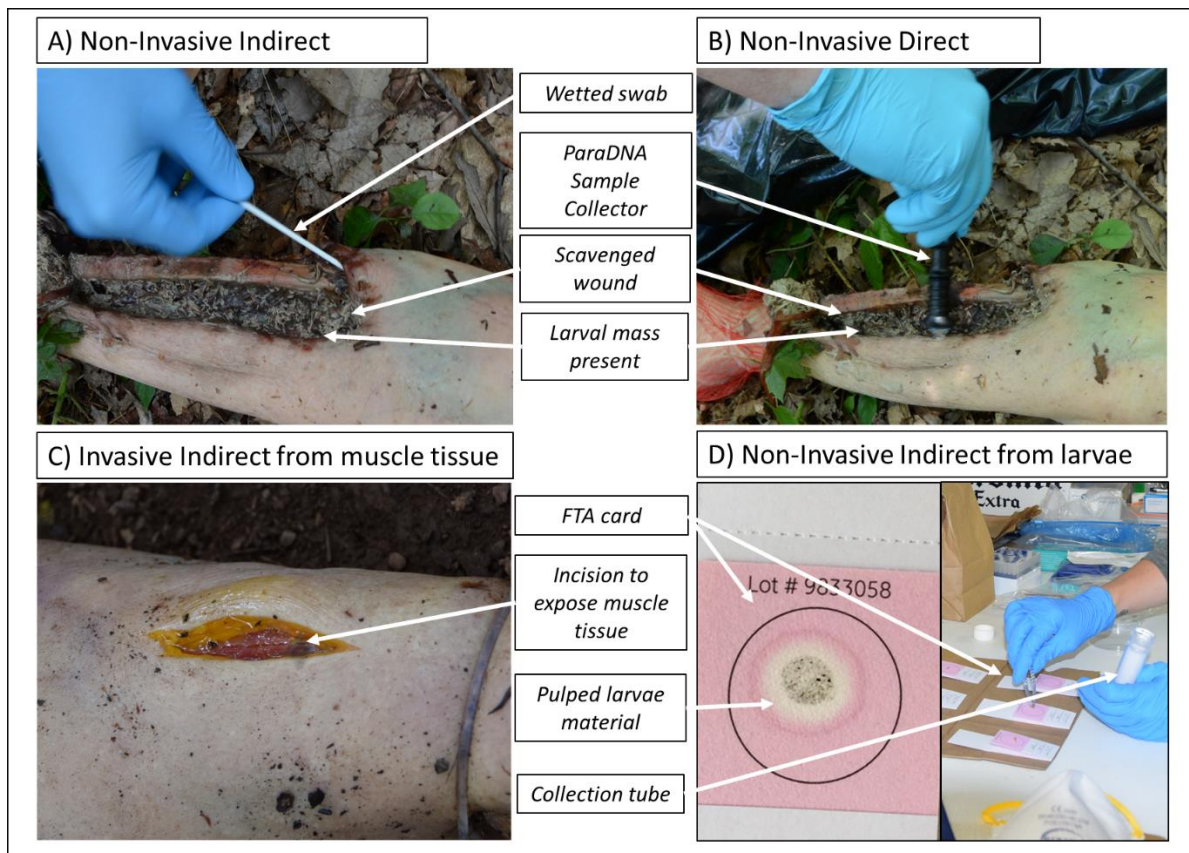
163 decomposition. The muscle tissue was cross hatched with the scalpel and swabbed with a dry cotton

164 swab for 30 seconds. The swab was then firmly pressed onto an indicating FTA card (GE Healthcare,

165 Whatman, WB120412) and left to dry in a small paper bag containing silica gel desiccant for 30 minutes

166 (n=30). Larvae samples were collected directly from the donor's remains and placed in a screw top 50
 167 ml plastic collection tube. The samples were frozen and transferred to a 1.5ml micro-centrifuge tube
 168 containing 75 µl of PCR grade water before being pulped using micro-pestles (Eppendorf, 0030120973).
 169 The solution was then transferred to an FTA card and left to dry in a paper bag containing silica gel for
 170 1 hour before sampling (n=6). When recovering material from FTA cards, the ParaDNA Sample
 171 Collector was scratched against the sample deposition site on the FTA card for 30 seconds to recover
 172 FTA fibres before being transferred to the PCR assay.

173



174
 175 **Figure 2.** Non-invasive indirect methods based on subsampling from a swab (a) and non-invasive direct
 176 methods based on sampling from the donor using the Sample Collector (b) were initially attempted. The
 177 latter was further divided into direct from skin surface and direct from scavenged wound. Further
 178 sampling included invasive indirect from muscle swab transferred to FTA card (c) and pulped larvae
 179 transferred to FTA card (d).

180

181 *Field-based DNA Analysis*

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

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

190

191 *Laboratory-based assessment of DNA amplification success and inhibition*

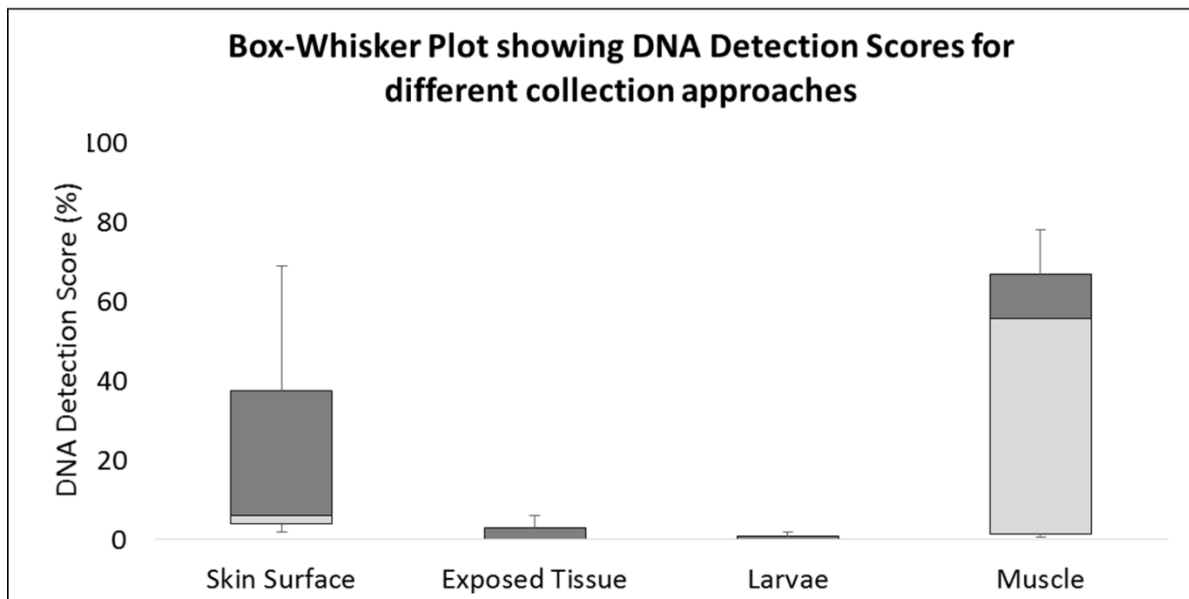
192 The impact of sample inhibition on DNA amplification was assessed by first spiking purified human
193 DNA into the Intelligence Test chemistry (final assay concentration at 2ng per well) effectively making
194 a positive control plate. Inhibition may be in the form of nucleases, proteases and lipids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

204

205 **Results and Discussion**

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

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

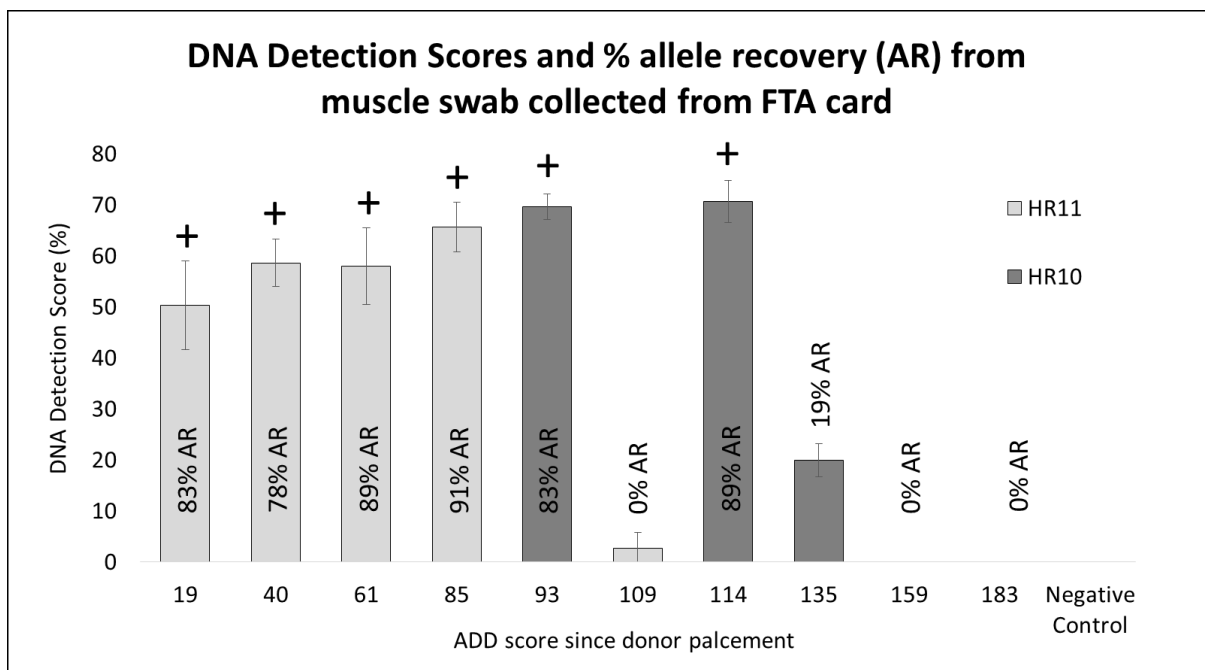


215 **Figure 3.** Box whisker plot showing DNA Detection Scores (%) generated by ParaDNA Intelligence
216 Test. Each plot shows the second quartile (light grey) and third quartile (dark grey) with the first and
217 fourth interquartile ranges provided by the error bars. The median value is the line separating the
218 second and third quartiles.
219
220

221 DNA amplification was also shown to be possible when collecting direct from the skin surface giving
222 a median DNA score of 4% (Figure 3). This is considered largely due to a single sample (HR6)
223 amplifying in two instances (Table 1). When collecting samples from this donor it was noted that the
224 donor exhibited some blistering on the skin surface which appeared to contain blood. As such, the
225 sampling action caused the blisters to rupture meaning that the blood-based material was collected as
226 well as skin (author's personal observation). If these two samples are removed from the data in Figure
227 3 then both skin surface and exposed tissue data are broadly equal. The data also show reduced
228 amplification in the exposed tissue samples with a median DNA score of 0% (Figure 3) which is
229 unexpected given that muscle tissue and suspected blood were visually observed at the time of collection.
230 Collection of human material from carrion fly larvae also failed to yield results in the field (median
231 DNA Score of 0%), although amplification was observed in one single sample (Figure 3). Possible
232 explanations for the poor results observed in the skin, exposed tissue and larvae treatments include low
233 template recovery using the Sample Collector, DNA degradation and/or PCR inhibition.

234

235 Previous research using the plastic sample collector has been shown to be successful at collecting
 236 material from a variety of different surfaces [19] and images of the sample collector taken immediately
 237 after the sampling clearly shows biological material is being recovered (authors personal observation).
 238 Also, sample collection direct from fish tissue (raw and cooked) has been shown to work [22] which
 239 would suggest that the plastic collection device is capable of lifting biological material. DNA
 240 fragmentation resulting from the release of cellular nucleases [23] is considered a more likely
 241 explanation. The destruction of high molecular weight DNA as ADD increases will create a smaller
 242 pool of potential DNA targets for amplification during PCR, and can be seen in the data collected from
 243 the muscle tissue which shows a reduction in the amount of DNA amplified as ADD increases (Figure
 244 4). This can be combatted by using PCR primers designed to amplify short DNA fragments, resulting
 245 in greater PCR efficiency and increased success when profiling degraded and inhibited samples [24,
 246 25]. The size of the PCR amplicons generated using the ParaDNA Intelligence Test cannot be
 247 ascertained as the system uses a melt curve detection approach to differentiate between alleles and the
 248 primer sequences are not in the public domain. As such it is not known to what extent amplicon length
 249 may be affecting the observed results.



250
 251 **Figure 4.** Data showing decreasing DNA Detection Scores as a function of increasing ADD. The
 252 number of alleles recovered (AR) is provided as a % of the maximum 12 alleles observed. No
 253 amplification was observed in negative FTA cards. Error bars denote 1 Standard Deviation. + Denotes
 254 instances when a consensus DNA profile based on the three replicates was generated
 255

256 Muscle data also show that when DNA is collected and amplified, the Allele Recovery (AR) rate is
257 ~80% up until ~100 ADD, five days post placement (Figure 4). When STR alleles were not positively
258 identified, the sample was given a reduced confidence score and the putative allele identity was 'masked'
259 by the software. Using this approach, it was necessary to build up a consensus DNA profile across the
260 three replicate samples that were collected from each donor. In such instances the full five STR profile
261 was generated together with Amelogenin. Across the sampling period there were no observed instances
262 of sample contamination, allele drop-in or discordant profiles between individuals. There were instances
263 in which the muscle collection approach yielded 0% DNA detection scores, although these were
264 towards the end of the sampling period (Figure 4). Together, these data suggest that DNA is recoverable
265 and a complete mini STR profile is obtainable from muscle when using the ParaDNA Field Unit and
266 Intelligence Test Chemistry.

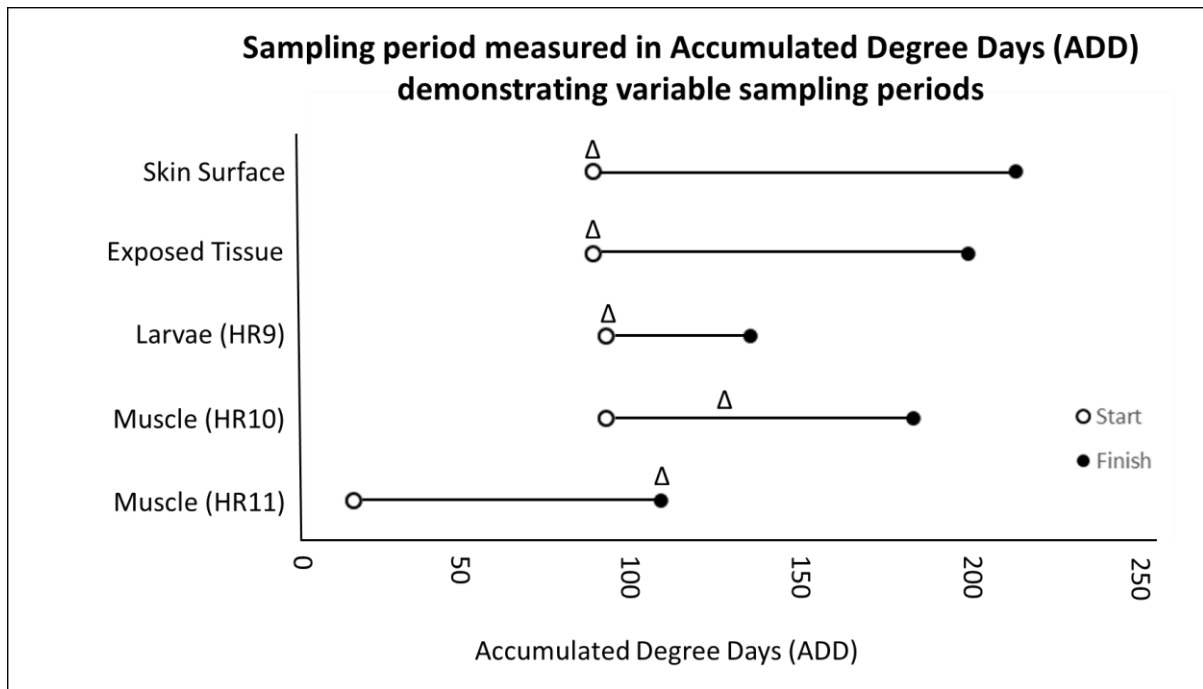
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268 The same pattern was not observed for the other sampling methods, possibly due to variation in the
269 sampling periods between the methods (Figure 5). Samples from HR11 were collected on the day the
270 donor was placed and sampling from HR10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

276

277

278



279
 280 **Figure 5.** Accumulated Degree Day (ADD) and range over which sample collection occurred for each
 281 treatment. Delta (Δ) indicates the first point in which a consensus DNA profile was not generated for
 282 each sampling method.
 283

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

298

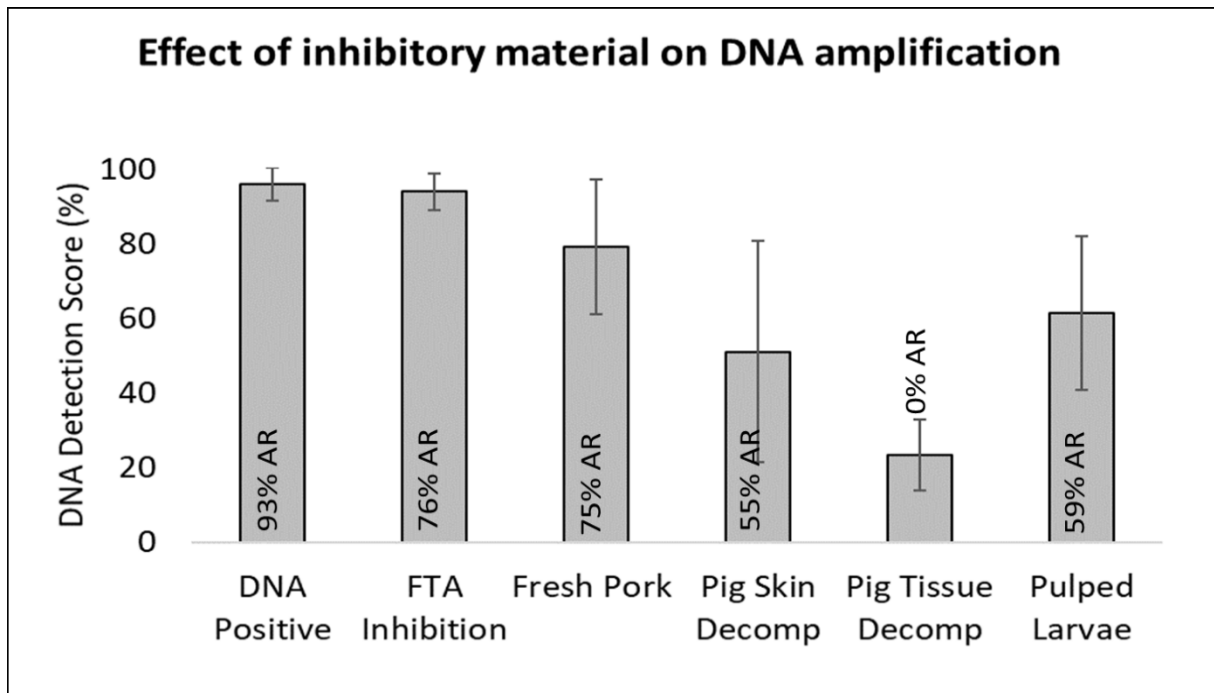


Figure 6. Data showing that DNA Detection Scores and allele recovery rates (AR) vary depending on what samples are collected. The pattern observed is similar to that observed when collecting human data. Error bars denote 1 Standard Deviation.

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301
302
303

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

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

324

325 **Summary**

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

346

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352

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