DNA Persistence and Preservation Following Environmental Insult

By

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DECLARATION

I declare that the work contained in this thesis has not been previously submitted for any other award from an academic institution. To the best of my knowledge and belief, the thesis contains no materials previously published or written by another person except where due reference is made.

Signed _____ Date _____

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ABSTRACT

This research was conducted to provide empirical evidence to supplement advice available to the forensic community for the collection of muscle tissue for forensic analysis. This type of collection is normally carried out to determine the identity of individuals following mass disasters, such as plane crashes or natural disasters.

DNA degradation was assessed in two model organisms, pig and rabbit (with human DNA as a control), over various time points. Rabbit recombination activating gene (RAG 1) was aligned to identify conserved regions in pig, rabbit and human. Primers were designed and optimised to create a 4-plex PCR multiplex that can amplify 70 bp, 194 bp, 305 bp and 384 bp in three species. The 4-plex multiplex was found to work efficiently in all three species down to 0.3 ng of DNA template. The multiplex was used to assess whether DNA degradation can be predicted by accumulated degree-days (ADD), which provides a measure of both time and temperature. A series of field studies were performed to assess DNA persistence in pig and rabbit soft muscle tissues using a combination of whole animals, suspended muscle tissues (insect activity free) and muscle fragments. Field studies were carried out in: August-September 2009; February-May 2010; May-June 2010; June-July 2010 and September-November 2010. Soft muscle tissue samples were collected at different ADD.

4-plex multiplex results showed that DNA was more persistent in pig tissues compared to rabbit tissues. In the September 2010 experiments, full multiplex amplification was obtained from rabbit until 137 ADD (whole carcases) and 210 ADD (body fragments and suspended tissues), while in the August 2009 experiments, full multiplex amplification was obtained until 112 ADD (whole carcases and body fragments) and until 141 ADD (suspended tissues). In the June 2010 experiments, full multiplex amplification was possible until 64 ADD.

Pig whole carcases which were placed in the field in February 2010, showed multiplex amplification until day 90 (603 ADD), followed by September 2010 (until day 44 (490 ADD)) and May 2010 (until day 27 (338 ADD)). During the September 2010 project, body fragments produced full amplification until muscles were collected (342 ADD), while in case of whole carcases and suspended tissues; the amplification was possible until 490 ADD. There was complete failure of amplification of 305 bp and 384 bp in pig whole carcases after 342 ADD, while in suspended tissues, the amplification of 305 bp and 384 bp was possible until 420 ADD.

The statistical analysis showed that amplification success of larger amplicons (194 bp, 305 bp and 384 bp) reduces with increase in ADD in pig and rabbit whole carcases, body fragments and suspended tissues while 70 bp was more persistence. The results showed that there was no significant difference in DNA persistence between whole carcases verses suspended tissues ($Z=0.57$, $p>0.05$) and whole carcases verses body fragments ($Z=1.71$, $p>0.05$), There was however a significant difference ($Z=2.31$, $p<0.05$) in DNA persistence in suspended tissues and body fragments with increase in ADD. The results from field experiments suggested that muscle tissues, if available, should be collected for DNA profiling, since even if degraded, a profile can be obtained. The results also suggested that the isolation of tissues from insect activity as quickly as possible (even if immediate storage is not possible) may be beneficial for DNA persistence. Seasonal variation in DNA persistence was observed due to maggot mass growth which increases carcase decomposition and ultimately effect on DNA persistence.

Controlled incubation experiments were also performed at 27 °C, 37 °C and 47 °C until 21 days to assess DNA persistence, as these temperatures were not available under field conditions. The results showed that the amplification of 70 bp was more persistent compared to larger amplicons (194 bp, 305 bp and 384 bp). The drop-out in amplification of larger amplicons occurred more rapidly in samples incubated under laboratory conditions compared to the field samples. The statistical analysis showed species, ADD and temperature have strong effect ($p<0.05$) on DNA persistence under controlled conditions.

The appearance of 70 bp amplicons in all samples collected from field and in most samples from controlled incubation experiments suggested that soft muscle tissues exposed to different environments can be used to perform SNP analysis.

The full 4-plex multiplex amplification obtained from rabbit and pig preserved and dehydrated samples suggested that 96% ethanol, cell lysis solution (with and without 1% sodium azide) and dehydration can be used to preserve fresh and partially decomposed soft muscle tissues at room temperature for one year. The drop-out in amplification of larger amplicons in tissues preserved in 10% buffered formalin suggested that formalin was not suitable for long term storage. This system should therefore be considered as an additional method during Disaster victim identification (DVI) work to preserve fresh and partially decomposed samples. This study also suggested that the developed multiplex (4-plex) can be used to assess DNA persistence in human decomposing bodies and in experimental studies.

I dedicate this thesis with love to my beloved late father and family

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List of Abbreviations

ADD	Accumulated degree-days
HMW DNA	High molecular weight DNA
PCR	Polymerase chain reaction
DVI	Disaster Victim Identification
INTERPOL	International Criminal Police Organization
RAG-1	Recombination activation gene 1
TRACES	Taphonomic Research in Anthropology: Centre for Experimental Studies
STRs	Short tandem repeats
VNTRs	variable number tandem repeats (VNTRs)
PMI	Post-mortem interval
SWGDAM	Scientific Working Group on DNA analysis Methods
SNPs	Single nucleotide polymorphisms

CHAPTER 1

INTRODUCTION

1.1 DNA Profiling

DNA profiling is a well-established scientific technique, used all over the world for criminal intelligence, paternity testing and mass disaster victim identification. It was first described in 1985 using variable number tandem repeats (VNTRs) (Jeffreys et al., 1985) but is now most commonly achieved using short tandem repeats (STRs) (Butler, 2005). DNA analysis can be performed on any biological sample, providing complete DNA degradation has not occurred (Graham et al., 2008).

1.2 DNA Degradation in Post-mortem Tissues

DNA degradation in living organisms starts rapidly after death (Pääbo et al., 2004).The endogenous nucleases produced by host cells or exogenous nucleases released by microorganisms cause DNA to degrade into smaller fragments. During later stages of cell death, the rupture of cell membranes help to produce a nutrient-rich fluid which accelerates the growth of environmental microorganisms such as bacteria and fungi on cadaverous tissues, which reduces the probability of obtaining high molecular weight (HMW) DNA (Hofreiter et al., 2001; Ogata et al., 1990). As the post-mortem interval (PMI) increases, DNA continues to degrade until no DNA remains (Cina et al., 1994). Environmental conditions such as temperature and humidity have been shown to exert more influence on the rate of DNA degradation than elapsed time since death. There is an inverse relationship between DNA yield and PMI, with degradation accelerated by increases in temperature (Robins and Furey, 2001) and low temperature is considered essential for the longevity of DNA molecules (Lindahl, 1993; Willerslev et al., 2004). Nucleotide base decomposition decreased about 10 – 25 fold with a 20 °C reduction in temperature (Hoss et al., 1996), although this figure is based on DNA in bone.

A dry environment also helps to reduce DNA degradation whilst high humidity or the presence of water as moisture encourages bacterial growth and also provides a substrate for hydrolytic enzymes to degrade DNA. Anaerobic bacterial fermentation in the intestines or in the soil and presence of humic acid make further contributions to pH reduction and therefore reduce DNA stability (Haglund, 1996).

Post-mortem DNA stability varies among tissues. Brain tissue appears to be one of the best sources for DNA analysis in post-mortem studies followed by muscle, blood, spleen and kidney, whereas liver is consistently a poor source of DNA (Haglund, 1996).

In a limited study (3 week) carried out in 1988, HMW DNA has been obtained for 3 weeks from biopsy samples of human brain, lymph nodes and skeletal muscles stored at 4 °C or -20 °C and until 1 week from spleen, kidney and thyroid (Bär et al., 1988).

DNA could be detected in more than 90% of the muscle samples after a post-mortem period of 3 days with subsequent storage at -20 ºC, and from 40 % of muscle specimens after a period of 8 days at room temperature under conditions of high humidity (Ogata et al., 1990). Successful amplification of microsatellites or short tandem repeats (STRs) was found in human autopsy samples with and without obvious signs of decomposition (Hoff-Olsen et al., 2001). Phengon et al., (2008) studied DNA persistence in muscle tissue of pig. Two-inch cubic chunks of pork meat were left in sea and tap water for 1 week, and air dried for two weeks. A 211 bp and a 289 bp *B*-actin fragments were amplified from tissue samples immersed in tap and sea water up to 1 week, while there was an incomplete or no amplification after day 4 in air dried samples. Amplification of a mitochondrial DNA (323 bp *cytochrome* b) fragment was possible until day 14.

DNA stability in human autopsy samples (kidney, liver, spleen, and pancreas tissue specimens) was assessed using the AmpF*l* STR® SGM plus® PCR amplification kit loci (D3S1358, VWA, D16S539, D2S1338, D81179, D21S11, D18S51, D19S433, TH01, FGA and gender marker amelogenin) in Poland (Niemcunowicz et al., 2007). The tissue samples were incubated at 4 ºC and 21 ºC in closed 40 ml containers and at 21 ºC in closed 40 ml containers filled with sand, garden peat, pond water or salt water and at 21 ºC in open 40 ml containers. The liver specimens immersed in pond water and sea water gave full profiles until day 49, while partial profiles were produced after day 7 in liver specimens stored in closed containers at 21 ºC. The spleen and pancreas specimens were partially typeable within 119 days and 126 days respectively. Faster decrease in typeability of AmpF*l* STR® SGM plus® kit loci was observed in all tissue specimens incubated in peat and sand which might be due to humic acid content, microbial action or acidic pH (Ranjard and Richaume, 2001).

In another experiment, DNA persistence in human muscle tissue specimens and blood stains was determined using STRs and single nucleotide polymorphisms (SNPs) (Onori et al., 2006). The muscle tissue and wet and dry blood stains were exposed to different environments such as open air, buried in soil, immersed in river and sea water. STR typing was possible until 1 week from buried muscle samples and those immersed in sea and river water. Allelic and locus drop-out phenomena were observed in all other

samples (dry and wet bloodstains and muscle tissue stored at open air) from the first week. However, SNPs typing gave positive results in muscle tissue samples stored in open air and in dry blood stains until 9 months (Onori et al., 2006).

The rate of DNA degradation is also influenced by the availability of certain chemical substances in teeth and bone tissues (Dobberstein et al., 2008). DNA stability in bone is enhanced by the presence of hydroxylapatite (Lindahl, 1993; Eglinton et al., 1991) which is also a major component of tooth matrix. Therefore, dental DNA is also better protected from degradation (Urbani and Kramer, 1999; Vonwurmb-Schwark et al., 2003).

Overall, studies suggest loss of amplification occurs due to the DNA being broken down into smaller fragments leading to the drop-out of larger target loci (Foran, 2006).

1.2.1 Accumulated Degree-Days (ADD)

Accumulated degree-days (ADD) is a measure of the cumulative total of the average daily temperatures. ADD can be calculated in different ways. The most basic way to calculate ADD is to simply take the average of every day. The ADD for a single day can be calculated by the rectangular method. This uses simple averaging and can be calculated using the following formula:

ADD = [(Maximum + Minimum Temperature)/2] - Minimum Threshold

Minimum threshold (base temperature) represents the temperature at which biological process such as bacterial or larval growth essentially stops (Larkin et al., 2010; Megyesi and Haskell, 2005). As freezing severely inhibits biological processes, zero °C has been used as the base temperature in decomposition studies of human remains (Megyesi and Haskell, 2005). Accumulated degree days (ADD) accounts for approximately 80% of the variation in decomposition of human remains and is very important when estimating the post-mortem interval (PMI) based on decomposition using a quantitative approach (Megyesi and Haskell, 2005). The effects of ADD on the quantity of DNA extracted from porcine skeletal muscles using three different methods of extractions have been previously determined by Larkin et al., 2010. Agarose gel based DNA quantification analysis showed an overall decrease in DNA yield from 0 ADD to 101 ADD in summer and up to 138 ADD in winter. The inconsistency in ADD and DNA yield in summer and winter specimens was thought to be due to several factors such as soil, humidity,

wind, sampling procedures, storage, bacterial and fungal growth. DNA quantification was made based on agarose gel electrophoresis, as agarose measured total DNA regardless of species, quantitative PCR (qPCR) using porcine specific primers was recommended (Larkin et al., 2010)

1.3 Methods of DNA Preservation

Following mass fatality incidents, DNA profiling, essential for identification and re-association of fragmented, burnt or decomposed corpses, is very difficult or impossible using traditional means such as fingerprinting and odontology. However, successful DNA recovery depends on appropriate collection and preservation of biological material from deceased individuals and the availability of reference samples (Graham et al., 2008). Inefficient preservation methods cause destruction of intact DNA to such an extent that data is not always available for victim identification (Bing and Bieber, 2001).

Biological samples may be successfully preserved using a number of physical and chemical treatments by adjusting temperature, ambient pH and salt concentrations (Dawson and Jacobs, 1998). Cryopreservation is the preferred method of DNA preservation which is accomplished by freezing samples over dry ice (-78 °C) or in liquid nitrogen (-196 °C) (Dessauer et al., 1995; Seutin et al., 1991). Sample storage at -20 °C also enhances DNA preservation. Although freezing is ideal to minimize decomposition, dried stains and skeletal remains can be stored at room temperature in a low humidity environment. (Budowle and Eisenberg., 2005).

Tissue fixation is another method of DNA preservation when freezing is not available. Previous studies (Johnson and Traweek, 1993; Pavelic et al., 1996; Bounin et al., 2005; Shi et al., 2004) showed that tissue fixation and embedding processes cause DNA fragmentation. The duration and type of fixative are the two most important factors affecting DNA integrity. Buffered formalin preserves DNA to a greater extent compared to unbuffered formalin, as unbuffered formalin oxidises to formic acid and creates an acidic environment that leads to the degradation of nucleic acids (Zsikla et al., 2004). Longer fixation time (more than 7 days) causes a higher degree of cross-links between biomolecules and a higher rate of DNA degradation resulting in small DNA fragments. DNA fragmentation causes a reduction of products that can be amplified using PCR (polymerase chain reaction) (Legrand et al., 2002).

Archival fixed and paraffin-embedded tissue (PET) enabled valuable genetic information to be obtained from preserved specimens up to 25 years but high quality genomic DNA was hard to obtain, as fixation often results in DNA fragmentation and sequence alterations. DNA obtained from fixed samples, has rarely produced DNA amplicons exceeding 300 bp (Gillio-Tos et al., 2007; Srinivasan et al., 2002).

The desiccation of tissues also impedes nuclease and microbial activity simply through water removal. Silica desiccant has been used to rapidly dry and store tissues. Silica is inexpensive, safe, inert and can easily be transported to the field, however, it makes the tissues brittle and more difficult to process for DNA extraction (Foran et al., 1997; Murphy et al., 2002; Roeder et al., 2004; Grassberger et al., 2005).

Heat drying of tissues also destroys bacteria and cellular enzymes that degrade DNA, and for sterilisation purposes of food, drying at 70 °C is considered sufficient (USDA, 2011). Dehydrated human soft muscle tissue samples have been used previously for successful PCR amplification using Identifiler® (Applied Biosystems) (Michaud, 2006; Allen-Hall and McNevin, 2012)

Ethanol has also been used in previous studies to preserve specimens at room temperature (Kilpatrick et al., 2001; Gillespie et al., 2002; Michaud and Foran, 2011). Alcohol storage preserves samples without DNA cross-linking which is important for successful DNA extraction. Ethanol is considered to be an effective long term tissue storage method that allows for successful DNA extraction from preserved specimens. An advantage of this method is that it also kills bacteria and fungi (Penna et al., 2001). Ethanol preservation is cost effective and readily obtained, making it an attractive candidate for field applications.

Storage buffer and tissue type are among the principal factors affecting DNA preservation (Dawson and Jacobs, 1998). Bone and teeth have resilient structures to prevent DNA degradation especially compared to soft tissue samples. However, the process of DNA extraction from hard tissues is laborious and time consuming (Budowle and Eisenberg, 2005).

Recently, two buffer solutions, Oragene™ and LST (lysis storage and transportation) were found to be effective for room temperature preservation of human soft muscle tissue samples for a period of 12 months and have been recommended for soft tissue preservation under field conditions, following mass fatality incidents (Graham et al.,

2008). Genofix, a new alcohol-based tissue fixative can be used for biopsy tissue preservation at room temperature up to one year and seven months and at -20°C for longer storage (up to 3 ½ years). This new and odourless tissue fixative has also been suggested to be useful in criminal investigations or mass disaster identifications for soft tissue samples preservations (Frégeau et al., 2001).

Different chemical solutions such as acetone, isopropanol, Carnoy's, ethanol (both at 70% and 100% concentrations), DMSO and formalin (10%) have been used effectively for specimen preservation from diverse species including insect species, baboons, fish and marine invertebrates (Bisanti et al., 2009; Chakraborty et al., 2006; Frantzen et al., 1998; Mandrioli et al., 2006 and Dawson and Jacobs, 1998).

Dehydration is the removal of water from an object and in physiological terms, it relates to a deficiency of fluid within an organism (Lavizzo-Mourey, 1987). In forensic context, dehydration refers to oven dried tissues (Michaud and Foran, 2011; Allen-Hall and McNevin, 2012).

1.4 Validation of Laboratory Procedures

Validation is the process of demonstrating that a certain laboratory procedure is reliable, robust and reproducible in the hands of the laboratory staff performing the procedure in that laboratory. A robust method is one where most of the samples give successful results and few, if any, need to be repeated. The method will be considered reliable when results obtained are accurate and correct. While reproducibility refers to getting the same results every time a sample is repeated.

In forensic laboratories, validation studies are routinely performed prior to the application of any new technique for use with evidentiary samples or when a substantial modification to existing techniques has been made (Greenspoon et al., 2006). In response to the increased demand for DNA analysis, Forensic laboratories are continuously developing enhanced technologies to process database and casework samples more efficiently and effectively (Wang et al., 2011). These advancements include real-time PCR for human DNA quantification (Greenspoon et al., 2006; Horsman et al., 2006; Nicklas et al., 2006; Swango et al., 2007; Barbisin et al., 2009). Multiplex STR kits for profiling of autosomal and Y-STR loci (Holt et al., 2002; Collin et al, 2004; Coticone et al., 2004; Krenke et al., 2005; Mulero et al., 2006; Chang et al., 2008), capillary electrophoresis instruments (Sgueglia et al., 2003; Butler 2004),

florescence imaging systems (Mikka 1999) and DNA data analysis programmes (Kadash 2004; Butler 2005; Greenspoon et al., 2006; Asamura et al., 2007; Robby 2007; Mulero et al., 2008). Most of these advanced techniques are based on PCR multiplex assays as the use of multiple loci enables a high power of discrimination without consuming a high DNA template in a single reaction (e.g., 1ng or even less of the starting material) (Butler 2007), and have been validated according to the guidelines of Scientific Working Group on DNA analysis Methods (SWGDAM) (Micka 1999; Holt 2002; Collins 2004; Coticone et al., 2004; SWGDAM 2004; Krenke et al., 2005; Swango et al., 2006; Mulero et al., 2008).

In addition, in Forensic DNA analysis, highly degraded samples have poor amplification of the larger sized alleles (300-500bp) in standard multiplex typing kits (Wallin 1998; Cotton et al., 2000; Benjamin et al., 2002). In severely decomposed samples, the DNA template can become highly fragmented and yield of complete target amplicons is greatly reduced. Thus a decay curve can be seen in multiplex kits with a wide range of amplicon sizes, where the peak height is inversely proportional to the amplicon length (Lygo et al., 1994; Wallin 1998; Cotton et al., 2000). In this case, the larger amplicons fall below the detection limits and often have low sensitivity., therefore a partial genetic profile can be seen) (Greenspoon et al., 2006). To solve this problem reduced sized primer sets called miniplexes have been developed to function with florescence based sequencers for the detection of degraded DNA samples (Butler 2003).

Generally there are two types of validation: developmental validation and internal validation. Developmental validation generally involves the testing of new STR kits or STR loci, primer sets and new technologies for STR allele detection. While internal validation is normally used to verify that already established procedures will work effectively in one's own laboratory (Butler 2001).

A lot of research has been done to observe the effect of temperature on DNA degradation (Neubauer et al., 1996; Sheu, 2006; Forterre, 1994). However, limited studies have been undertaken to describe the effect of temperature on DNA degradation in terms of accumulated degree-days (ADD). Therefore, this study was performed to assess DNA persistence in muscle tissue in relation to ADD in a temperate climate, which might be extended to other types of climate.

Similarly, no work is described in the literature that assesses the effect of fragmentation of a body on the rates of DNA degradation or looks at the effectiveness of different preservation techniques when working with partially decomposed muscle tissue.

The specific aims of this research are

1- Temperature (measured in ADD) can be used to predict time frame within which DNA will be recovered from muscle tissues.

DNA degradation in muscle tissues was assessed in relation to ADD. The use of ADD to assess DNA degradation and persistence in muscle tissues will, if the hypothesis that ADD is the critical environmental factor in predicting time frame within which DNA will be recovered, give a better understanding of the likelihood of successfully retrieving DNA from post-mortem muscle tissue.

2- DNA degradation in muscle tissue is slower in fragmented remains than in whole bodies.

If there is a significant difference between whole and fragmented bodies then this may provide valuable information for disaster victim identification (DVI) work, allowing samples with the highest probability of containing sufficient DNA to be collected. In fragmented remains the process of putrefaction will be limited since the fragments have been separated from the gut contents.

3- Optimal DNA preservation methods can help to preserve partially degraded DNA in muscle.

The preservation techniques that are currently described in the literature use fresh muscle tissues. In real DVI cases the muscles tissue will not be fresh, but partially decomposed. Assessing the suitability of current methods of preservation with decomposing samples will also provide useful information for Disaster Victim Identification (DVI) scientists.

In a second phase of research, partially decomposed rabbit (45 and 79 ADD) and pig (79 ADD and 210 ADD) soft muscle tissue samples were preserved in 96% ethanol, 10% buffered formalin and Qiagen cell lysis solutions (with and without 1% sodium azide) for a period of one year. The efficiency of these preservative solutions to preserve DNA in partially decomposed tissues was determined.

4- Storage of heat dried soft tissues can preserve DNA for longer time.

The presence of water in the form of moisture cause DNA degradation due to the presence of bacteria and hydrolytic enzymes, therefore, heat dried soft tissues were stored at 4 °C for one year and DNA persistence was assessed.

5- Development Multiplex PCR based Assay for pig, rabbit and human.

6- Assess DNA degradation in pig and rabbit soft muscle tissue samples incubated at 27 °C, 37 °C and 47 °C for 21 days.
The DNA degradation results obtained from soft muscle tissues of decomposing pig and rabbit were compared to the tissues incubated under controlled conditions to assess comparative rate of DNA degradation.

To realise these aims, rabbit carcasses were exposed to environmental insults during different times of the year. Soft muscle tissue samples were collected from the carcasses at different ADD. DNA extraction was performed on all samples. The rate of DNA degradation was assessed in relation to ADD.

CHAPTER 2

MATERIALS and METHODS

2.1 Experimental Design

Throughout this study, rabbit (*Oryctolagus cuniculus*) and pig (*Sus scrofa*) were used as model animals: the use of pigs was approved by the University's Animal Research Ethics Committee.

Rabbit carcasses were obtained as a product of standard pest control procedures in the UK. They were collected from Walkers Game, Gainthorpe, Lincolnshire, UK from a licensed dealer. Domesticated pig was chosen as an animal model due to its similarity with the human in terms of body weight and basal metabolic rate. These animals were purchased from farms after being killed using a bolt gun by a licensed individual.

To assess DNA persistence in muscle tissue in relation to ADD, pig and rabbit carcasses were exposed to the environment at different seasons at the University's experimental field site called TRACES (Taphonomic Research in Anthropology: Centre for Experimental Studies). Carcasses were placed in direct contact with the ground (Figure 2.1, 2.2, 2.3 and 2.4) and covered by a wire cage to inhibit scavenger access. TRACES is located on the outskirts of Burnley and is surrounded by farmland.

2.2 Field Studies

A series of field studies were performed using a combination of whole animals, animal portions and muscle pieces. Field studies were carried out in: August-September 2009; February-May 2010; May-June 2010; June-July 2010; and September-November 2010. The environmental temperature and humidity was recorded using a data-logger (EasyLog® Lascar, UK). Muscle tissue samples collected during field experiments were placed in labelled sterile plastic bags and immediately placed on ice for transport to the laboratory. Samples were stored at -20 °C until DNA extraction. The carcases were photographed on each visit.

Figure 2.1 Shown above is an example of the rabbit whole carcases and body fragments at TRACES.

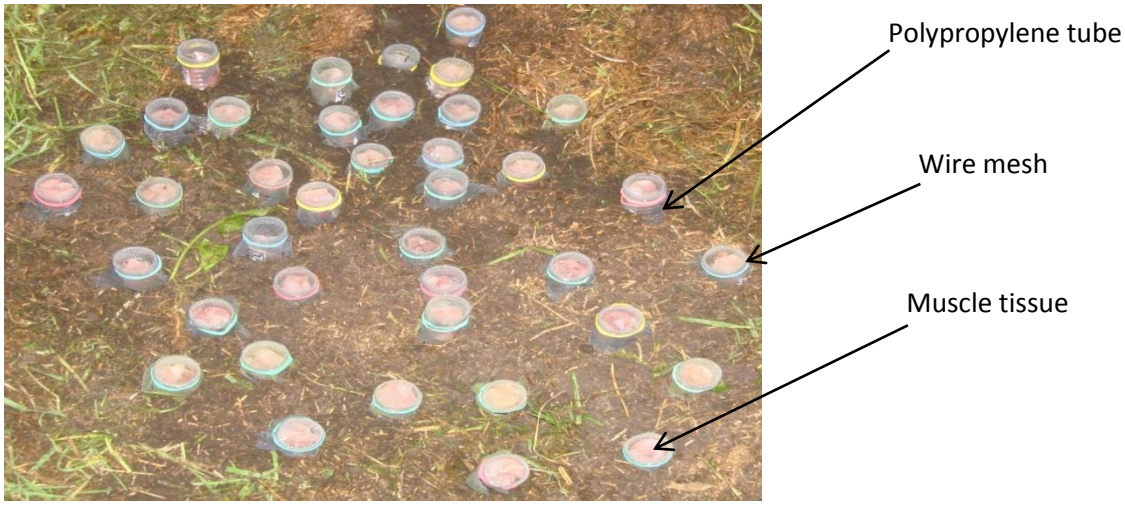

Figure 2.2 Shown above is an example of rabbit suspended muscle tissues (insect activity free) at TRACES (1 cm^3 muscle tissues were placed inside 250 ml polypropylene tubes and covered by wire mesh and kept near the carcasses).

Figure 2.3 Shown above is an example of the pig whole carcases at TRACES.

Figure 2.4 Shown above is an example of the pig body fragments at TRACES**.**

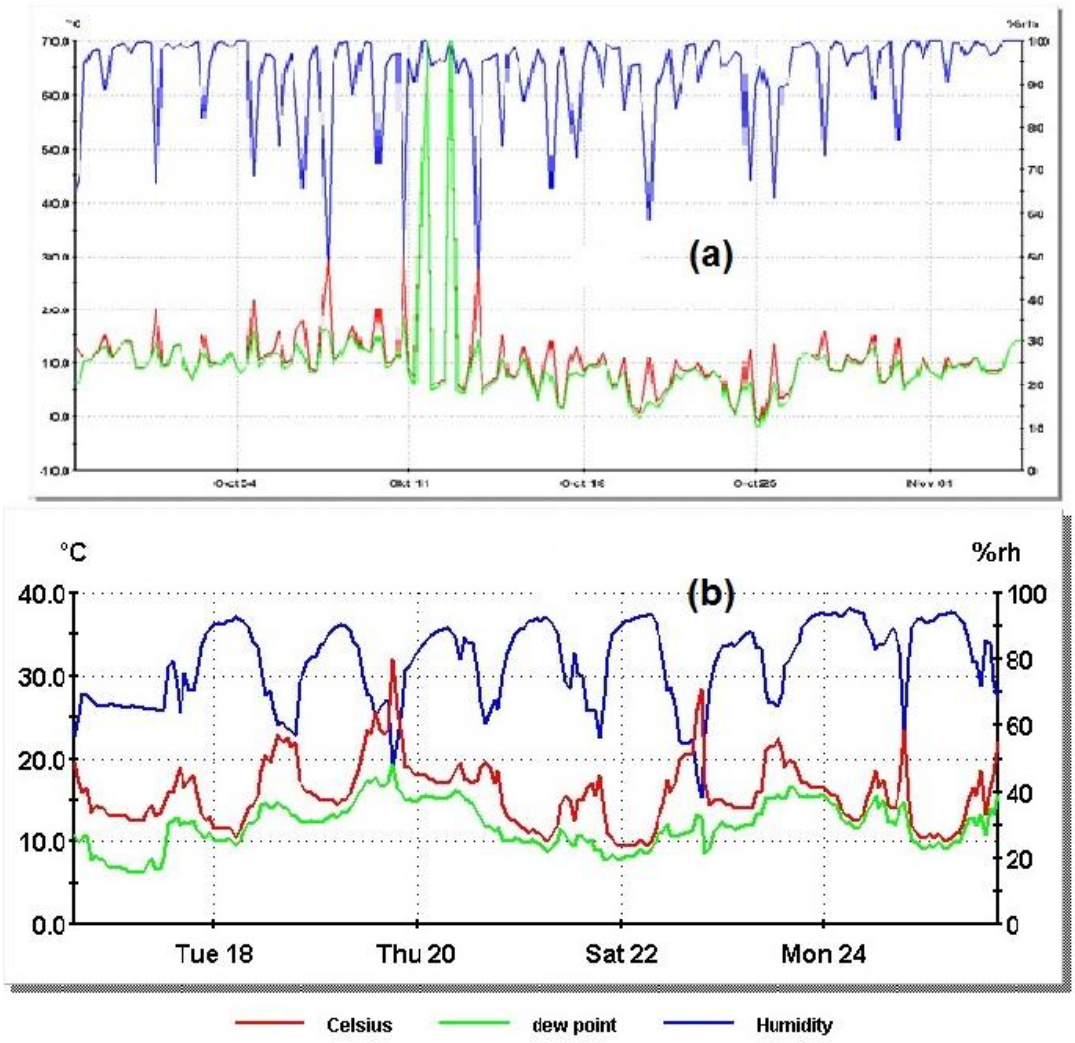

Figure 2.5 Shown above are examples of ambient temperature and humidity records using data-logger during pig and rabbit field experiments.

Table 2.1 Shown below is the summary of the field projects performed at different time points using pig and rabbits to assess DNA persistence in muscle tissues experiments.

Field Projects	Species		ADD	Project Length (days)
	Rabbit	Pig		
August 09	Whole carcasses Body fragments Suspended tissues	–	14-520	44
Feb 10	_	Whole carcasses	34-603	90
May 10	_	Whole carcasses	77-338	27
June 10	whole carcasses	_	13-64	7
Sept 10	whole carcasses Body fragments Suspended tissues	Whole carcasses Body fragments Suspended tissues	49-490	44

2.2.1 August-September 2009

This project was started on 16th August 2009 and completed on 29th September 2009. The experiment was designed to determine the relative rate of decomposition and DNA persistence in whole carcases, body fragments and insect activity free tissues (suspended soft muscle tissues) of rabbit. The whole carcases and body fragments were completely decomposed after one week (112 ADD) with no soft muscle tissue samples to collect at day nine. Suspended tissues were collected until day 44 (520 ADD).

2.1.1.1 Whole Carcasses

A total of 12 triplicate sets of rabbit carcasses (36 carcasses) were placed in direct contact with the ground.

2.1.1.2 Body Fragments

A total of 12 triplicate sets of rabbit rear legs (36 rear legs) from 18 rabbits were separated from the rest of the body and were placed alongside the whole carcasses in direct contact with the ground.

2.1.1.3 Suspended Muscle Tissues

A total of 33 soft muscle tissue samples; each approximately 1 cm^3 were placed inside 50 ml polypropylene tubes that were open to air but protected from insects by a wire mesh. These tubes were inserted into the ground near the whole carcasses.

2.1.1.4 Positive Control

A total of 6 samples from 3 rabbits were collected to act as positive control at day zero. These samples were stored at -20 °C until DNA extraction.

2.1.1.5 Sample Collection

Soft muscle tissue samples (1 cm^3) were collected in triplicate from whole carcasses, body fragments and suspended tissues (that were isolated from insect activity). Samples were collected at 14, 48, 81 and 112 ADD from whole carcases and body fragments. There were no soft tissues to collect after day 7 (112 ADD). Suspended tissues were collected at 14, 48, 81, 112, 141, 172, 228, 257, 350 and 520 ADD.

Table 2.2 Shown below are the accumulated degree-days and humidity records obtained during August-September 2009 rabbit field experiments.

Date of sample collection	Sample collection				Accumulated degree-days (ADD)	Relative Humidity (rh%)
	Number of days	Whole carcasses	Body fragments	Suspended muscle		
17/08/2009	1	+	+	+	14	79
19/08/2009	3	+	+	+	48	69
21/08/2009	5	+	+	+	81	76
23/08/2009	7	+	+	+	112	69
25/08/2009	9	-	-	+	141	86
27/08/2009	11	-	-	+	172	73
04/09/2009	17	-	-	+	228	85
08/09/2009	21	-	-	+	257	91
14/09/2009	29	-	-	+	350	92
29/09/2009	44	-	-	+	520	94

"+" denotes sample collection and "-" represents that samples were not available.

2.2.2 February-May 2010

In this project, whole pig carcases were used to determine the DNA persistence and decomposition rate in pig whole carcases during winter. The total duration of this project was 90 days (11th February-12th May 2010). Accumulated degree-days (ADD) were calculated based on ambient temperature records.

2.2.2.1 Whole carcasses

Five pig carcases were placed at TRACES in winter.

2.2.2.2 Sample collection

Soft muscle tissue samples (approximately 1cm^3) were collected in triplicate from pig whole carcases at 34, 57, 88, 185, 302, 367, 418, 494, 554 and 603 ADD.

Table 2.3 Shown below are the accumulated degree-days obtained during February 2010 pig field experiments.

Date of sample collection	Number of days	Sample collection from whole carcasses	Accumulated degree-days (ADD)
25/02/2010	14	+	34
04/03/2010	21	+	57
12/03/2010	29	+	88
24/03/2010	41	+	185
09/04/2010	57	+	302
16/04/2010	64	+	367
23/04/2010	71	+	418
29/04/2010	77	+	494
06/05/2010	84	+	554
12/05/2010	90	+	603

''+'' denotes sample collection and ''-'' represents that samples were not available.

2.2.3 May-June 2010

To determine the rate of DNA degradation in muscle tissues during summer, pig carcases were exposed to the outside environment at TRACES for a period of 27 days between May 22 to June 18, 2010. There was complete skeletisation in 33 days, at which point no soft muscle tissues were present.

2.2.3.1 Whole Carcases

A total of 24 whole pig carcases were exposed to the outside environment at TRACES. Half of the carcases (12) were slashed on the neck to determine the rate of DNA degradation and decomposition in slashed verses unslashed animals (controls).

2.2.3.2 Sample Collection

Soft muscle tissue samples (approximately $1cm^3$) were collected from pig carcases at 77, 159, 203, 249, 295 and 338 ADD (day 27). Five different parts (neck, right foreleg, left foreleg, right leg and left leg) of the pig carcases were selected for sample collection.

Table 2.4 Shown below are the accumulated degree-days obtained during May 2010 pig field experiments.

Date of sample collection	Number of days	Sample collection Whole carcases	Accumulated degree-days (ADD)
28/05/2010	6	+	77
04/06/2010	13	+	159
07/06/2010	16	+	203
11/06/2010	20	+	249
15/06/2010	24	+	295
18/06/2010	27	+	338

''+'' denotes sample collection.

2.2.4 June 2010 (Summer Season)

In this project, rabbit carcases were exposed to the outside environment to assess DNA persistence in soft muscle tissues of rabbit during summer for a period of 21 days (14^{th} June -5^{th} July 2010).

2.2.4.1 Whole Carcases

36 rabbit carcases (12 triplicate sets) were placed at TRACES.

2.2.4.2 Sample Collection

Soft muscle tissue samples (1 cm^3) were collected at 0, 13 and 64 ADD (day 4) from five different parts (neck, right foreleg, left foreleg, right leg and left leg) of rabbit carcases to assess relative DNA degradation. Soft muscle tissues were decomposed within a week and there were no samples to collect at day 8.

Table 2.5 Shown below are the accumulated degree-days (ADD) obtained during June 2010 rabbit field experiments.

Date of sample collection	Number of days	Sample collection		Accumulated degree-days (ADD)
		Positive controls	Whole carcases	
14/06/2010	0	+	+	0
15/06/2010	1	-	+	13
18/06/2010	4	-	+	64
22/06/2010	8		-	106

''+'' denotes sample collection and ''-'' represents that samples were not available.

2.2.5 September-November 2010

2.2.5.1 Whole Carcasses

Three pigs and 60 rabbit whole carcases were placed at TRACS during autumn.

2.2.5.2 Body Fragments

Rabbit rear legs and pig body fragments (rear legs, front arms and neck) were placed near the whole carcases under separate wire cages.

2.2.5.3 Muscle Tissues

Rabbit and pig $1cm^3$ soft muscle tissue samples were put inside each of 50 ml polypropylene tubes that were open to air but protected from insects by a wire mesh. These tubes were left near whole carcasses.

2.2.5.4 Sample Collection

Muscle tissue samples were collected from five different parts (neck, right foreleg, left foreleg, right leg and left leg) of the pig and rabbit whole carcases. Samples were also collected in triplicate from both body fragments and insect activity free muscle tissue of rabbit and pig carcases. Day zero samples were collected to work as positive controls and for controlled incubation (27 °C, 37 °C and 47 °C) experiments (Section 2.5). Day zero positive controls and partially decomposed tissues of rabbit (45 ADD and 79 ADD)

and pig (79 ADD and 210 ADD) were also collected for DNA preservation experiments (Section 2.6). The temperature (ambient) and humidity were recorded by data loggers.

Table 2.6 Shown below are the accumulated degree-days and humidity records obtained during September 2010 pig field experiments.

Date of sample collection	Sample collection				Accumulated degree-days (ADD)	Relative Humidity (rh%)
	Positive Controls	Whole carcases	Body fragments	Suspended muscle tissues		
06/10/2010	+	+	+	+	106 ADD	99
08/10/2010	-	+	+	+	137 ADD	90
12/10/2010	-	+	+	+	210 ADD	79
15/10/2010	-	+	+	+	254 ADD	97
19/10/2010	-	+	+	+	288 ADD	92
27/10/2010	-	+	+	+	342 ADD	96
04/11/2010	-	+	-	+	420 ADD	99
11/11/2010	-	+	-	+	490 ADD	98

‘‘+’’ denotes sample collection and ‘‘-’’ represents that samples were not available.

Table 2.7 Shown below are the accumulated degree-days and humidity records obtained during September 2010 rabbit field experiments.

Date of sample collection	Sample collection				Accumulated degree-days (ADD)	Relative Humidity (rh%)
	Positive Controls	Whole carcases	Body fragments	Suspended muscle tissues		
27/09/2010	+	-	-	-	0	57
01/10/2010	-	+	+	+	49	65
04/10/2010	-	+	+	+	79	92
06/10/2010	-	+	+	+	106	99
08/10/2010	-	+	+	+	137	90
12/10/2010	-	+	+	+	210	79
15/10/2010	-	+	+	+	254	97
19/10/2010	-	-	+	+	288	92
27/10/2010	-	-	-	+	342	96
04/11/2010	-	-	-	+	420	99
11/11/2010	-	-	-	+	490	98

‘‘+’’ denotes sample collection and ‘‘-’’ represents that samples were not available.

2.3 DNA Extraction

DNA extraction was carried out using DNeasy® Blood and Tissue kit according to the manufacturer's instructions. Initial experiments were carried out using 200 mg tissue but results suggested that 25-30 mg tissue provided consistent results. Henceforth, muscle tissue samples (approximately 25 mg - 30 mg) were cut into small pieces using a sterile scalpel (Swann-morton®, UK) and placed into 1.5 ml Eppendorf tubes (ELKay, UK). Buffer ATL (180 µl) and proteinase k (20 µl) from the DNeasy® Blood and Tissue kit were added to the sample. The samples were mixed thoroughly by vortexing and incubated at 56 °C until the tissues were completely digested (overnight). During incubation, samples were also vortexed occasionally. After incubation, buffer AL (200 µl) was added to the samples and mixed by vortexing. Then (200 µl) ethanol (96%) were added and mixed again thoroughly by vortexing.

The samples were briefly centrifuged for 15 s to remove drops from inside the lid of the microcentrifuge tube. The mixture was transferred in to a DNeasy® Mini spin column placed in a 2 ml collection tube, centrifuged at 13200 *g* for 1 min. The flow-through and collection tube were discarded. The DNeasy® mini spin columns were placed into a new 2 ml collection tube and 500 µl of buffer AW1 were added. The samples were again centrifuged at 13200 *g* for 1 min; flow-through and collection tubes were discarded. Buffer AW2 (500 µl) was added into spin column after placing them into a new collection tube. The samples were centrifuged at 13200 *g* for 3 min and flow-through and collection tube was discarded.

The mini spin column was placed in a clean 1.5 ml centrifuge tube and 200 µl AE buffer was added. The samples were incubated for 1 min at room temperature and then centrifuged at 13200 *g* for 1 min. The samples were labelled and stored at -20 °C until further use.

2.3.1 Agarose Gel Electrophoresis (AGE)

The quality of the extracted DNA samples was assessed using agarose gel electrophoresis (AGE). AGE was conducted using a 2% (w/v) SeaKem® LE agarose gel in a tray tank (12 cm × 6 cm) which was submerged under 1X TAE buffer (per 1000 ml: 4.84 g Tris Base, 1.14 ml glacial acetic acid and 2 ml 0.5 EDTA, pH 8.0). DNA samples were prepared as follows: 7 µl of extracted DNA samples were separately placed into tubes, 3 µl 6 X gel loading buffer (ABgene) was added into each

DNA sample. These samples were briefly centrifuged and loaded in to the wells. Similarly, 5 µl (0.05 µg/µl) of 100 bp DNA ladder (ABgene) with Reddy Run dye was also loaded into the gel. The gel was run at 50 V for 40 min, stained in 0.5 µg/ml ethidium bromide and visualized using a UV transilluminator (Bio Doc-It™ imaging system, USA).

2.4 DNA Quantification

2.4.1 Real-Time PCR base DNA Quantification

2.4.1.1 DNA Quantification of Rabbit and Pig Day Zero Samples using GoTaq®

DNA samples that were extracted from day zero (positive controls) muscle tissues of rabbit and pig were quantified using ABI 7500 real-time PCR machine (Applied Biosystems) to establish amounts of DNA for subsequent experiments.

The total reaction volume was 12.5 µl, which comprised of 6.25 µl of 2x GoTaq® qPCR Master Mix (Promega®, UK), 4.75 µl H_2O, 0.25 µl (0.2 µM) of each forward and reverse primer for 70 bp product (Section 2.7.1) and 1 µl of DNA from pig and rabbit positive controls. DNA dilutions (68.25 ng, 22.75 ng, 7.58 ng, 2.52 ng, 0.84 ng, 0.28 ng, 0.09 ng and 0.031 ng) were made using commercially available human DNA standard (273 ng/µl, Promega®, UK).

The reaction was prepared as a master mix. A MicroAmp™ optical 96-well reaction plate (Applied Biosystems) was placed on its base (MicroAmp™ splash free 96 well base) and 11.5 µl of master mix were loaded separately into each well. Then 1 µl of each dilution (human DNA standard) was loaded into corresponding well: each standard and unknown sample was set up in triplicate. 1 µl of day zero DNA sample was loaded in corresponding wells separately from pig and rabbit. After loading DNA samples, the plate was sealed with an optical adhesive cover (Applied Biosystems). The plate was centrifuged with 96 well plate centrifuge machine (Applied Biosystems) to remove air bubbles and drops from the sides of the tubes.

The plate was placed into the ABI 7500, which was already prepared for running DNA quantification. The thermal Cycler protocol was performed according to the manufacturer's instructions (Promega®, UK): stage 1, 95 °C for 2 min for 1 cycle; stage

2 at 95 °C for 15 s followed by 60 °C for 1 min for 40 cycles DNA concentration for each sample was estimated in ng/µl.

2.4.1.2 DNA Quantification of DNase Digested Human DNA using Quantifiler®

DNase I digested DNA (Section 2.9.5) was quantified using Quantifiler® Human DNA Quantification Kit (Applied Biosystems) according to the manufacturer's instructions on the ABI 7500 real-time PCR machine (Applied Biosystems). DNA standards (50 ng-0.02 ng/µl) were prepared using human DNA provided in the kit and run in duplicate. 12.5 µl reaction mixture was prepared for each unknown sample by mixing 5.25 µl human primer mix, 6.25 µl PCR mix (provided in the kit) and 1 µl of DNA from each time point (0, 5, 10, 15, 20, 30, 45, 60, 90, 120 and 180) min. The cycling parameters were; 95 °C for 10 min (1 cycle), 95 °C for 15 s and 60 °C for 1 min (40 cycles). DNA concentration for each sample was estimated in ng/µl.

2.4.1.3 DNA Quantification of Field Study Samples Using PicoGreen®

A PicoGreen dsDNA quantification kit, containing Quant-iT™ PicoGreen dsDNA reagents and Lambda DNA standard (100 µg/ml in TE) was purchased from Invitrogen™, UK. 96 well flat bottom black plates were obtained from Thermo Fisher Scientific, UK. Tris-EDTA buffer solution (TE buffer, 1.0 M Tris-HCL pH 8.0, containing 0.1M EDTA) and nuclease free water was purchased from Sigma-Aldrich®, UK.

Before being added to DNA standards and unknown samples, PicoGreen solution was diluted 200 fold using 1 x TE; 50 µl PicoGreen stock solution was added to 9.95 ml of 1 x TE buffer. DNA standard supplied with the kit (100 µg/ml) was diluted 50 fold using 1 x TE to make a working solution of 2 µg/ml; 15.6 µl DNA standard solution was added to 764 µl of 1 x TE buffer.

The working solution (2µg/ml) was used to make a series of dilutions ranging from 0.05 to 1000 ng/ml of lambda DNA using TE (Table 2.8). Equal volumes (150 µl) of DNA dilutions and working PicoGreen dsDNA quantification reagents (Table 2.8) were mixed and incubated at room temperature for 1 min. Following incubation, 300 µl aliquots were pipetted into microplate wells in duplicate. Unknown samples were prepared by mixing 3 µl DNA with 147 µl 1x TE and 150 µl PicoGreen working solution and pipetted (300 µl) into microplate wells in triplicate.

Fluorescence was determined using TECAN GEnios Pro plate reader at absorption and emission wavelengths of 485 and 535 nm respectively. Data was obtained in the form of RFU (Relative Fluorescent Unit) on a spread sheet. The reading from a blank well was subtracted from each well before analysing the data.

Table 2.8 Shown below are the DNA standard dilutions to generate standard curve using Quant-iT™ PicoGreen dsDNA kit for DNA quantification.

Volume of diluted PicoGreen (µl)	Volume of 2 µg/ml DNA (µl)	Volume of TE (µl)	Final DNA Concentration (ng/ml)
150	150	0	1000
150	120	30	800
150	60	90	400
150	30	120	200
150	15	135	100
150	6	144	40
150	3	147	20
150	1.5	148.5	10
150	0.15	149.85	1
150	0.075	149.925	0.5
150	0	150	0

2.5 DNA Degradation under a Controlled Environment

2.5.1 Experimental Design

Muscle tissue samples (1 cm^3) were obtained from the pig and rabbit carcases (Section 2.2.5.4) at day zero (0 ADD). These samples were placed individually inside each of 50 ml polypropylene tubes and were incubated at 27 °C, 37 °C and 47 °C for a period of 21 days. Temperature and humidity were recorded using USB data loggers (Figure 2.7 and 2.8).

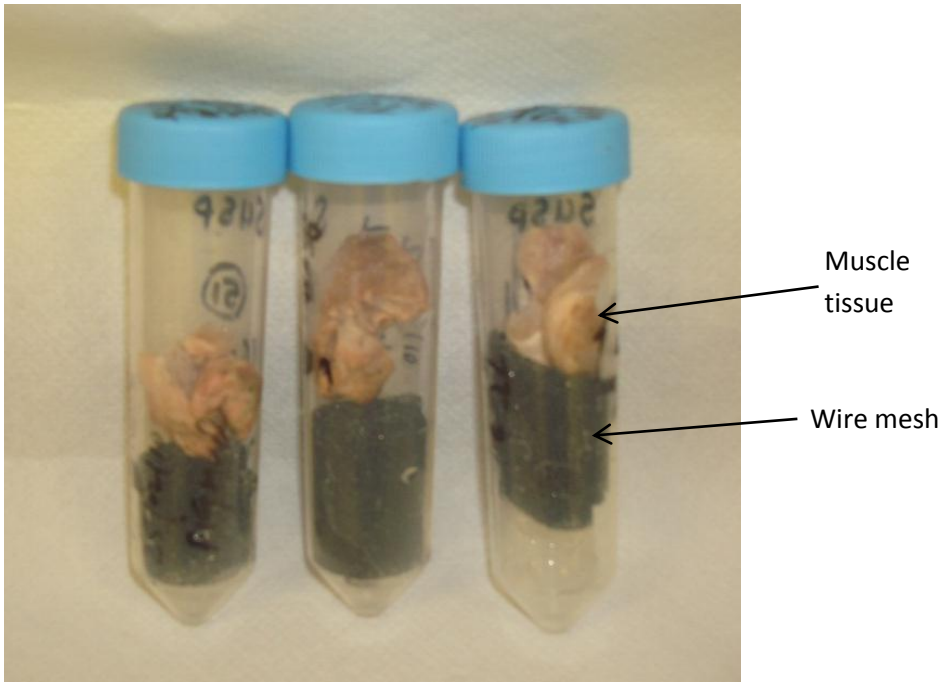

Figure 2.6 Shown above is an example of the pig soft muscle tissue samples used for incubation at 27 °C, 37 °C and 47 °C. Wire mesh was placed to prevent tissue samples from becoming submerged in water. An extra piece of wire mesh was placed above the tissue sample during field experiments to prevent insect access (Figure 2.2).

2.5.2 Sample Collection

25-30 mg soft muscle tissue samples of pig and rabbit were collected at day 1, 3, 5, 7, 9, 11, 13, 15, 17, 19 and 21. These samples were extracted immediately.

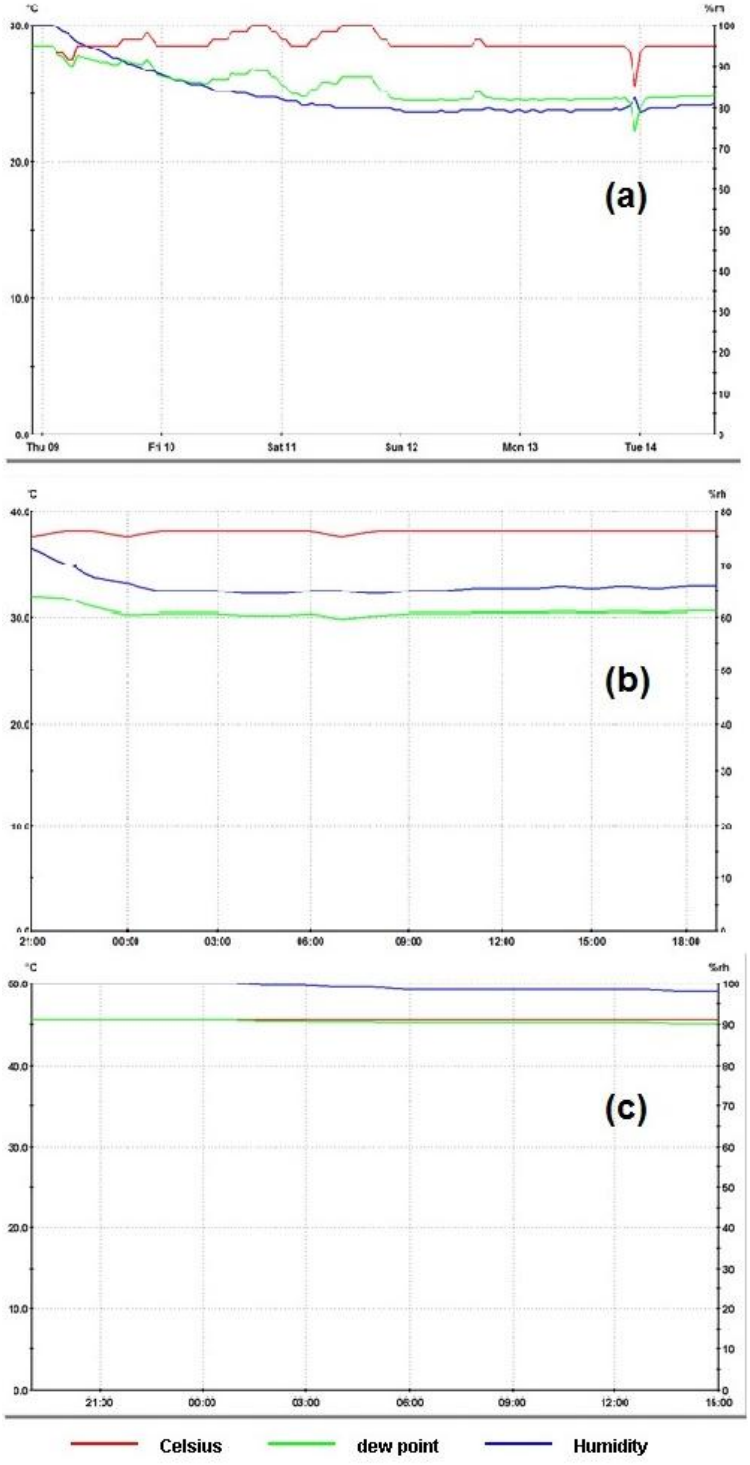

Figure 2.7 Shown above is an example of data recorded during rabbit controlled incubation experiment using USB data logger, a) 27 °C, b) 37 °C and c) 47 °C.

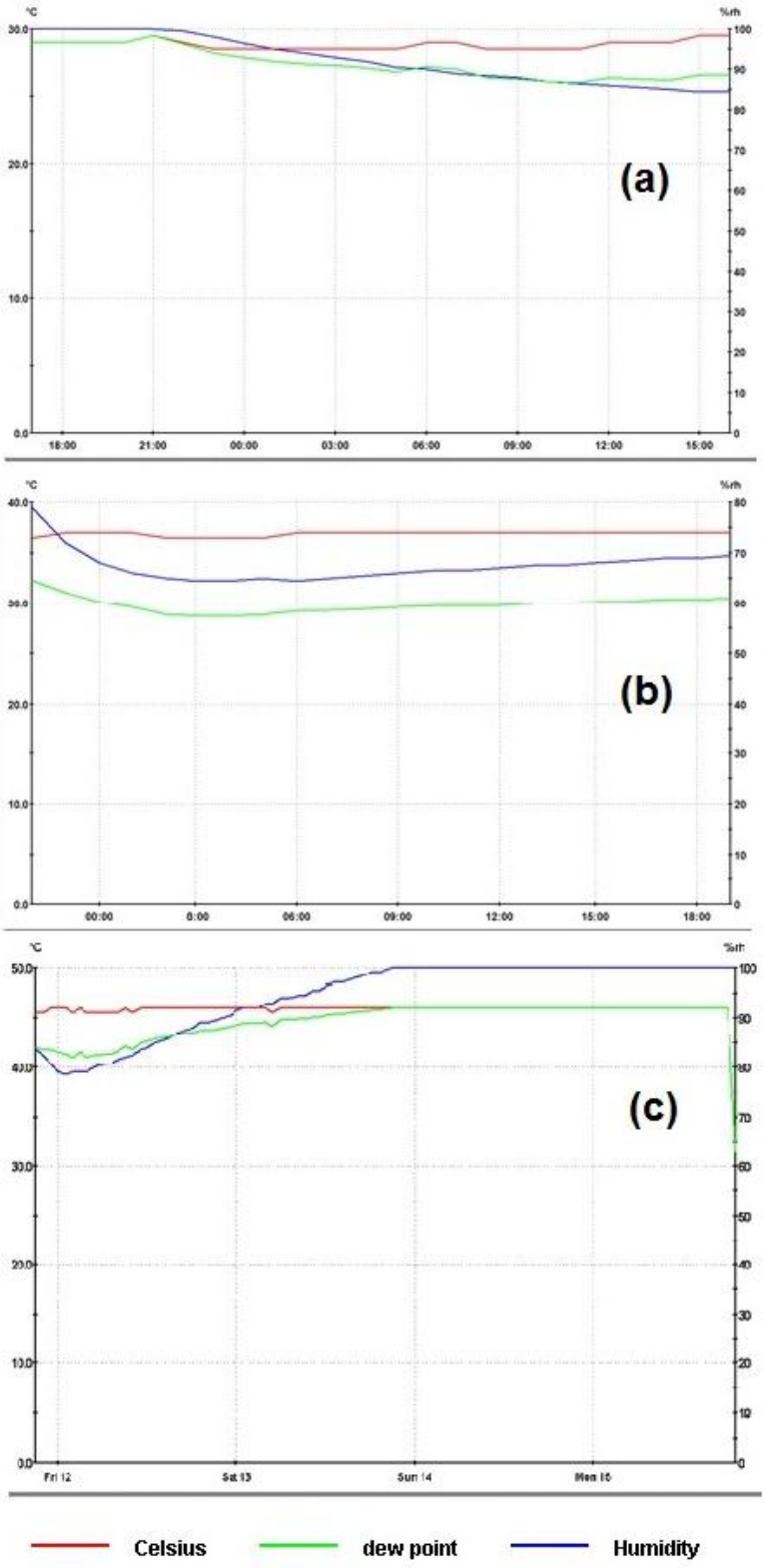

Figure 2.8 Shown above is an example of data recorded during pig controlled incubation experiment using USB data logger, a) 27 °C, b) 37 °C and c) 47 °C.

2.6 DNA Preservation in Soft Muscle Tissue

2.6.1 Preservation Methods

Room temperature DNA preservation methods for soft muscle tissue samples were identified through a review of the available literature: 96% ethanol, 10% buffered formalin, cell lysis solution with and without 1% sodium azide (Muralidharan and Wemmer 1994; Schultz et al., 1999). Buffered formalin (10%), ethanol (96%) and sodium azide was purchased from Sigma-Aldrich®, UK, while cell lysis solution was obtained from Qiagen®, UK.

2.6.2 Preservation of Samples

Pig and rabbit soft muscle tissue samples were used for DNA preservation study. The samples (1 cm^3) were collected at different time points from pig (79 and 210 ADD) and rabbit (45 and 79 ADD) whole carcases which were placed in direct contact with the ground at TRACES (Section 2.2.5.4) and were covered by a wire mesh to prevent scavenger access. Samples were also collected at 0 ADD (day 0) to serve as positive controls for DNA preservation.

2.6.3 Experimental Design

Samples weighing approximately 1 g and 0.5 g were placed into 50 ml polypropylene tubes containing 5 ml and 0.25 g samples were placed into 1 ml of preservative solutions (ethanol, formalin, cell lysis solution (with and without sodium azide)) . Day zero soft muscle tissue samples were also preserved in the same way. These samples were placed at room temperature and average daily temperature was recorded using a USB data logger.

2.6.4 Sample Collection

25-30 mg samples were collected from preserved soft muscle tissues of pig and rabbit at different time points (1, 6 and 12 months). Appropriate volumes (125 µl for 1 g, 250 µl for 0.5 g and 100 µl for 0.25 g) of cell lysis solutions (with and without sodium azide) were also collected at 6 months and one year for DNA extraction.

2.7 Development of a PCR Multiplex to Assess DNA Degradation

The aim was to design a multiplex that would contain amplicons that span the amplicon size range of current STR kits, so that DNA degradation could be assessed. A critical element of the PCR was that it should work with pig, rabbit and also human DNA.

2.7.1 Design of PCR Primers

Sequence data for a nuclear gene (RAG-1) from rabbit, pig and human was aligned to identify conserved regions for primer design: primers that would amplify 70 bp, 194 bp, 305 bp and 384 bp amplicons from pig, rabbit and human were identified. These primers were designed using the publicly available software Primer3 (primer3_www.cgi) and the oligonucleotide property calculator software (http://www.basic.northwestern.edu/biotools/oligocalc.html).

The annealing temperatures for all primer pairs were 60 °C ± 3 °C and GC content were in the range of 40-60%. A 4-plexset of primer pairs were checked collectively for the presence of any complementarity and hairpin formations using Autodimer software (www.cstl.nist.gov/div831/strbase/AutoDimerHomepage/DownloadPage.htm).

The self-complementarity of individual primers was assessed using the National Centre for Biotechnology Information (NCBI) Primer-BLAST (basic local alignment search tool) program (www.ncbi.nlm.nih.gov/Entrez/).

2.7.2 Primer Synthesis

Primer pairs both with and without fluorescein dye modifications at 5′ end of forward primers were synthesized by Invitrogen™ and were delivered desalted and lyophilized. 100 micromolar (µM) stock solutions were prepared by adding the appropriate volume of 1X TE buffer (1.0 M Tris HCl, 0.1 M EDTA, pH 8.0; Sigma, UK) and stored at -20 °C, while an aliquot of a 10 µM working solution was kept at 4 °C for regular use.

2.7.3 PCR Optimisation

2.7.3.1 PCR Primer Optimization for Singleplex Reactions

Singleplex reactions were tested using designed primer sets to assess locus-specific amplification. Each primer pair (70 bp, 194 bp, 305 bp and 384 bp) was optimized for at

different temperatures (Table 2.8) using varying levels of $MgCl_2$ and primer concentrations. PCR was carried out in a GeneAmp® 2700 (Applied Biosystems).

Human genomic DNA was obtained from Promega®, UK (273 ng/µl, 10 mM Tris-HCl (pH 8.0), 1mM EDTA), Dilutions of different DNA concentrations (2.80 ng, 0.93 ng, 0.31 ng, 0.10 ng and 0.03 ng/µl) were made using 5 µl of human genomic DNA with appropriate volumes of 1X TE buffer. These dilutions were stored at -20 °C for further use. 1 µl of each DNA template (2.80 ng, 0.93 ng, 0.31 ng, 0.10 ng and 0.03 ng) was used in separate PCRs for a total reaction volume of 15 µl containing: appropriate concentration (0.05 µM to 0.4 µM) of each primer (70 bp, 194 bp, 305 bp, 384 bp), 7.5 µl of 2X Reddy MiX™ PCR master mix (ABgene® UK) containing Thermoprime plus DNA polymerase (1.25 units), Tris-HCl- pH 8.8 (75 mM), $(NH4)_2$ SO4 (20 mM), Tween® 20 (0.01% v/v) and each dNTP (0.2 mM) and 1.5 mM $MgCl_2$. 0.3 µl (0.5 mM) $MgCl_2$ was also added to get a final concentration of 2.0 mM. The remaining volume for each primer pair was adjusted using dH_2O and a master mix was prepared. Each primer pair was tested using different temperatures (54 ºC-64 ºC), PCR conditions and cycling parameters (Table 2.9 and 2.10).

Table 2.9 Cycling conditions/ PCR Programmes for PCR primer optimisation

	Steps	Program A	Program B	Program C	Program D	Program E	Program F
Stage 1	Initial denaturation	94 °C 3 min	94 °C 3 min	94 °C 3 min	94 °C 3 min	94 °C 3 min	94 °C 3 min
Stage 2	Denaturation	94 °C 1 min	94 °C 1 min	94 °C 1 min	94 °C 1 min	94 °C 1 min	94 °C 1 min
	Annealing	54 °C 1 min	56 °C 1 min	58 °C 1 min	60 °C 1 min	62 °C 1 min	64 °C 1 min
	Extension	72 °C 1 min	72 °C 1 min	72 °C 1 min	72 °C 1 min	72 °C 1 min	72 °C 1 min
Stage 3	Final Extension	72 °C 10 min	72 °C 10 min	72 °C 10 min	72 °C 10 min	72 °C 10 min	72 °C 10 min
Stage 4	Hold[a]	12 °C	12 °C	12 °C	12 °C	12 °C	12 °C

[a] Hold is the final step for PCR till samples are removed from the PCR cycler.

Singleplex PCR programmes were run for 33 cycles, while in case of 4-plex reactions, 30 cycles were used along with different initial denaturation temperature (95 °C for 5 min for Hot Start).

Table 2.10 Shown below are primers concentrations (Set 1-5) used for the optimisation of singleplex and multiplex PCR reactions**.**

PCR Primers (bp)	Primers Concentration (µM)				
	Set 1	Set 2	Set 3	Set 4	Set 5
70	0.2	0.1	0.5	0.05	0.05
194	0.2	0.2	0.2	0.1	0.1
305	0.3	0.3	0.3	0.15	0.15
384	0.4	0.4	0.4	0.2	0.4

2.7.3.2 Optimised Singleplex PCR

PCR for each primer pair was set up individually using following PCR conditions: 94 °C for 3 min, followed by 94 °C for 1 min, 58 °C for 1 min, 72 °C for 1 min for 33 cycles and final extension at 72 °C for 10 min, and hold at 12 °C until samples were removed from the thermocycler.

2.7.3.3 Multiplex PCR optimization

Multiplex (4-plex) PCR optimisations were carried out through primer concentration adjustment and empirical performance testing in an effort to generate maximal sensitivity, balanced peak heights and specific signals for 4 primer pairs in a single reaction. Primers with different concentrations (Set 1-5, Table 2.10) were mixed in a single reaction and optimised using different temperatures (54 ºC to 64 ºC), PCR conditions and cycling parameters (Table 2.9). In a total reaction volume of 15 µl PCR (7.5µl 2X AmpliTaq Gold® PCR master mix (Applied Biosystems);, 250 U (0.05 U/µl) DNA polymerase, 1X GeneAmp® PCR Gold Buffer, 30 mM Tris/HCl, pH 8.05, 100 mM KCl, dNTP, 400 µM each, 5 mM $MgCl_2$, 0.6 µl of primer mix (forward primers 5´ fluorescein labelled), 5.9 µl of dH_2O and 1 µl of human genomic DNA dilutions were used. Dilutions (2.80 ng, 0.93 ng, 0.31 ng, 0.10 ng and 0.03 ng) were made for pig and rabbit day zero positive controls DNA samples which had been quantified using ABI 7500 real-time PCR (Section 2.4.1.1).

2.7.3.4 4-plex Multiplex PCR for Rabbit, Pig and Human Degraded DNA Samples

4-plex Multiplex PCR amplification was performed for pig, rabbit and human DNA samples using following PCR conditions: stage 1; 95 °C for 5 min, stage 2; 94 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min for 30 cycles and final extension at 72 °C for 10 min, and hold at 12 °C until samples were removed from the thermocycler.

2.7.3.5 Gel Electrophoresis of PCR Products

The PCR products of singleplex and multiplex (4-plex) amplification were analysed using a 2% (w/v) SeaKem® LE agarose gel in a tray tank (12 cm × 6 cm) which was submerged under 1X TAE buffer (Section 2.3.1). A 100 bp DNA ladder (ABgene) was used as size standard. 6 µl of amplified PCR products and 5 µl (0.05 µg/µl) of size marker were loaded on the gel. The gel was run at 50 V for 40 min, stained in 0.5 µg/ml

ethidium bromide and visualized using a UV transilluminator (Bio Doc-It™ imaging system, US).

2.8 Capillary Electrophoresis

2.8.1 ABI 310 Prism® Genetic Analyser

The optimisation of singleplex and multiplex PCR products was assessed using an ABI 310 Prism® Genetic Analyser (Applied Biosystems). DNA fragment analysis was carried out using a 47 cm long capillary using POP™- 4 polymer (Applied Biosystems). Electrophoresis running buffer was used at 1X concentration. The GSPOP 4 (1ml) F.md4 run module with dye set DS-32 (filter set F): 5-FAM (blue), JOE (green), NED (yellow) and ROX (red) was used with the following parameters: run temperature 60 °C, syringe pump time 150 s, pre-run voltage 15 kV, pre-run time 120 s, injection time 5 s, injection voltage 15 kV, run voltage 15 kV and run time 30 min.

The PCR products obtained from optimized singleplex PCR reactions (Section 2.7.3.1) were run on ABI 310 Genetic Analyser in a total volume of 11.5 µl: 10 µl of Hi- Di™ formamide, 0.3 µl GeneScan™ Rox-500 internal size standard (Applied Biosystems) and 1µl of PCR product was used. The samples were mixed, briefly centrifuged and then incubated at 95 °C for 5 min. The samples were allowed to cool at 4 °C prior to being run on ABI 310 genetic analyser.

2.8.2 ABI 3500 Prism® Genetic Analyser

Due to the ability to increase throughput of sample processing, DNA fragment analysis was also performed using a ABI 3500 Prism® Genetic Analyser (Applied Biosystems) using a 50 cm capillary array and 3500 POP-6™ polymer. The FragmentAnalysis50_POP6 run module was used in combination with the dye set DS-32 (filter set F): 5-FAM (blue), JOE (green), NED (yellow) and ROX (red) with the following parameters: run temperature 60 °C, pre-run voltage 15 KV, pre-run time 180 s, injection time 10 s, injection voltage 1.6 kV, run voltage 15 kV and run time 2400 s.

Each sample for fragment analysis was prepared by adding 1 µl of PCR product to 10 µl of Hi-Di™ formamide (Applied Biosystems) containing 0.3 µl GS500 ROX size standard (Applied Biosystems). The samples were heated at 95 °C for 5 min, and snap-cooled at least 3 min prior to CE.

2.8.3 Analysis of DNA Profiles

The data obtained from capillary electrophoresis (CE) was analysed using GeneMapper™ ID version 3.2 (ABI 310) and GeneMapper® Software v4.1 (ABI 3500), Applied Biosystems (UK).The parameters for the analysis of the multiplex amplicons were kept constant for each run and were described in Table 2.11.

Table 2.10 Shown below are the parameters for the analysis of PCR fragments.

Parameters	Values
Analysis Range	Full Range
Size Call Range	All Sizes
Size Calling Method	Local Southern
Peak Detection	50 RFU
Baseline Window	51 pts (points)
Minimum Peak Half Width	2 pts
Polynomial Degree	3 pts
Peak Window Size	15 pts
Slope Threshold for peak start/end	0-0

2.9 Validation of Multiplex (4-plex) PCR for analysis of DNA Degradation

2.9.1 DNA samples

Human genomic DNA G3041 (273 ng/µl) was obtained from Promega®, UK. Pig and rabbit positive control DNA samples were extracted from soft muscle tissue samples collected at day zero using DNeasy® Blood and Tissue kit according to the manufacturer's instructions. These samples were then quantified in triplicate with the SYBR-Green DNA quantification kit (Promega®, UK) using the manufacturer's recommended protocol using an ABI 7500 real-time PCR machine (Applied Biosystems), (Section 2.4.1.1). After quantification DNA samples were stored at -20 °C.

2.9.2 Sensitivity Study

The sensitivity study was carried out to determine the template DNA range as recommended by the Scientific Working Group on DNA analysis Methods (SWGDAM) for the developmental validation of commercial STR kits (Krenke 2002; Greenspoon et al., 2006; SWGDAM 2004; Asamura et al., 2007). Human genomic DNA (G3041) was amplified using template amounts of 2.80 ng, 0.93 ng, 0.31 ng, 0.1 ng and 0.03 ng. Pig and Rabbit positive control samples (day zero) were also amplified using the same amounts of DNA template (2.80 ng. 0.93 ng, 0.31 ng, 0.1 ng and 0.03 ng) (Section 2.7.3.4). CE was performed on all the samples using ABI 310 genetic analyser (Section 2.8.1).

2.9.3 Stochastic Effects

When PCR amplification is performed with very few DNA template molecules, peak height imbalance (in heterozygote loci) and allelic dropout can occur. To determine the dropouts of PCR amplicons, 18 samples were amplified using a template range of 0.03 ng to 0.1 ng and run on ABI 310 genetic analyser (Section 2.8.1).

2.9.4 Reproducibility and precision

Data from different runs on the ABI 310 genetic analyser were evaluated to determine the consistency of the PCR amplicons during different time points. The consistency of sizing was assessed by looking at the average size and standard deviation of all PCR amplicons for each sample injected.

2.9.5 Degraded DNA Study

The effect of DNA degradation on the amplification efficiency of the 4-plex multiplex was examined according to the protocol described by Swango et al., (2006). A degradation series was prepared by digesting high molecular weight (177 µg/ml) DNA with DNase I (Applied Biosystems) for progressive lengths of time. A reaction containing 15.7 µg DNA, 10X DNase buffer (100 mM Tris, 25 mM $MgCl_2$, 5 mM $CaCl_2$, pH 7.6) and nuclease-free water to bring the reaction volume to 110 µl, was prepared. 10 µl was removed and used as a control sample (0.143µg/µl). DNA in the remaining solution was digested by adding 1.25 µl of DNase I (2U/µl) and incubated at 37 °C. From this solution, 10 µl was removed at 5, 10, 15, 20, 30, 45, 60, 90, 120, and 180 min time points and DNase I activity stopped by mixing 2 µl of DNase inactivation reagent and incubated at 65 °C for 15 min. Samples were then centrifuged at 10,000 x g for 2 min and supernatant transferred to a fresh tube. The degree of DNA degradation was assessed by running 2 µl of digested products on 2% agarose gel using 100 bp DNA ladder (BioLabs®, UK). DNA quantification was performed using the Human Quantifiler® kit according to the manufacturer's instruction (Section 2.4.1.2). PCR multiplexing was performed using 0.6 ng DNA template (Section 2.7.3.4) and 1µl of PCR product was run on ABI 3500 genetic analyser (Section 2.8.2).

2.9.6 Field DNA Samples

Rabbit soft muscle tissue samples were collected at day 1 and day 7 from whole carcases and body fragments (Section 2.2.1), DNA extracted using DNeasy® Blood and Tissue kit (Section 2.3) and 1 µl DNA used for multiplex amplification and amplification success determined by running 1 µl of PCR product on the ABI 310 genetic analyser (Applied Biosystems).

2.9.7 Species Specificity

The specificity of this technique for pig, rabbit and human was checked by including the DNA samples of various insect species (*Calliphora vicina*, *Calliphora vomitoria*, *Protophormia terraenovae*, *Lucilia sericata*, *Lucilia illustris* and *Lucilia caesa*) that are commonly observed on decomposing bodies. Multiplex amplification was performed using both control DNA samples of pig, rabbit and human along with insect species.

2.9 8 Data Analysis

ADD for all field experiments were calculated from the temperature records obtained from data-loggers and weather station near TRACES. Statistical analysis (regression and ANOVA) analysis was performed to access the rate of DNA degradation among different experimental groups in relation to ADD.

CHAPTER 3

DESIGN and VALIDATION of 4-PLEX MULTIPLEX PCR-BASED ASSAY

3.0 Introduction

To assess the degradation of DNA in the model organisms (pig and rabbit) used in this study, it was necessary to develop a new PCR-based assay. It was decided that the best approach was to use a multiplex PCR reaction that would generate PCR products of a similar size to the amplicons typically generated using commercial STR kits. This would allow the degree of DNA degradation within samples to be assessed over a size range that is relevant to DNA profiling.

3.1 Aims of the Study

The specific aims when developing the multiplex were:

1. To develop a 4-plex assay that will amplify PCR products between 70 bp and 384 bp and that the products could be detected using conventional capillary electrophoresis;
2. To select primers that would amplify human, rabbit and pig DNA with equal efficiency;
3. To validate the multiplex assessing reproducibility, sensitivity and performance with high molecular weight and degraded DNA in human, rabbit and pig samples.

3.2. Multiplex Design

3.2.1 Selection of Loci

Single copy recombination activating gene 1 (RAG-1) is involved in somatic V(D)J rearrangement of B and T cell lymphocytes, essential for the development of a normal immune system and its functions. The RAG-1 gene has nearly constant base composition across taxa, without any asymmetry in directional patterns (David et al., 1989; Carlson et al., 1991; Bernstein et al., 1996).

This gene was a suitable target for multiplex amplification due to high levels of similarity in conserved regions between rabbit and human (90%), rabbit and pig (86%) and pig and human (88%).

3.2.2 PCR Primer Design

RAG-1 sequence data for rabbit, pig and human were downloaded from GenBank, aligned using BioEdit version 7.0.5.3 (Hall, 2011) and regions of high similarity selected (Appendix 1).

The 4 primer pairs were designed to generate amplicons of 70 bp, 194 bp, 305 bp and 384 bp across pig, rabbit and human. The predicted annealing temperature of all these primers was 60 ± 3 °C except for the reverse 384 bp primer, which was predicted to be 56 °C (Table 3.1). The G+C content of each primer were kept between 40-60%. Each forward primer was labelled with fluorescein dye at the 5′ end.

Initially the primers were optimised in single PCR reactions and then combined in a multiplex. For the first round of optimisation the primers were amplified using different temperatures (54 °C – 64 °C), primer concentration (0.05 to 0.4 µM) and DNA template amount (0.03 ng to 75.25 ng). The PCR products were assessed using agarose gels.

Table 3.1 Shown below are 4 PCR primer sets for conserved regions of pig, rabbit and human.

Nuclear gene	NCBI Reference	PCR primers Forward and Reverse (5'-3')	PCR Annealing Temperature (C)	Amplicon Length (bp)
Recombination activating gene RAG-1	M77666.1	CCT CAA AGT CAT GGG CAG C	60	70
		GAC TCT CCA GGT CAG TAG G	60	
		GCT GTT TGC TTG GCC ATC CG	63	194
		GTG CTG GAA GAC ACA TTC TTC	60	
		ATG AGG TCT GGC GTT CCA AC	60	305
		TGG TCA TGA GCT TCC TGG CA	60	
		GAG CAA TCT CCA GCA GTC CT	60	384
		GCT AAA CTT CCT GTG CAT GA	56	

Human genomic DNA G3041 (273 ng/µl) was obtained from Promega®, UK. Pig and rabbit positive control DNA samples were extracted from muscle tissue samples collected at day zero. The $MgCl_2$ concentration was kept at 2.0 mM for singleplex and 2.5 mM for multiplex PCR reactions.

The 4 primer pairs were found to function optimally at concentrations of 0.05 µM, 0.1 µM, 0.15 µM and 0.4 µM for 70 bp, 194 bp, 305 bp and 384 bp respectively (Table 3.1 and Figures 3.1 and 3.2).

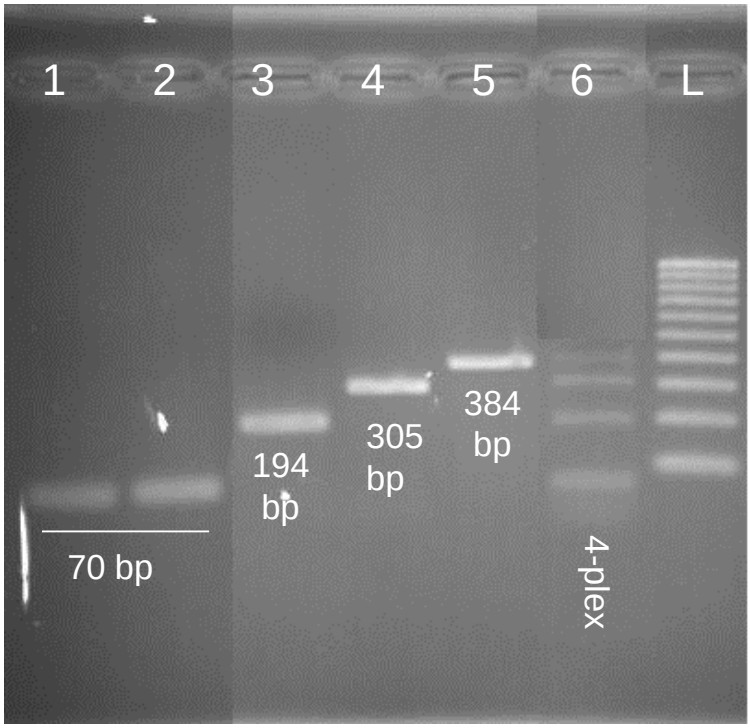

Figure 3.1 Shown above is optimisation of PCR primers, singleplex (Lane 1-5), 4-plex multiplex (Lane 6) and 100 bp DNA ladder (L). The gel is a composite of different agarose gels.

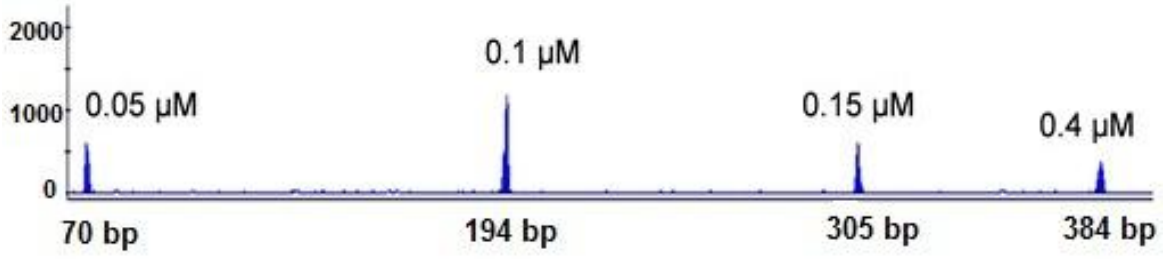

Figure 3.2 Shown above is of electropherogram of 4-plex optimisation of rabbit positive control DNA (day zero). Primer concentrations (µM) are also shown.

3.2.3 ABI 310 Genetic Analysis

The optimised 4-plex PCR reaction was assessed for any non-specific amplification that would lead to extra peaks and interfere with target peaks. Positive control DNA of all three species was evaluated on the ABI 310 (Section 2.8). All four products produced a specific peak for the targeted loci without any additional peaks. There were no extra peaks observed in positive control samples of human, pig and rabbit (Figure 3.2).

3.2.4 Reproducibility and precision

Results from multiple analyses of the positive control samples, DNase I digested samples and samples collected from the field showed full 4-plex multiplex amplification when independently run on the ABI 310 (Figures 3.2, 3.3, 3.4, 3.5, 3.6, 3.7 and 3.8). The standard deviation of the amplicon sizes for multiple runs of the same and different samples was below one base pair for all amplicons. The 70 bp fragment was detected at 64.32 bp ± 0.69 bp; 195 bp at 194.54 bp ± 0.04 bp, 305 bp ± 0.5 bp and 384 bp at 383.9 bp ± 0.05 bp. Different values were also seen when using the ABI 3500 genetic analyser; 70 bp (65.17 bp ± 0.02 bp), 194 bp (195.66 bp ± 0.03 bp), 305 bp (307.38 bp ± 0.60 bp) and 384 bp (384.69 bp ± 0.15 bp). All samples were run in triplicate.

3.2.5 Sensitivity Study

4-plex multiplex PCR reactions were set up using serial dilutions of positive control DNA samples of human (Promega®, UK), rabbit and pig (extracted from day zero soft muscle tissues) which had been quantified using ABI 7500 real-time PCR, using the 70 bp amplicon (Applied Biosystems) (Section 2.4) was carried out. The 4-plex multiplex was found to work efficiently in triplicate samples of all three species down to 0.3 ng of DNA template. There was full amplification of 4-plex in rabbit and pig triplicates down to 0.1 ng DNA template and partial 4-plex profiles with 0.03 ng albeit with small peak heights. In human, there was dropout of 384 bp with 0.1 ng DNA template and complete failure of 4-plex amplification with 0.03 ng DNA templates (Figures 3.3, 3.4 and 3.5).

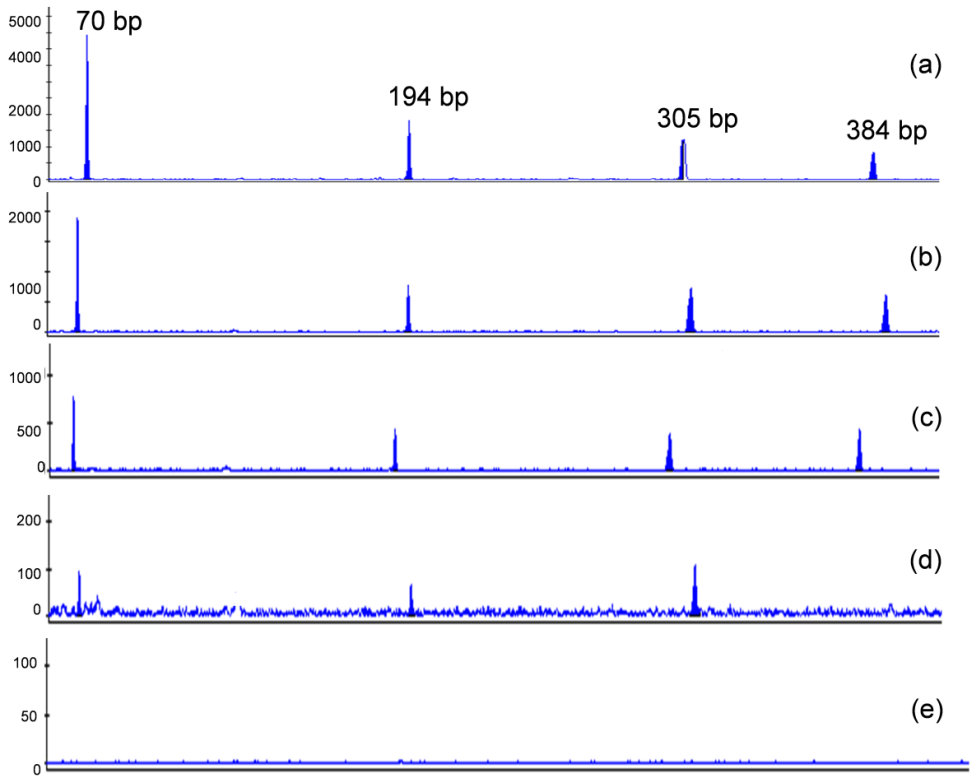

Figure 3.3 Shown above is the electropherograms represents 4-plex PCR with human positive control DNA samples (a) 2.80 ng (b) 0.93 ng (c) 0.31 ng (d) 0.1 ng and (e) 0.03.ng.

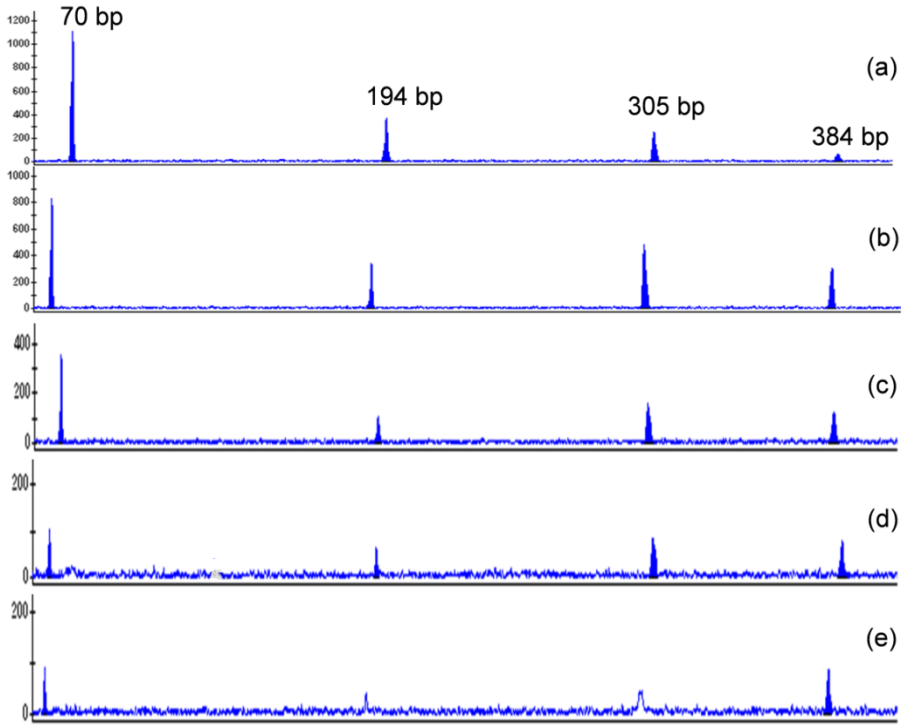

Figure 3.4 Shown above is the electropherograms represents 4-plex PCR with pig positive control DNA samples (a) 2.80 ng (b) 0.93 ng (c) 0.31 ng (d) 0.1 ng and (e) 0.03 ng.

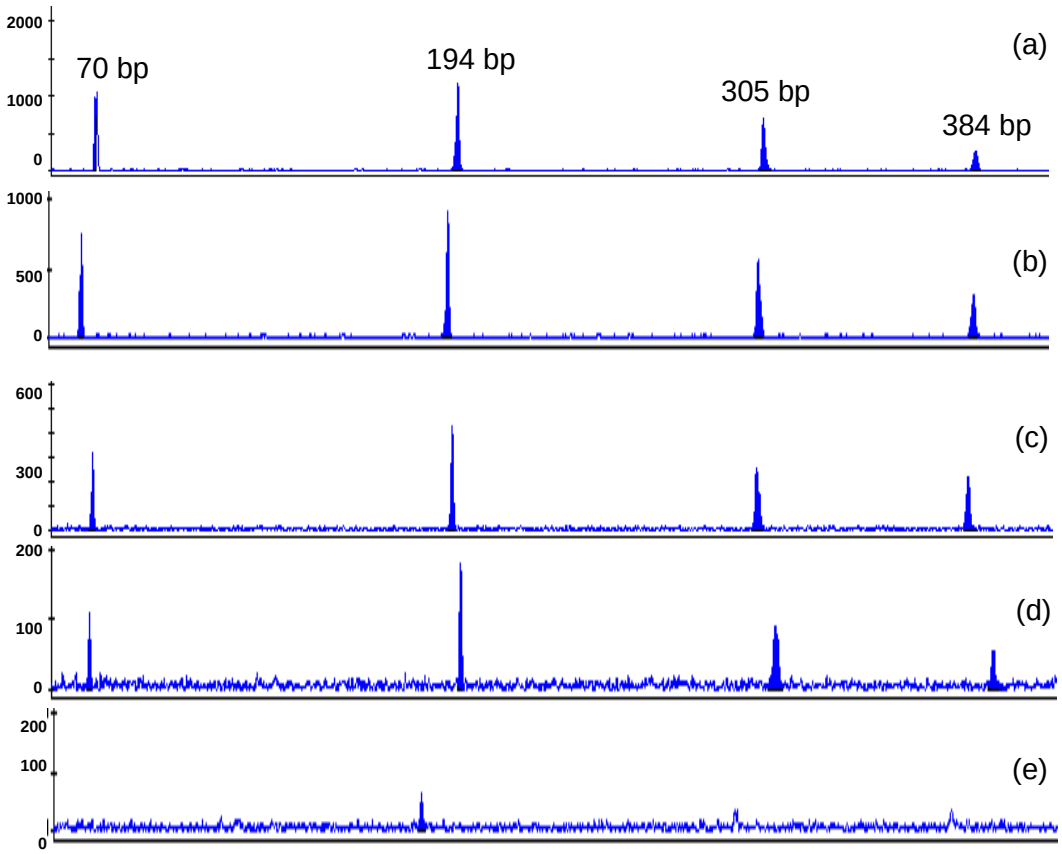

Figure 3.5 Shown above is the electropherograms represents 4-plex PCR for rabbit positive control DNA samples (a) 2.80 ng (b) 0.93 ng (c) 0.31 ng (d) 0.1 ng and (e) 0.03 ng.

3.2.6 Degraded DNA study

To assess the ability of the multiplex (4-plex) assay to quantify DNA in degraded samples, a 10 point degradation series was prepared by treating aliquots of high molecular weight genomic DNA (Promega®, UK) with DNase I for increasing periods of time ranging from 5 to 180 min (Section 2.3.5). The result was a degradation series exhibiting incremental increase in the extent of DNA degradation. 0.6 ng DNA template (based on real-time quantification) (Applied Biosystems) was amplified for the 4-plex multiplex. Volumes were adjusted in order to use 0.6 ng within each amplification and when no DNA was present, maximum volume of sample was used. Complete 4-plex profiles were obtained reproducibly until 30 min of DNase I digestion. The peak heights were higher for those samples with 10 min DNase I activity compared to 30 min. There was complete failure of amplification in all DNA samples digested with DNase I for a period of 45 min or above (Figure 3.6).

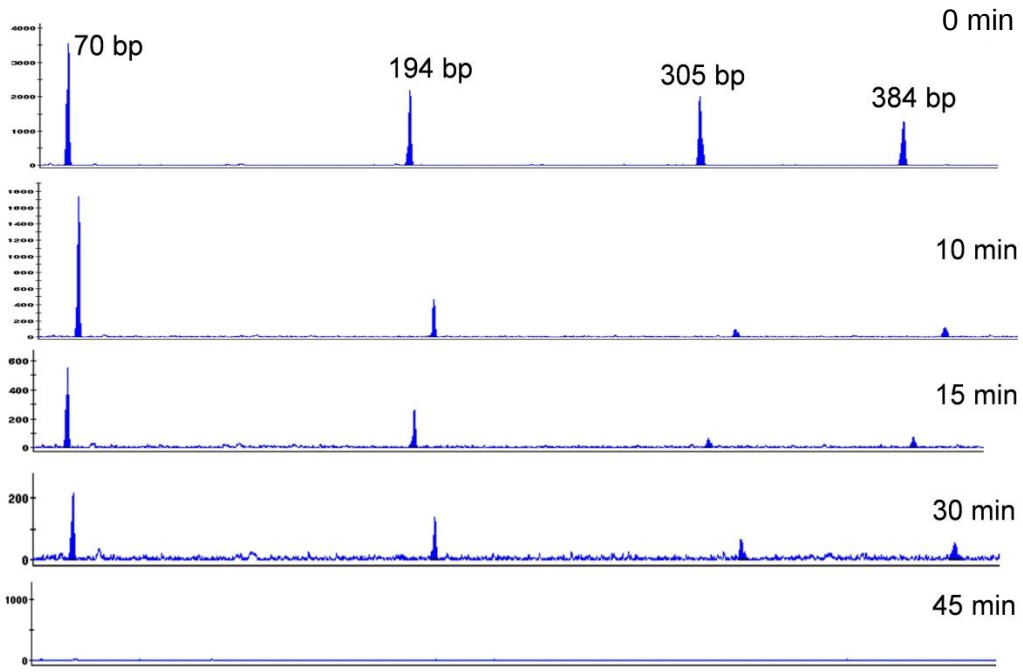

Figure 3.6 Shown above is the electropherograms represents 4-plex PCR from the artificially degraded DNA (DNase I digestion) at 0, 10, 15, 30 and 45 min time points**.**

3.2.7 Field DNA samples

DNA was obtained from muscle tissue samples of rabbit whole carcases and body fragments, collected at 13 ADD and 112 ADD. This multiplex assay amplified all the 4 expected products for degraded DNA samples collected at day 7 (Figure 3.7). Additional field samples were analysed in Chapter 4.

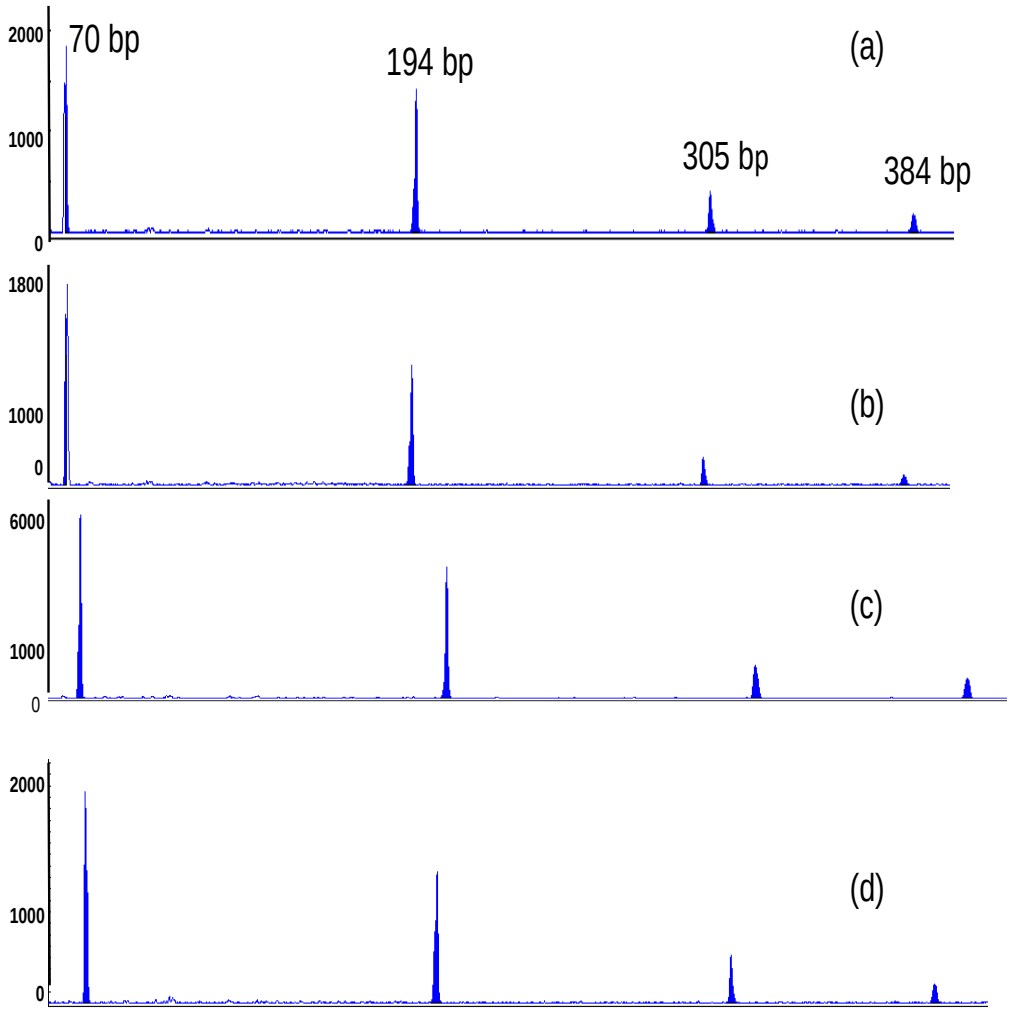

Figure 3.7 Shown above is the electropherograms of rabbit soft muscle tissue samples collected from a and b) body fragments at 13 ADD and 112 ADD, c and d) whole carcases at 13 ADD; d) 112 ADD.

3.2.8 Species Specificity

Various insect species (*Calliphora vicina*, *Calliphora vomitoria*, *Protophormia terraenovae*, *Lucilia sericata*, *Lucilia illustris* and *Lucilia caesar*) play an important role in carcass decomposition (Bachmann and Simmons 2010.) To determine the specificity of multiplex for pig, rabbit and human, and that DNA was not being amplified from insect species. PCR was carried out using approximately 6 ng DNA templates of various insect species, whilst 0.6 ng was used in human, rabbit and pig. No detectable DNA profiles were observed in any of the insect species (Figure 3.8).

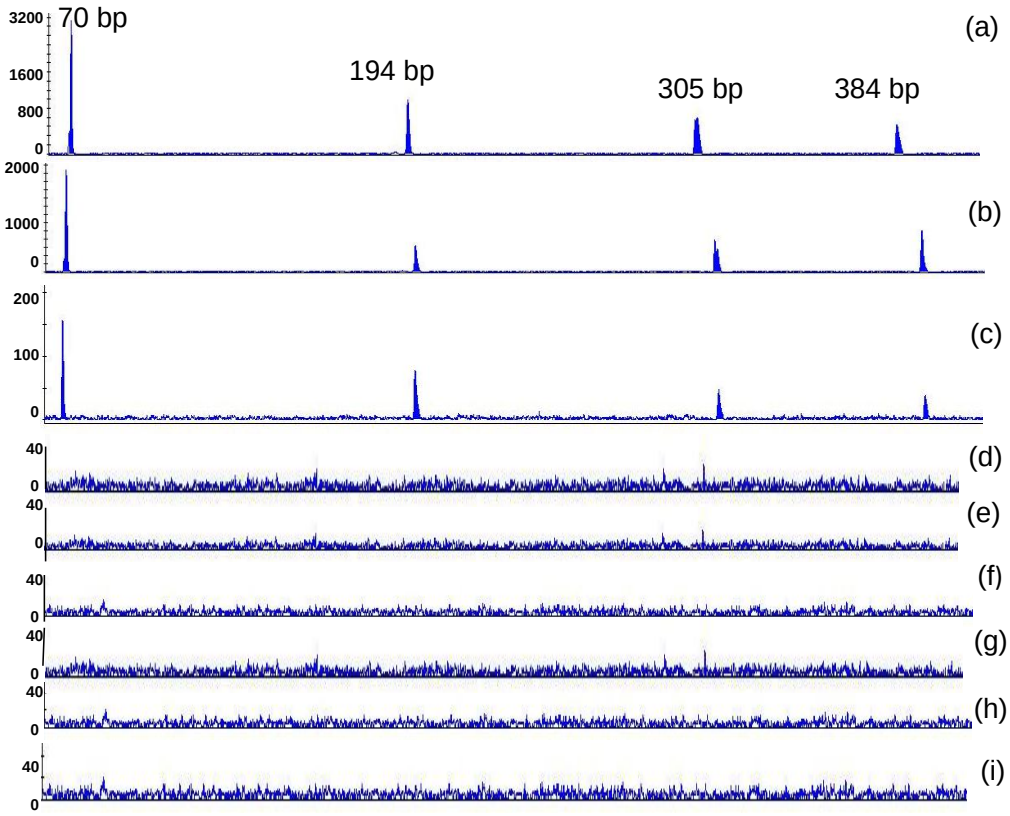

Figure 3.8 Shown above is the amplification of human (a), pig (b) rabbit (c) and insect species (*Calliphora vicina* (d), *Calliphora vomitoria* (e), *Protophormia terraenovae* (f), *Lucilia sericata* (g), *Lucilia illustris* (h) and *Lucilia caesar* (i) using 6 ng DNA template.

3.3 Conclusion

The multiplex (4-plex) PCR technique to determine DNA persistence in pig, rabbit and human was validated. The multiplex requires a very low quantity of DNA template, efficiently amplifies the product from samples that have been exposed to the environment, and produced the expected sized fragment from highly degraded DNA samples. It was found that full 4-plex profiles for human, pig and rabbit can be obtained using a template amount of 0.3 ng. This multiplex can be used in forensic analysis to assess the DNA persistence in human decomposing bodies and in experimental studies.

CHAPTER 4

DNA PERSISTENCE in MUSCLE TISSUES

4.0 Introduction

DNA typing is an important method for the identification of human remains that are found at a crime scene or a mass disaster situations (Jobling and Gill 2004; Butler 2005). In degraded human remains, analysis of DNA recovered from degraded tissue is a great challenge and would result in partial or no DNA profiles (Rerkamnuaychoke 2000; Hoff-Oslon et al., 2001).

Sample collection for DNA analysis is determined by each crime scene or mass disaster scenario. Generally material should be collected to avoid exogenous contamination and body cross-contamination. When bodies are not highly decomposed, preferable samples include deep red muscle tissues (Prinz et al., 2007). Whilst DNA preservation is better in hard tissues, DNA recovery from muscular tissue is simpler and more convenient (Phengon et al., 2008).

The environment has a strong influence on DNA persistence in muscle tissues. The rate of DNA decay increases with increase in temperature; however, there are few systematic studies that have examined the correlation between environmental temperature and DNA persistence. In addition to temperature extremes, temperature fluctuations and seasonal variations also contribute to DNA degradation (Robins and Furry, 2001). In this study, DNA persistence in muscle tissues was assessed in relation to both time and temperature (ADD). Rabbit and pig were used as experimental models and their carcases were exposed to environmental conditions at different seasons of the year. Average daily temperature and humidity was recorded. DNA extraction followed by quantification and PCR using the developed 4-plex multiplex (Chapter 3) was used to assess recovered DNA.

4.1 Aims and Objectives

To test the following hypotheses:

- Temperature (measured in accumulated degree-days) is the dominant environmental factor that governs DNA degradation in a temperate environment.
- DNA degradation in muscle tissue is slower in fragmented remains than whole bodies.
- Insect activity increases carcass decomposition, which results in rapid DNA degradation.

4.2 Results

4.2.1 Samples

Soft muscle tissue samples were collected from pig and rabbit carcasses that were exposed to the natural environment at TRACES at different time points of the year (Section 2.1). Tissue samples were placed out in the form of whole animals, fragmented animals and small (approximately 1 g) tissue fragments in a plastic tube protected from insects by wire mesh, but open to the atmosphere. DNA extraction was carried out using DNeasy® Blood and Tissue kit and agarose gel electrophoresis was performed to assess the recovered DNA (Section 2.2.1 and 2.2.2). Samples were extracted in triplicate

DNA quantification was performed using Quant-iT™ PicoGreen® dsDNA reagents (Invitrogen™, UK) on the TECAN GEnios Pro Micro plate Reader (Section 2.7). Florescence intensity was determined for known DNA standards ranging from 0 to 1000 ng/ml. Over this range the intensity increased in a linear fashion. A linear regression analysis was performed using Microsoft Excel and the value of r^2 in all cases was at least 0.99. DNA standards were run in duplicate and a standard curve was generated for DNA concentrations and RFU values, an example is shown in Figure 4.1.

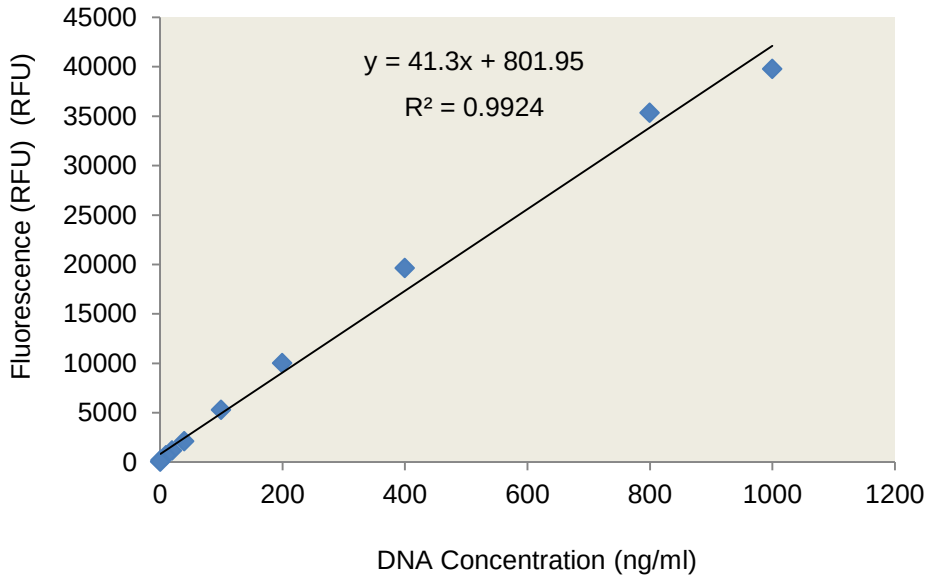

Figure 4.1 Shown above is an example of standard curve for PicoGreen® DNA quantification.

Table 4.1 Shown below is the summary of the 4-plex multiplex amplification of pig and rabbit DNA samples obtained from field experiments at different time points.

Projects	Rabbit		Pig	
	Full 4-plex profile	Partial 4-plex profile	Full 4-plex profile	Partial 4-plex profile
August 09	W=112 ADD BF=112 ADD S=141 ADD	S=72 ADD	-	-
Feb 10	-	-	W=494 ADD	W=554-603 ADD
May 10	-	-	W=295 ADD	W=338 ADD
June 10	W=13 ADD	W=64 ADD	-	-
Sept 10	W=137 ADD BF=137 ADD S=210 ADD	W=210-254 ADD BF=254-288 ADD S=254-490 ADD	W=342 ADD BF=342 ADD S=420 ADD	W=420-490 ADD S=490 ADD

W=whole carcasses, BF=body fragments, S=suspended muscles.

4.3 August-September 2009

This experiment acted as a pilot and was designed to see the relative rate of DNA persistence in rabbit whole carcases, body fragments (rear legs separated from rest of the body) and suspended muscle tissues (approximately 1 g of soft muscle tissues put inside 50 ml polypropylene tubes which were open to air and were protected from insects by covering the top with wire mesh (Chapter 2, Figure 2.2). ADD was calculated for every visit to the field for sample collection. The humidity during this project was 57-95% and average daily temperature was in a range of 13-19 °C.

4.3.1 Rabbit Whole Carcases and Body Fragments

It was observed that the whole carcases and body fragments were completely decomposed after day 7. DNA was present in all samples collected from whole carcases and body fragments (Figures 4.2 and 4.3) up to day 7 (112 ADD). Maggots were present from day 3.

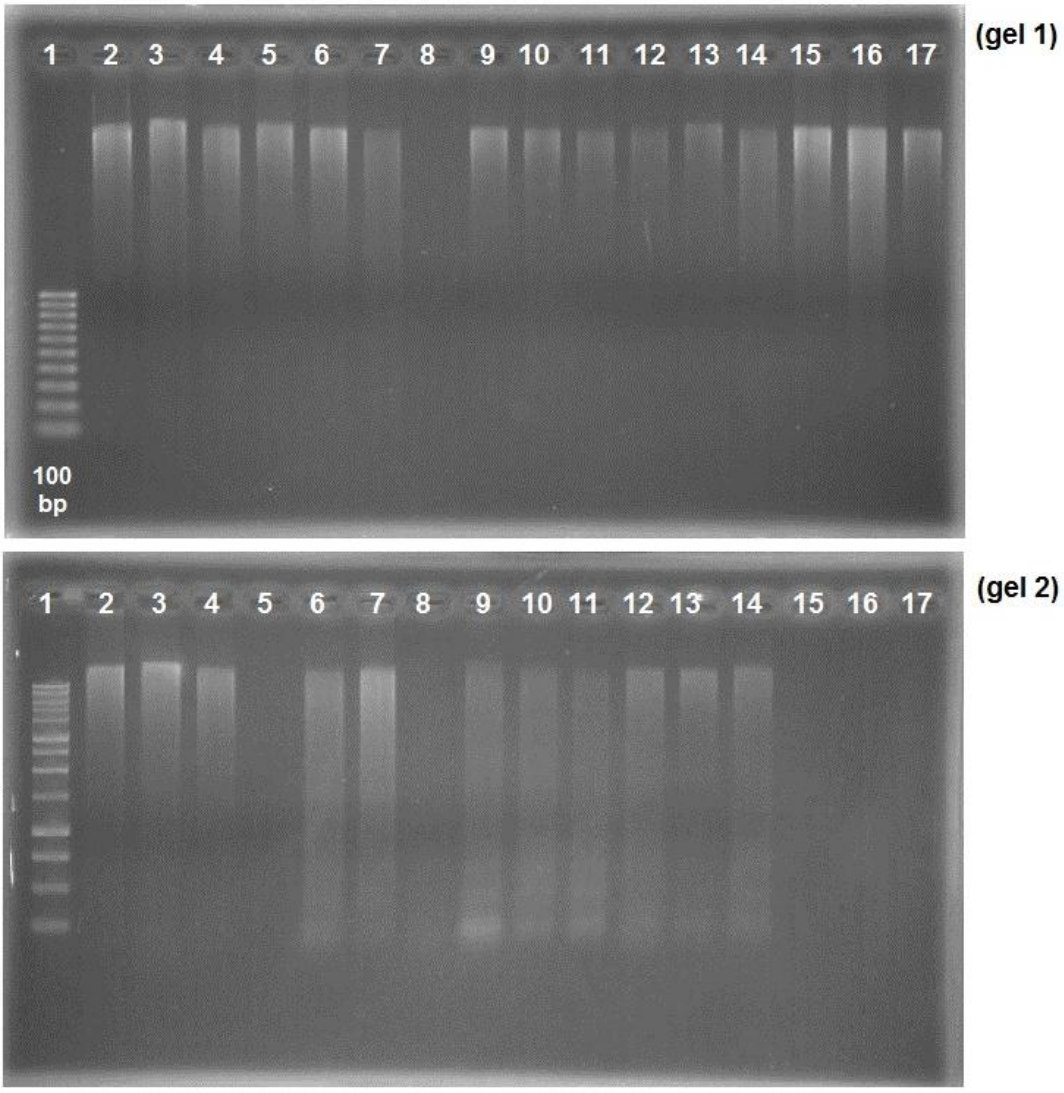

Figure 4.2 Shown above are agarose gels containing rabbit DNA obtained from whole carcases and body fragments during August-September 2009 field experiments; gel 1 represents 100 bp DNA ladder (Lane 1), day zero (positive controls) (Lane 2 - 7), extraction negative control (Lane 8), rabbit samples at day 1 (14 ADD) whole carcases (Lane 9-11), body fragments (Lane 12-14) and suspended tissues (Lane 15-17).

Similarly, gel 2 shows 100 bp DNA ladder (Lane 1), day zero (positive controls) (Lane 2-4), extraction negative control (Lane 5), rabbit samples at day 7 (112 ADD) whole carcases (Lane 6-8), body fragments (Lane 9-11) and suspended tissues (Lane 12-14).

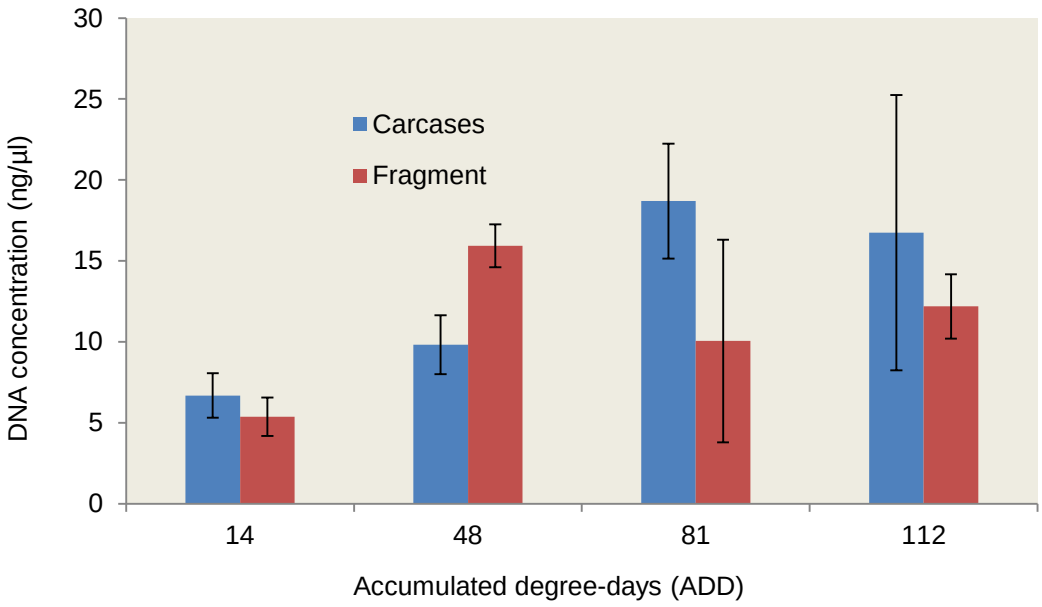

Figure 4.3 Shown above is DNA quantification using PicoGreen® for samples from rabbit whole carcases and body fragments**.** The error bars indicate the standard error (SE) from three replicates. A total of 200 µl was extracted from each sample and 3 µl was used for quantification.

4.3.2 Rabbit Suspended Muscle Tissues

The suspended muscle tissues persisted for a longer time period and the collection of samples was possible until day 44 (520 ADD). These samples were not directly affected by insect activity as they were covered with a wire mesh that let air and water pass, but prevented insect access. Therefore, there was no maggot mass seen but a layer of fungus was observed at the top of tissues after day 29 (320 ADD) and the muscle became soft, sticky and smelly with the passage of time especially after day 21(257 ADD). DNA was present in muscles until day 11 (172 ADD) (Figures 4.4 and 4.5).

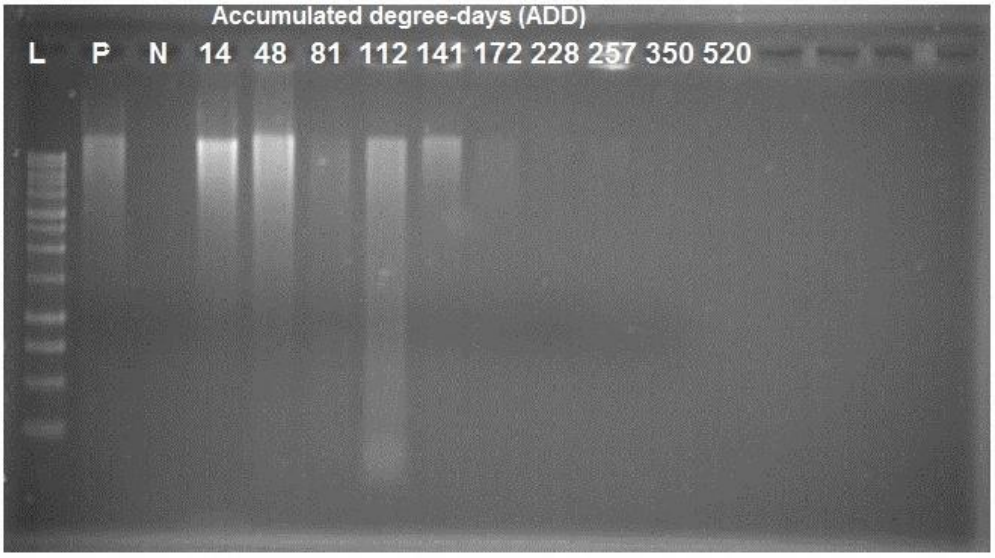

Figure 4.4 Shown above is a representative example of DNA from rabbit suspended tissues at different ADDs (14-520), 100 bp DNA ladder (L), positive control (P) and negative control (N) are also shown.

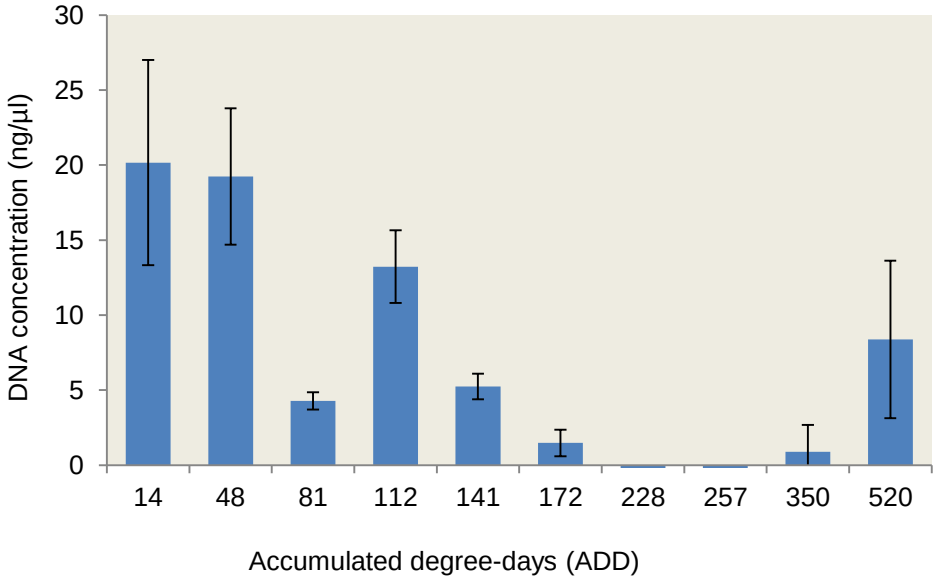

Figure 4.5 Shown above is the DNA quantification using PicoGreen® for rabbit suspended muscle tissues in August 2009. The error bars indicate the standard error from three replicates. A total of 200 µl was extracted from each sample and 3 µl was used for quantification.

4.3.3 4-plex Multiplex Amplification

PicoGreen quantification generally quantifies double stranded DNA irrespective of the species of origin. In field experiments, growth of microorganisms and accumulation of maggot mass on whole carcases and body fragments was observed at the later stages of decomposition and could contribute DNA. Therefore, to determine the amount of endogenous DNA in the samples, the 4-plex multiplex amplification was performed to determine the amplification success of the four amplicons (70 bp, 194 bp, 305 bp and 384 bp) in pig and rabbit muscle tissue samples collected at different time points. Based on PicoGreen quantification 0.6 ng DNA template DNA was used.

4.3.3.1 Rabbit Whole Carcases and Body Fragments

Full 4-plex multiplex amplification was obtained from triplicate muscle tissue samples collected from whole carcasses and body fragments until day 7 (112 ADD) (Figure 4.6 and Table 4.2)**.** There were no tissues available after day seven.

Table 4.2 Shown below are the results of 4-plex multiplex amplification of rabbit whole carcasses, body fragments and suspended muscle tissues collected during August 2009 project.

Days	Accumulated degree-days (ADD)	Rabbit muscle tissues			
		70 bp	194 bp	305 bp	384 bp
1	14	+++	+++	+++	+++
3	48	+++	+++	+++	+++
5	81	+++	+++	+++	+++
7	112	+++	+++	+++	+++
9	141	+++	+++	+++	+++
11	172	+--	---	---	---
17	228	---	---	---	---
21	257	---	---	---	---
29	350	---	---	---	---
44	520	---	---	---	---

‘‘+’’ denotes the presence and ‘‘-’’ represents the absence of PCR amplicons in samples during 4-plex multiplex amplification. The whole carcasses and body fragments were completely decomposed after day 7 (112 ADD) while suspended tissues were available to collect until 520 ADD.

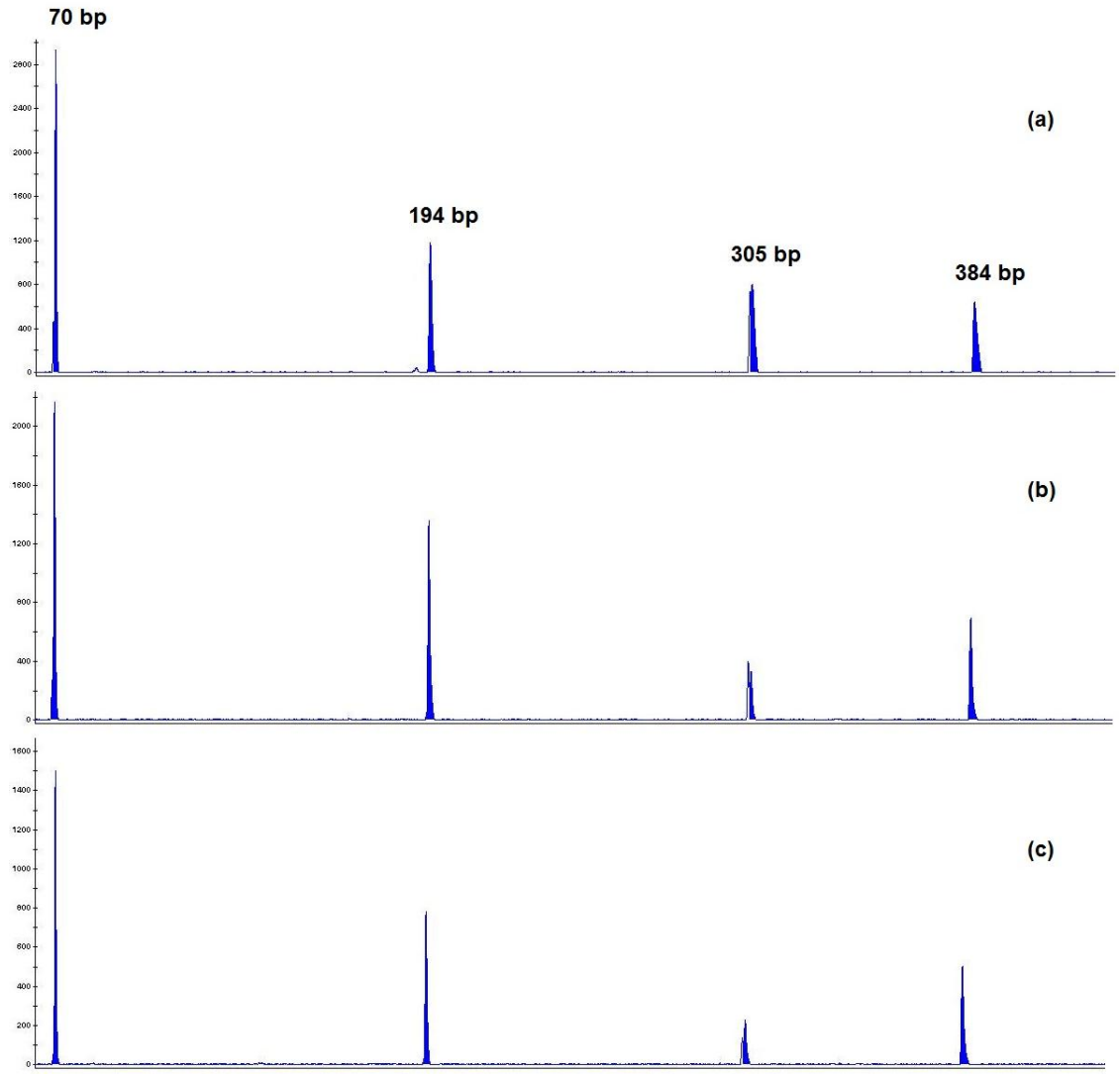

Figure 4.6 Shown above are examples of electropherograms of b) rabbit whole carcasses at day 7 (112 ADD) and c) rabbit body fragments at day 7 (112 ADD). Human positive control (a) also shown.

4.3.3.2 Rabbit Suspended Muscle Tissues

Rabbit suspended muscle tissues produced full multiplex amplification until day nine (141 ADD). At day 11 (172 ADD) only the 70 bp product was amplified in one sample (Figure 4.7). There was complete failure of amplification of the 4-plex after day 11 (Table 4.2).

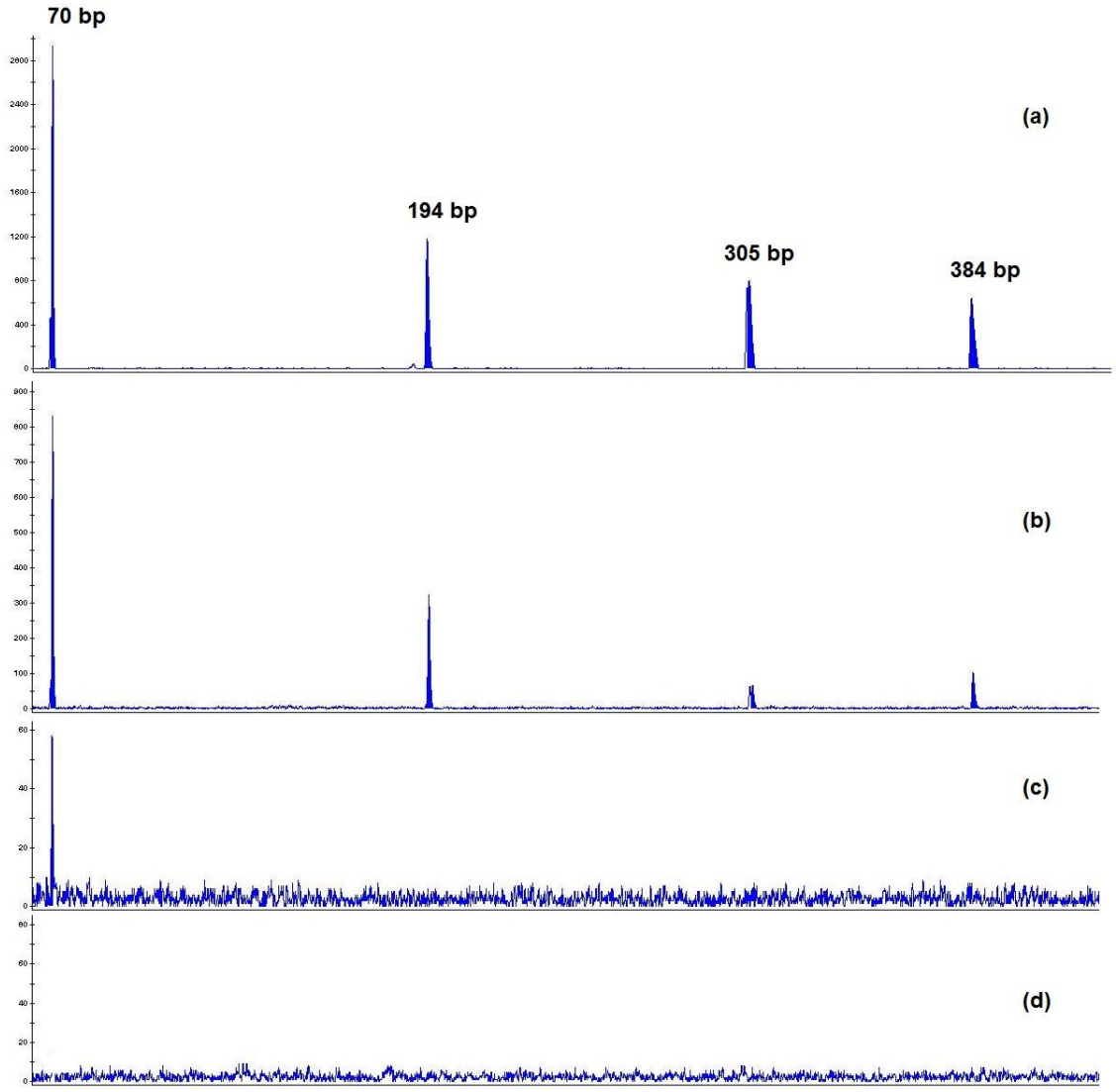

Figure 4.7 Shown above are examples of electropherograms of rabbit suspended tissues from August 2009; b) rabbit suspended tissues at day 9 (141 ADD) c) rabbit suspended tissues at day 11 (172 ADD); and d) suspended tissues at day 17 (228 ADD). Human positive control (a) also shown.

4.4 February-May 2010

This project was designed to evaluate the DNA persistence in pig whole carcases. Due to similarity to human body weight and basal metabolic rate, pig carcases have been used as an experimental model to determine the quality of post-mortem DNA profiling (Gunawardane et al., 2009) and estimation of post-mortem interval using ADD (Larkin et al., 2010).

4.4.1 Pig Whole Carcases

Five pig carcasses were left in the field for decomposition and to determine DNA persistence in muscle tissue. The weather was very cold and the average temperature on the day of delivery of the carcases to the field was 1.4 °C. On subsequent visits for muscle tissue collection, the bodies were found to be covered with ice. The skin of the carcasses was dry and there was no attraction of flies and no maggot mass growth. In April, with increase in daily temperature, there was insect activity. Muscles were present on the carcasses until last collection at 603 ADD (90 days) (Figure 4.8).

There was no skeletonisation or mummification observed in any of the winter carcasses. Collection of soft muscle tissue samples was stopped at 90 days since no DNA was visible on agarose gels. PicoGreen quantification showed DNA present in soft tissues until 401 ADD (Figure 4.9).

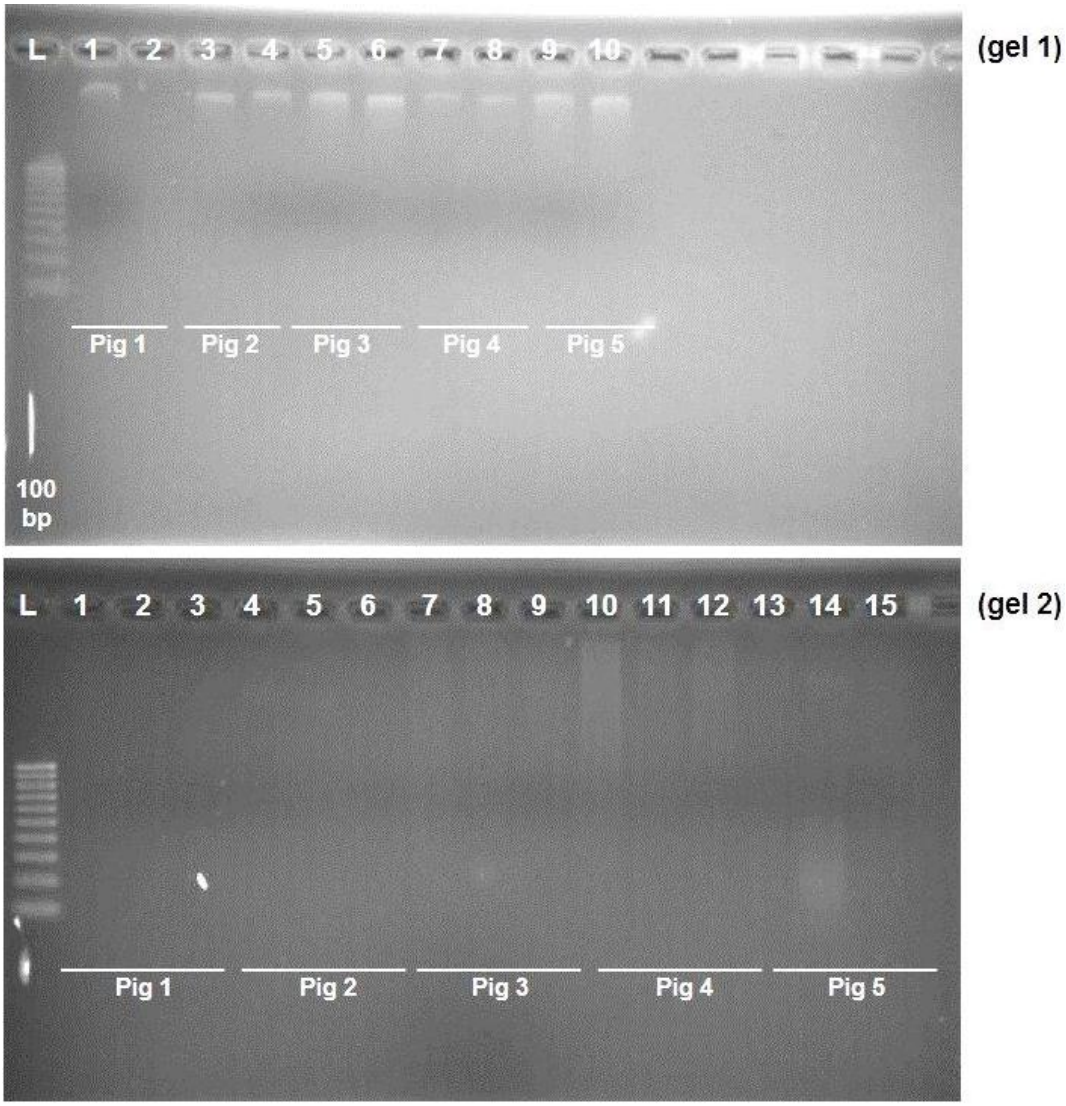

Figure 4.8 Shown above is an example of agarose gel electrophoresis of pig soft muscle tissue samples collected during February 2010 , gel 1) at day 14 (34 ADD) and gel 2) at day 71 (418 ADD). 100 bp DNA ladder (L) also shown. All samples were collected in triplicate but day 14 samples (gel 1) were run on gel in duplicate. This picture was inverted to make DNA visible.

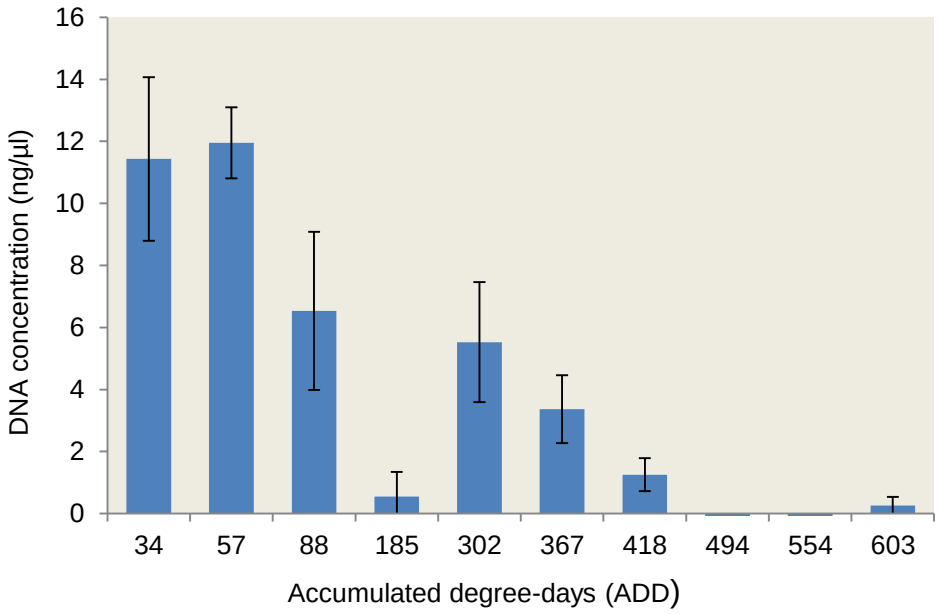

Figure 4.9 Shown above is the DNA quantification using PicoGreen® for pig whole carcases for February 2010. The error bars indicate the standard error from three replicates. A total of 200 µl was extracted from each sample and 3 µl was used for quantification.

4.4.2 Multiplex Amplification

Pig whole carcases showed full 4-plex multiplex amplification in all triplicate samples until day 77 (494 ADD). Drop-out in 194 bp, 305 bp and 384 bp fragments was observed at day 84 (554), whilst amplification of 70 bp was possible until day 90 (603 ADD) (Figure 4.10 and Table 4.3).

Table 4.3 Shown below are the results of multiplex (4-plex) amplification of pig soft muscle tissues collected during February 2010 project.

Days	Accumulated degree-days (ADD)	Pig whole carcases			
		70 bp	194 bp	305 bp	384 bp
14	34	+++	+++	+++	+++
21	57	+++	+++	+++	+++
29	88	+++	+++	+++	+++
41	185	+++	+++	+++	+++
57	302	+++	+++	+++	+++
64	367	+++	+++	+++	+++
71	418	+++	+++	+++	+++
77	494	+++	+++	+++	+++
84	554	+++	++-	+--	+--
90	603	+++	++-	+--	+--

‘‘+’’ denotes the presence and ‘‘-’’ represents the absence of PCR amplicons in triplicate samples during multiplex (4-plex) amplification.

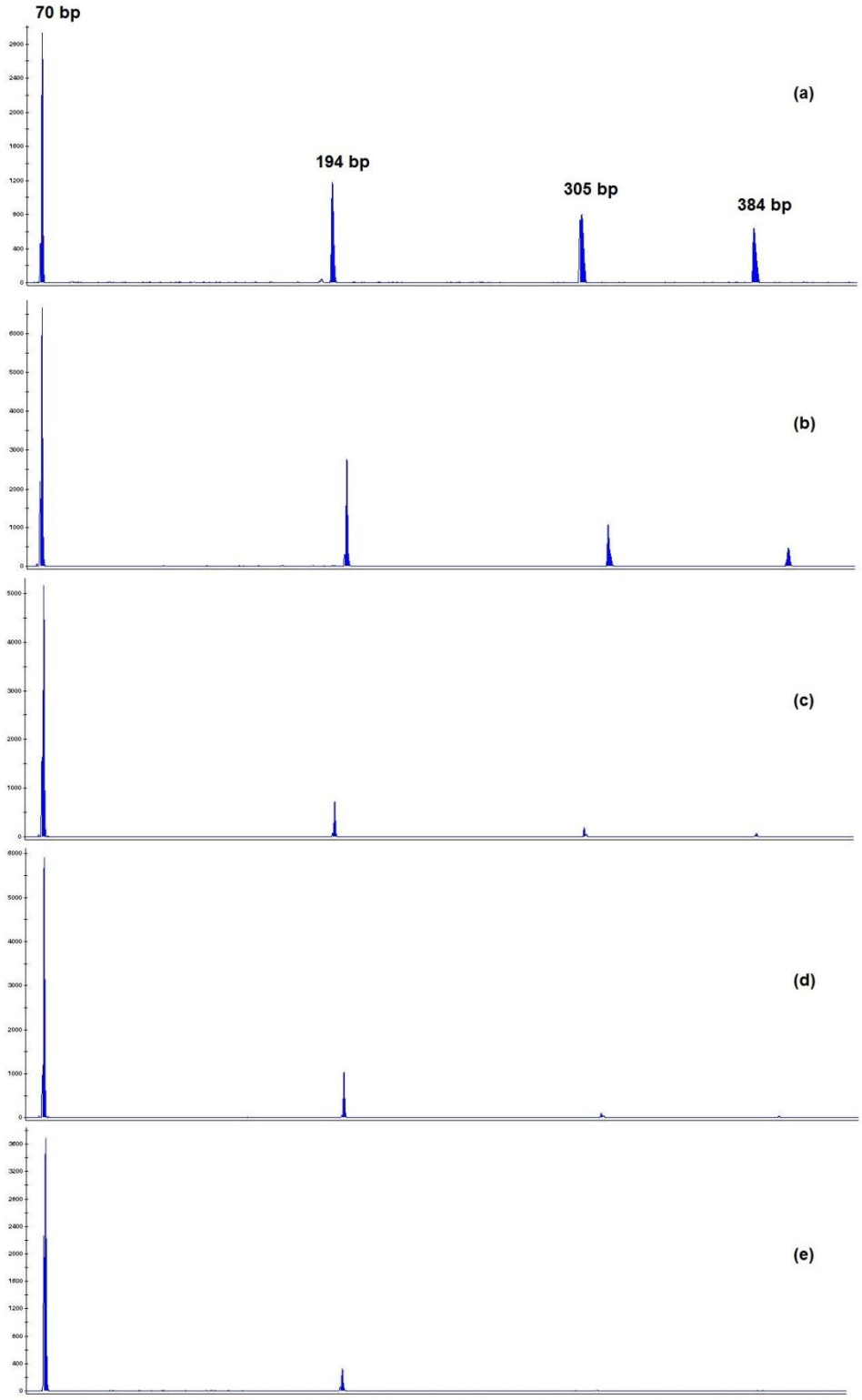

Figure 4.10 Shown above are examples of electropherograms of pig soft muscle tissues collected during February 2010 project at b) 494 ADD, c) 554 ADD, d and e) 603 ADD. Human positive control (a) also shown.

4.5 May-June 2010

During this project 24 pig whole carcases were placed at TRACES. Half of the carcases (12) were slashed on the neck as part of a different study. Samples were taken from different positions on the pig: right and left rear legs, forelegs and neck.

4.5.1 Pig Whole Carcases

Decomposition of pig whole carcases was faster in summer (May-June) and skeletonisation was complete in 27 days (338 ADD), after which there was no soft muscle tissue to collect. PicoGreen quantification showed DNA persistence until 159 ADD. High ambient temperature (up to 23 °C) and insect activity at the start of experiment led to rapid maggot mass growth in the decomposing bodies. After 159 ADD, there was no DNA except at 338 ADD (Figure 4.11, 4.12, 4.13, and 4.14) which could be bacterial.

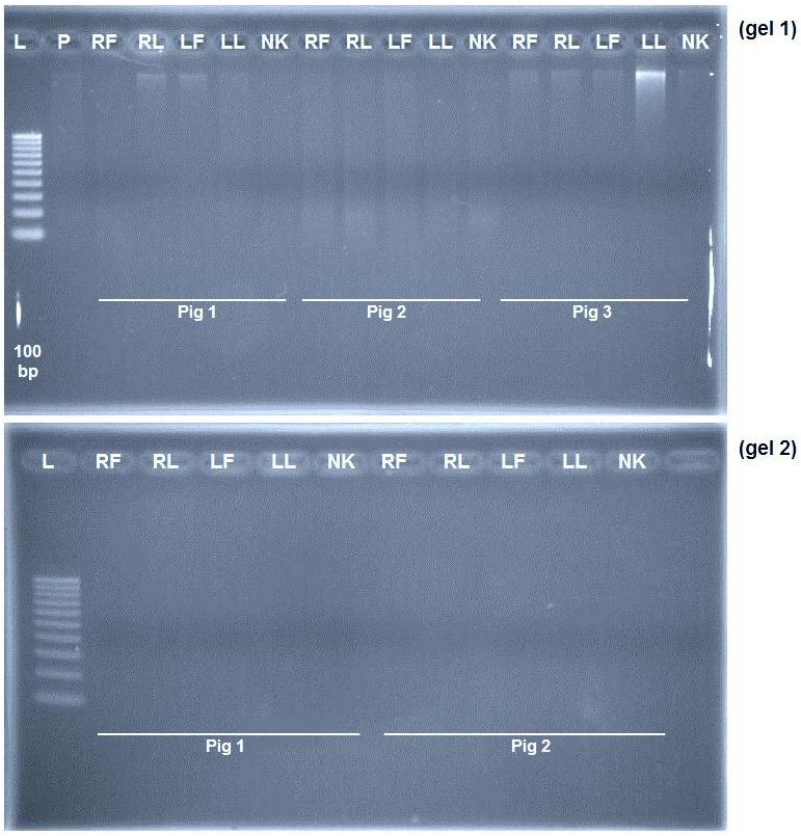

Figure 4.11 Shown above are examples of agarose gels with DNA from pig muscle tissue samples collected during May 2010, gel 1) at day 6 (77 ADD) and gel 2) at day 16 (203 ADD) from different body parts, right foreleg (RF), right leg (RL), left foreleg (LF), Left leg (LL) and neck (NK). 100 bp DNA ladder (L) and positive control (P) also shown. This figure was inverted to improve resolution.

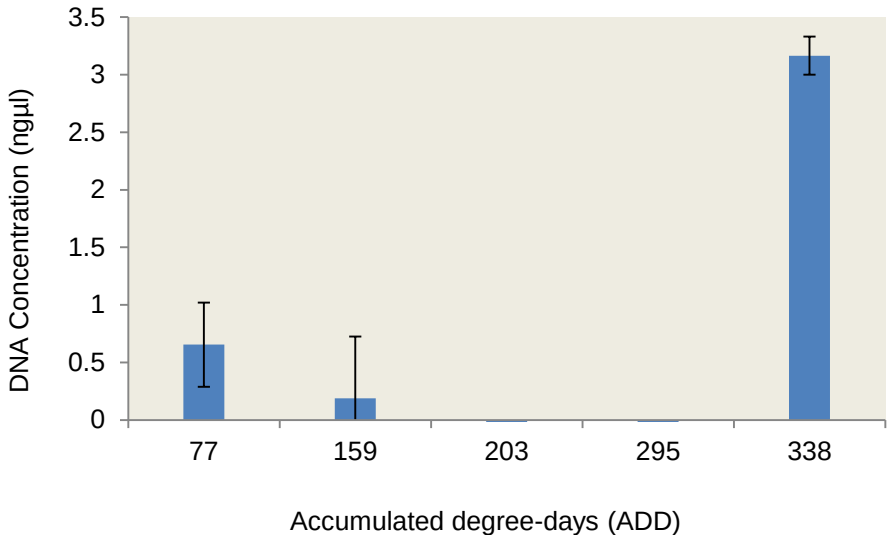

Figure 4.12 Shown above is the DNA quantification using PicoGreen® for pig whole carcases for May-June 2010. The error bars indicate the standard error from three replicates. A total of 200 µl was extracted from each sample and 3 µl was used for quantification.

4.5.2 4-plex Multiplex Amplification

Pig soft muscle tissue samples collected from different parts of whole carcases showed dropout in amplification of 194 bp, 305 bp and 384 bp at day 27 (338 ADD) (Figure 4.15 and Table 4.4).

Table 4.4 Shown below are the results of multiplex (4-plex) amplification of pig soft muscle tissues collected from whole carcases during May 2010 project.

Days	Accumulated degree-days (ADD)	Pig May 2010 project			
		70 bp	194 bp	305 bp	384 bp
6	77	+++	+++	+++	+++
13	159	+++	+++	+++	+++
16	203	+++	+++	+++	+++
24	295	+++	+++	+++	+++
27	338	+++	+++	+--	+--

‘‘+’’ denotes the presence and ‘‘-’’ represents the absence of PCR amplicons in samples during multiplex (4-plex) amplification.

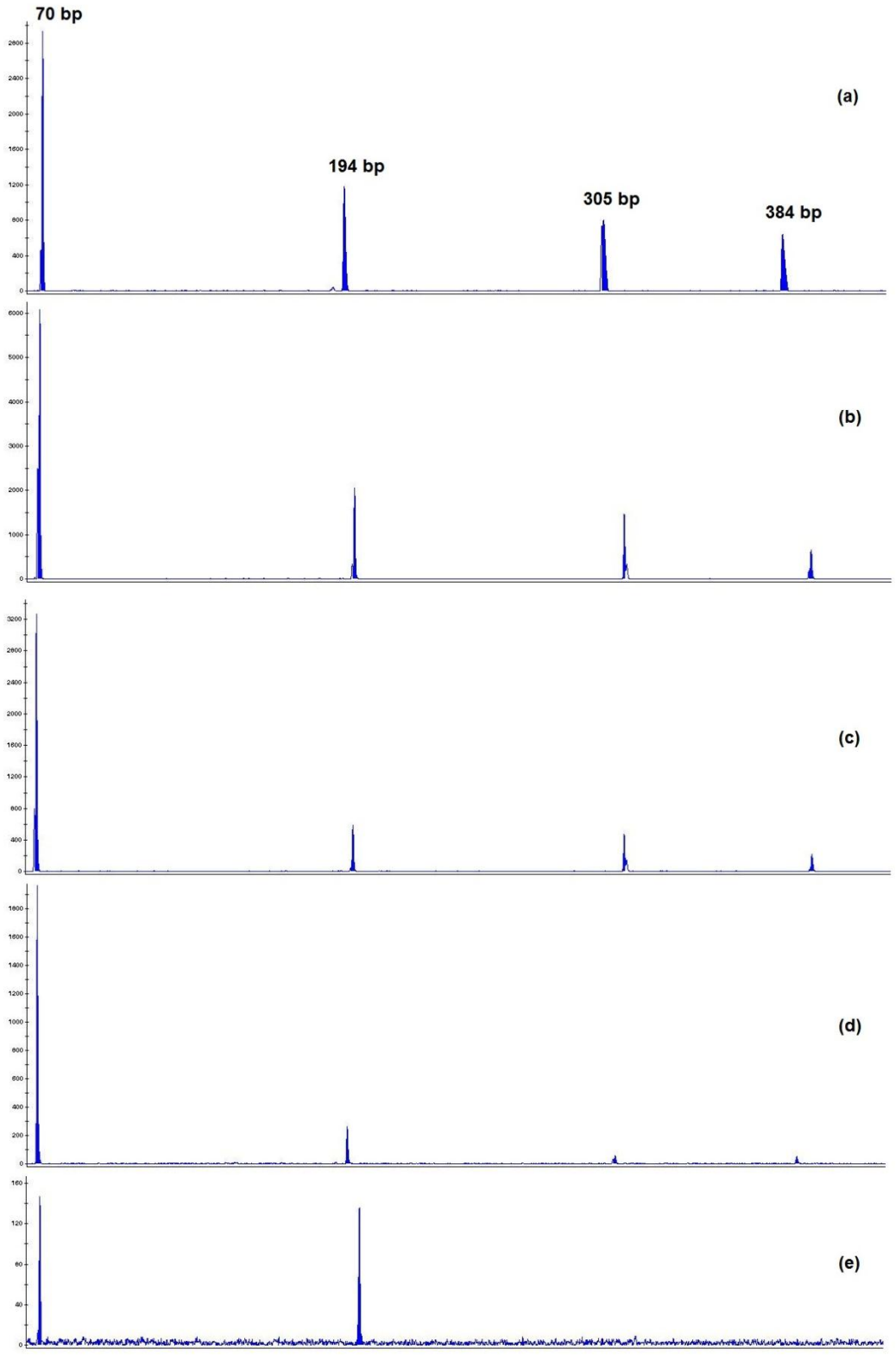

Figure 4.13 Shown above are examples of electropherograms of pig soft muscle tissues collected during May 2010 at b) 159 ADD, c) 203 ADD, d and e) 338 ADD. Human positive control (a) also shown.

4.6 June 2010

In this project, 36 rabbit whole carcases were placed at TRACES for 21 days to access DNA degradation in soft muscle tissues during season.

4.6.1 Rabbit Whole Carcases

The skeletonisation of rabbit carcases was faster in summer. All rabbit bodies were found to be dried up at day 8 (122 ADD) and there was no muscle tissue present at 122 ADD. This happened due to no rainfall (dry weather) and high temperature (13.2 °C-16.8 °C). DNA was present as long as soft muscle tissues were available to collect (Figure 4.16)

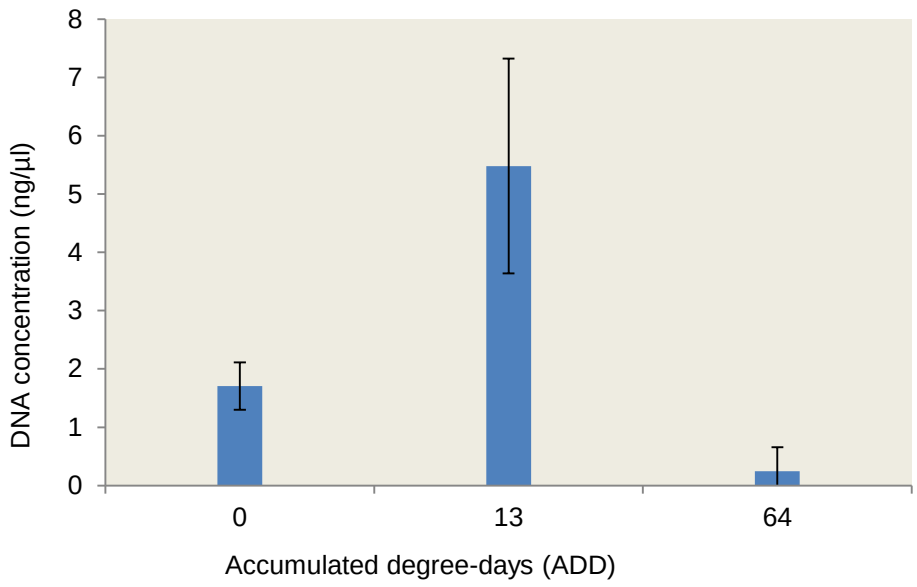

Figure 4.14 Shown above is the DNA quantification using PicoGreen® for rabbit whole carcases for June 2010 field project. The error bars indicate the standard error from three replicates. A total of 200 µl was extracted from each sample and 3 µl was used for quantification.

4.6.2 Multiplex Amplification

In rabbit tissues amplification of 70 bp and 194 bp was obtained until day 4 (55 ADD), while larger amplicons showed dropout in some samples (Figure 4.17 and Table 4.5). There were no muscle present at day 8 (122 ADD).

Table 4.5 Shown below are the results of multiplex (4-plex) amplification of rabbit soft muscle tissues collected during June 2010 project.

Days	Accumulated degree-days (ADD)	Rabbit whole carcases			
		70 bp	194 bp	305 bp	384 bp
1	13	+++	+++	+++	+++
4	64	+++	+++	+--	+--

‘‘+’’ denotes the presence and ‘‘-’’ represents the absence of PCR amplicons in samples during 4-plex multiplex amplification.

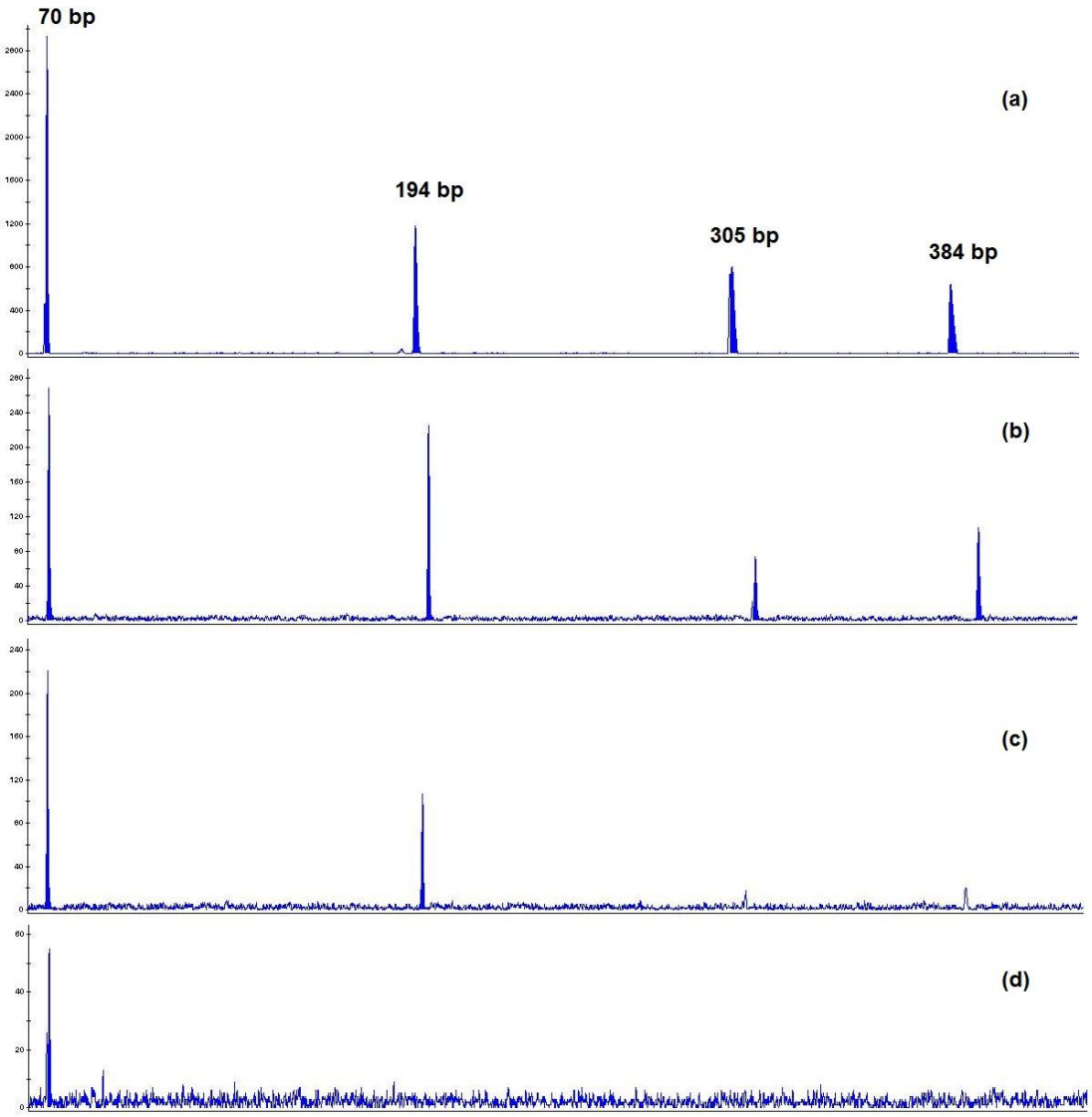

Figure 4.15 Shown above are examples of electropherograms of rabbit soft muscle tissues collected during June 2010 at b) day 1 (11 ADD), c and d) day 4 (55 ADD). Human positive control (a) also shown.

4.7 September-October 2010

This was the comparative study to determine the relative rate of DNA persistence in whole carcases, body fragments and suspended soft muscle tissue samples of pig and rabbit in the same season under similar field conditions. For the rabbit degradation experiment, 33 whole carcases, 36 body fragments (rear legs) and 39 suspended soft muscle tissue samples were placed at TRACES. In case of pigs, 3 whole carcases, 15 body fragments (3 neck, 6 forelegs, and 6 rear legs) and 39 suspended tissues were used.

It was observed that the rate of decomposition and hence the DNA degradation was faster in rabbit whole carcases, body fragments and suspended tissues compared to pig (Figure 4.18, 4.19, 4.20, 4.21 and 4.22). The insect activity was less at early stages of decomposition (Figure 4.26 (79 ADD and 210 ADD)) but with increase in PMI, high maggot mass growth was observed (Figure 4.26 (342 ADD and 420 ADD))..

4.7.1 Rabbit Whole Carcases

DNA quantification of muscle tissue samples collected from whole carcases showed that DNA was present until 254 ADD (Figure 4.18, 4.19 (gel 1) and 4.20)

4.7.2 Rabbit Body Fragments

DNA was present in rabbit body fragments until 210 ADD. (Figure 4.19 (gel 2) and 4.21).

4.7.3 Rabbit Suspended Tissues

Rabbit suspended muscle were available to collect for longer time points but severe DNA degradation was observed after 210 ADD (Figure 4.19 (gel 3) and 4.22).

0 ADD

79 ADD

137 ADD

210 ADD

254 ADD

288 ADD

Figure 4.16 Shown above is decomposition of rabbit whole carcases and body fragments at different ADD during September 2010 field experiments.

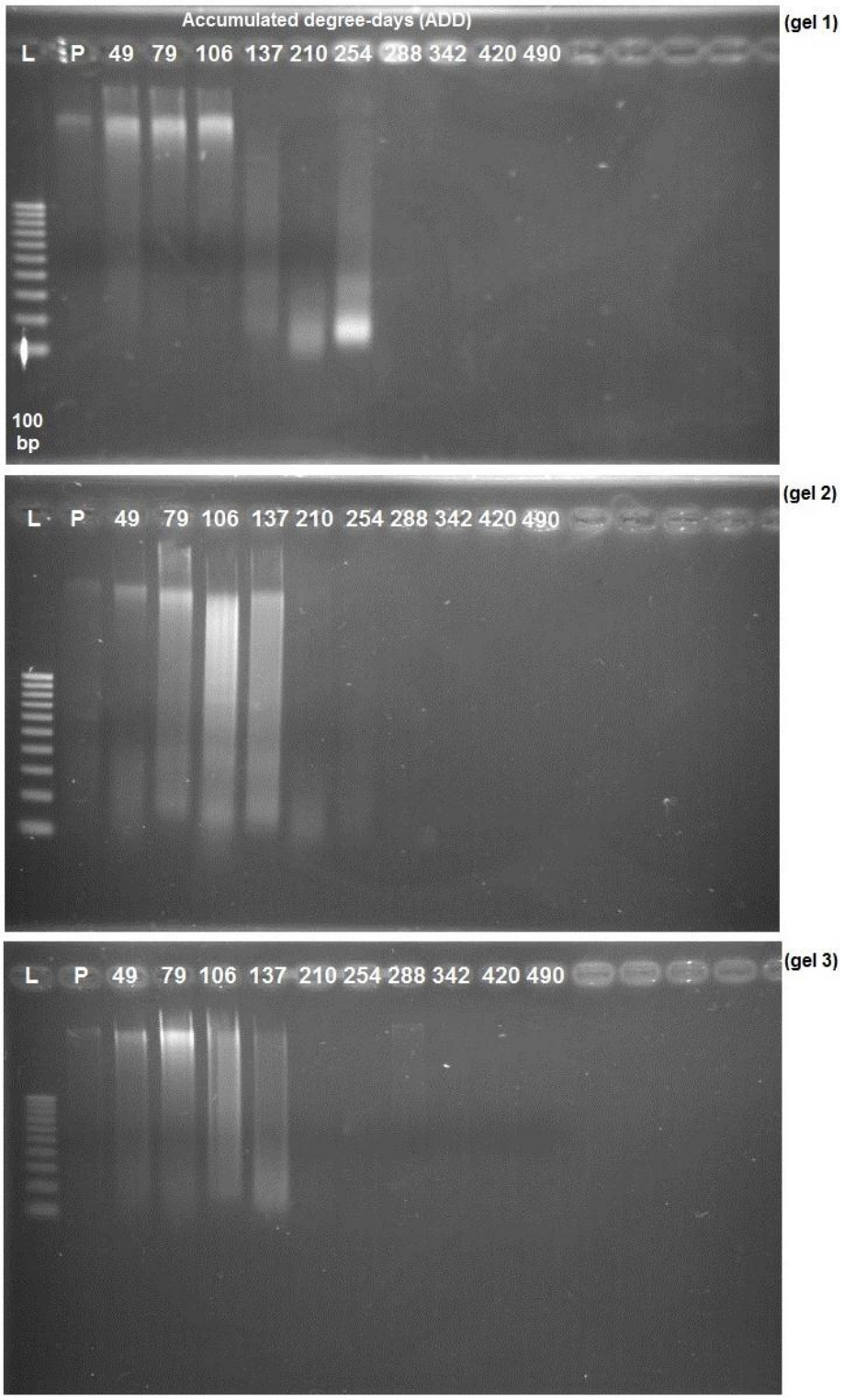

Figure 4.17 Shown above are examples of agarose gels of rabbit DNA samples collected at different ADD during September 2010, gel 1) with whole carcases, gel 2) body fragments and gel 3) suspended soft muscle tissues 100 bp DNA ladder (L) and positive control (P) are also shown.

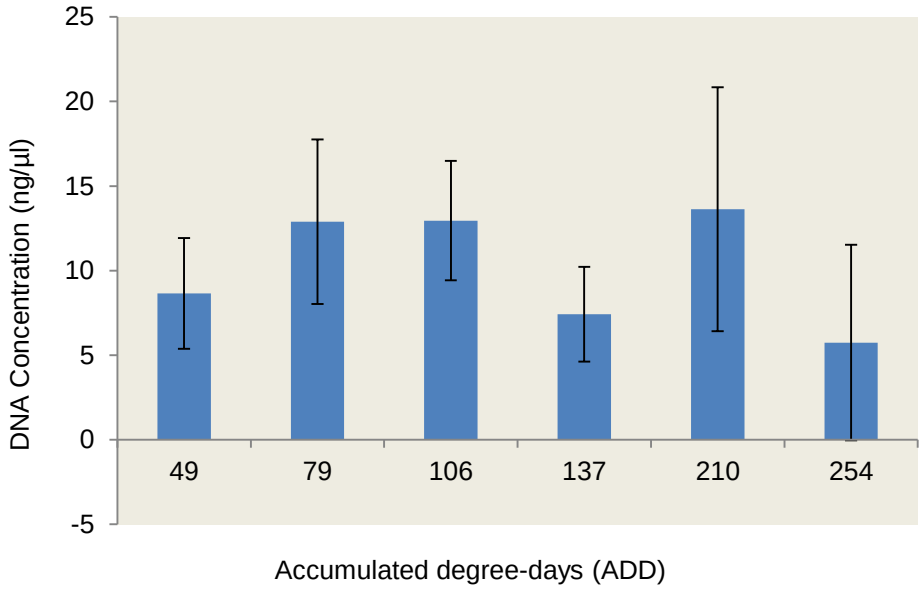

Figure 4.18 Shown above is the DNA quantification using PicoGreen® for rabbit whole carcases in September 2010. The error bars indicate the standard error from three replicates. A total of 200 µl was extracted from each sample and 3 µl was used for quantification.

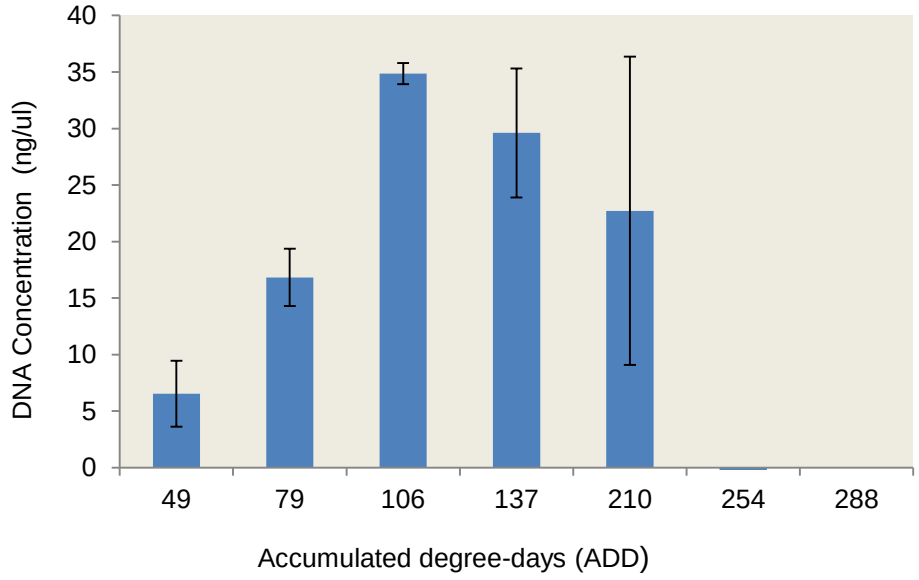

Figure 4.19 Shown above is the DNA quantification using PicoGreen® for Rabbit body fragments in September 2010. The error bars indicate the standard error from three replicates. A total of 200 µl was extracted from each sample and 3 µl was used for quantification.

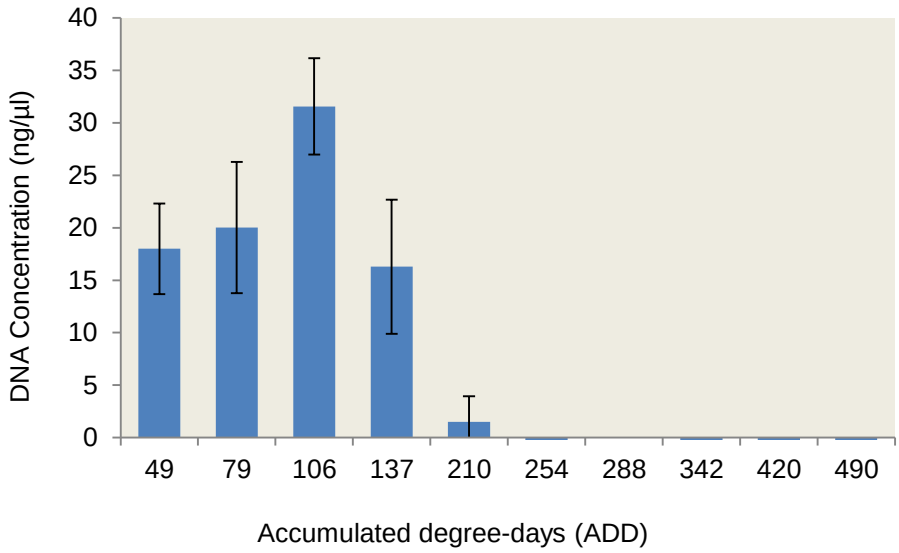

Figure 4.20 Shown above is the DNA quantification using PicoGreen® rabbit suspended muscle tissues in September 2010. The error bars indicate the standard error from three replicates. A total of 200 µl was extracted from each sample and 3 µl was used for quantification.

4.7.4 4-plex Multiplex Amplification of Rabbit Whole Carcases

Based on DNA quantification data, rabbit soft muscle tissue samples having no DNA were selected for 4-plex multiplex amplification. The dropout in amplification of 194 bp was observed at day 18 (254 ADD). There was complete failure of amplification of larger amplicons in whole carcases at day 15 (210 ADD) and day 18 (254 ADD (Figure 4.23 and Table 4.6).

Table 4.6 Shown below are the results of multiplex (4-plex) amplification of rabbit soft muscle tissues collected from whole carcases during September 2010 project.

Days	Accumulated degree-days (ADD)	Rabbit whole carcases			
		70 bp	194 bp	305 bp	384 bp
4	49	+++	+++	+++	+++
7	79	+++	+++	+++	+++
9	106	+++	+++	+++	+++
11	137	+++	+++	+++	+++
15	210	+++	+++	---	---
18	254	+++	+--	**---**	**---**

‘‘+++’’ denotes the presence and ‘‘---’’ represents the absence of PCR amplicons in triplicate samples during multiplex (4-plex) amplification.

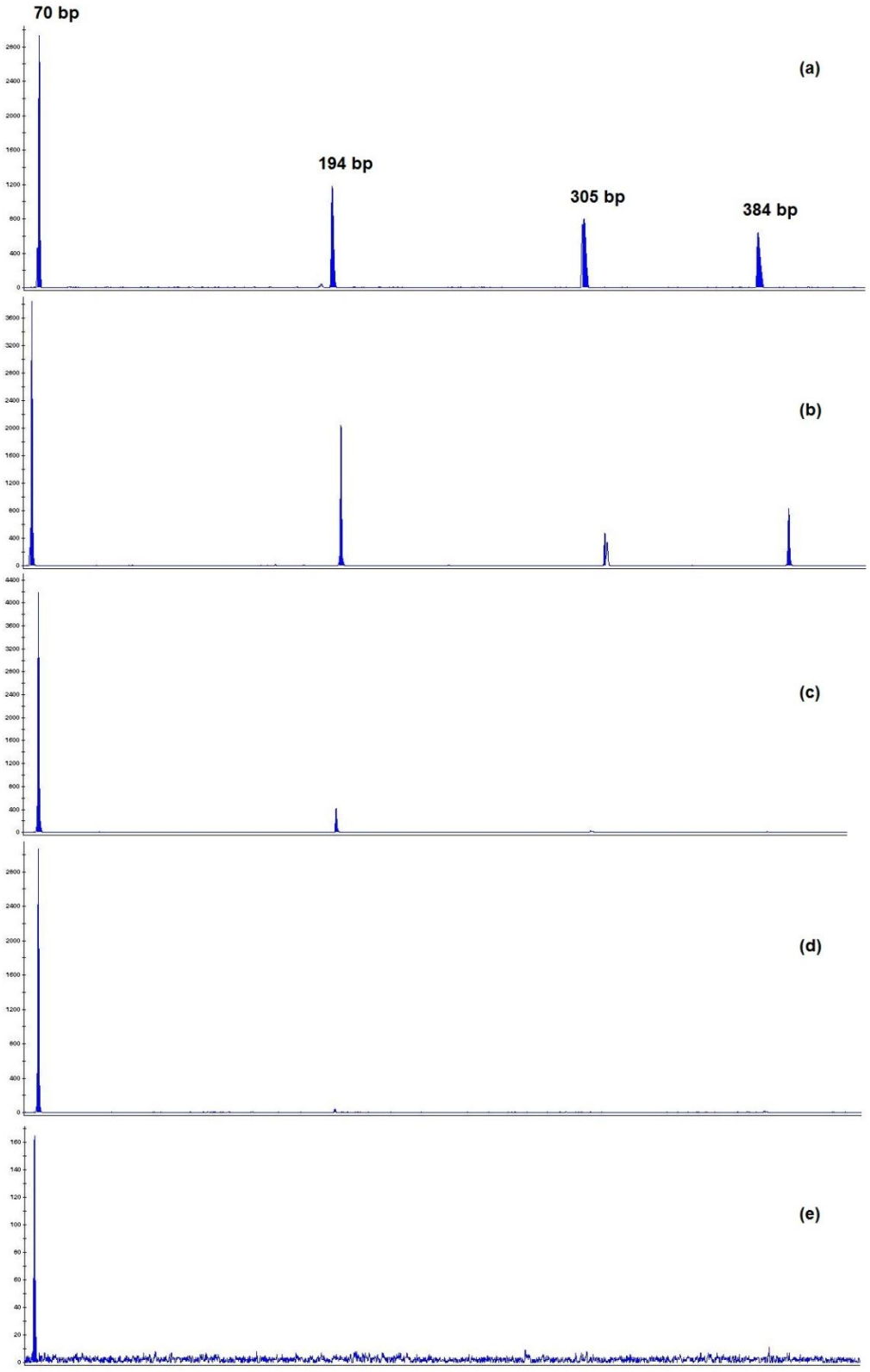

Figure 4.21 Shown above are examples of electropherograms of rabbit DNA obtained from whole carcases during September 2010 project at b) day 11 (137 ADD), c) day 15 (210 ADD), d and e) day 18 (254 ADD). Human positive control (a) also shown.

4.7.5 4-plex Multiplex Amplification of Rabbit Body Fragments

Rabbit body fragments showed full multiplex amplification of 70 bp until 288 ADD. For larger amplicons, partial profiles at 210 ADD and 288 ADD and complete failure of amplification at 254 ADD was observed (Figure 4.24 and Table 4.7).

Table 4.7 Shown below are the results of multiplex (4-plex) amplification of rabbit body fragment tissues collected during September 2010 project.

Days	Accumulated degree-days (ADD)	Rabbit body fragments			
		70 bp	194 bp	305 bp	384 bp
4	49	+++	+++	+++	+++
7	79	+++	+++	+++	+++
9	106	+++	+++	+++	+++
11	137	+++	+++	+++	+++
15	210	+++	+++	++-	++-
18	254	+++	+--	**---**	**---**
22	288	+++	++-	+--	+--

‘‘+++’’ denotes the presence and ‘‘---’’ represents the absence of PCR amplicons in triplicate samples during multiplex (4-plex) amplification.

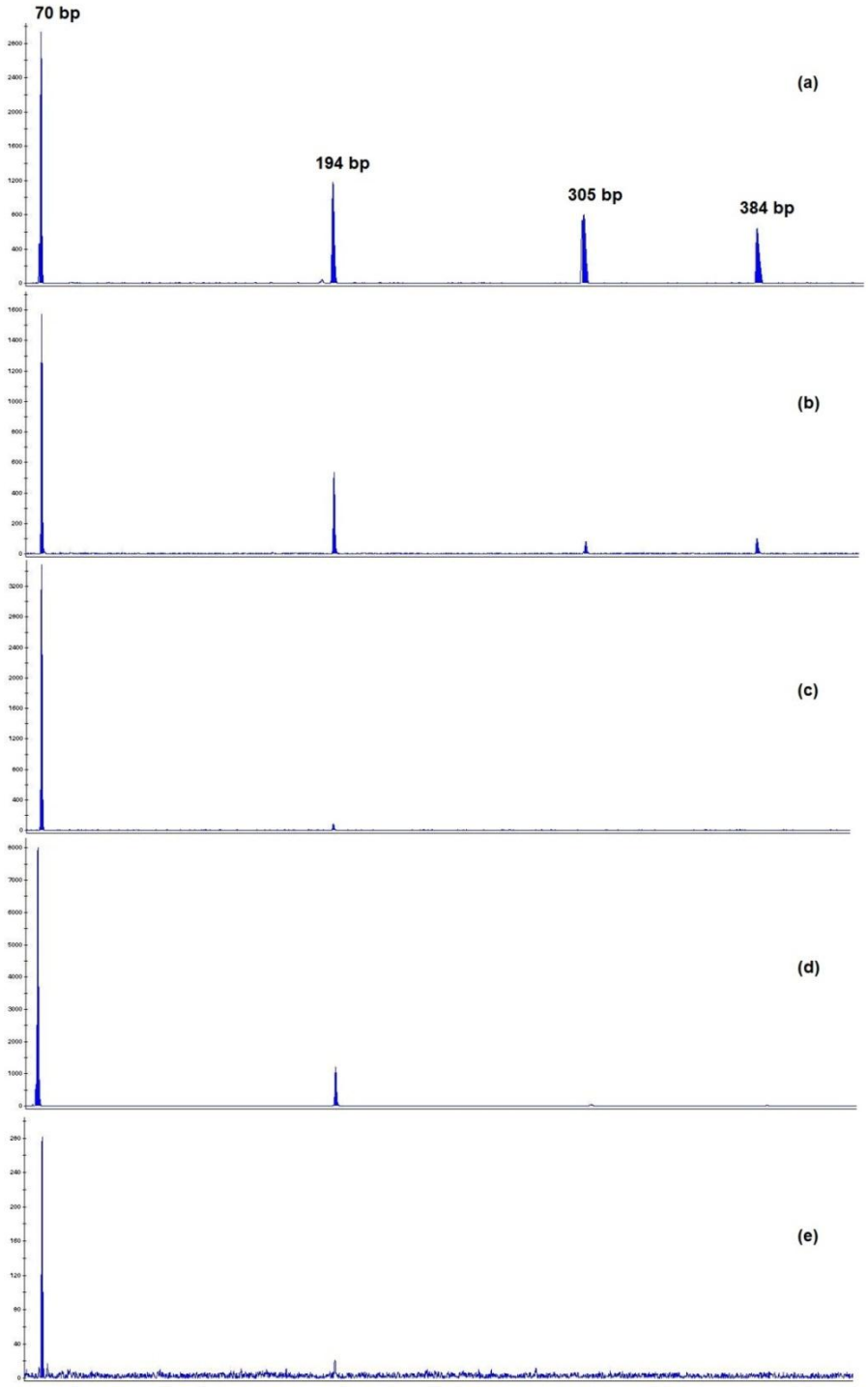

Figure 4.22 Shown above are examples of electropherograms of rabbit DNA obtained from body fragments during September 2010 project at b) day 15 (210 ADD), c) day 18 (254 ADD), d and e) day 22 (288 ADD). Human positive control (a) also shown.

4.7.6 Rabbit Suspended Tissues

Rabbit suspended tissues showed consistent multiplex amplification for 70 bp until 490 ADD There was complete failure of amplification of the 194 bp and larger amplicons after 210 ADD except one sample that amplified for 194 bp at 420 ADD (Figure 4.25 and Table 4.8).

Table 4.8 Shown below are the results of multiplex (4-plex) amplification of rabbit suspended tissues collected during September 2010 project.

Days	Accumulated degree-days (ADD)	Rabbit suspended tissues			
		70 bp	194 bp	305 bp	384 bp
4	49	+++	+++	+++	+++
7	79	+++	+++	+++	+++
9	106	+++	+++	+++	+++
11	137	+++	+++	+++	+++
15	210	+++	+++	+++	+++
18	254	+++	---	**---**	**---**
22	288	+++	---	**---**	**---**
30	342	+++	---	**---**	**---**
38	420	+++	+--	**---**	**---**
44	490	+++	---	**---**	**---**

‘‘+++’’ denotes the presence and ‘‘---’’ represents the absence of PCR amplicons in triplicate samples during multiplex (4-plex) amplification.

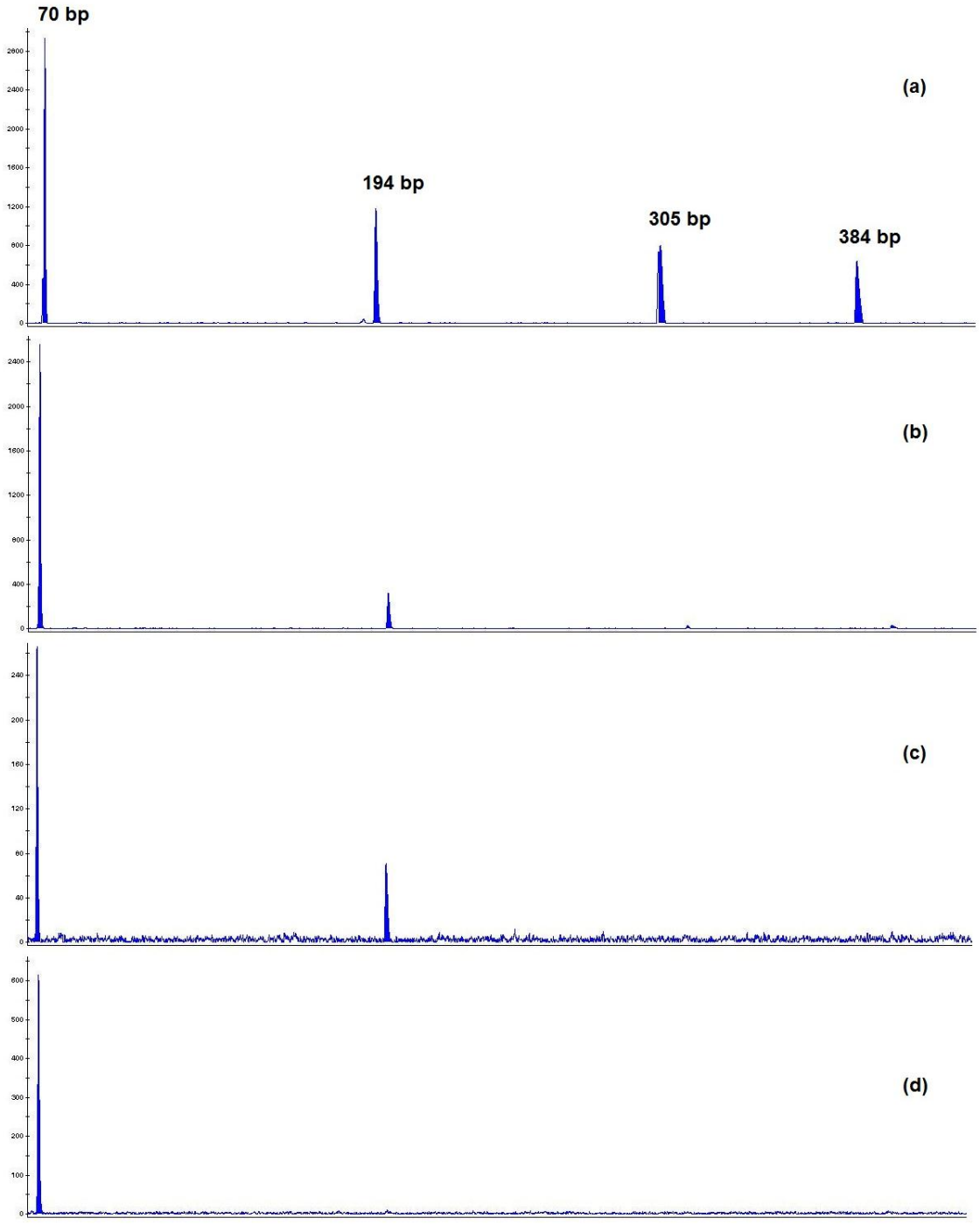

Figure 4.23 Shown above are examples of electropherograms of rabbit DNA obtained from suspended tissues during September 2010 at b) day 15 (210 ADD), c) day 38 (420 ADD), and d) day 44 (490 ADD). Human positive control (a) also shown.

4.8 Pig Whole Carcases

DNA quantification showed that DNA was present until 490 ADD in pig whole carcases, although there was sudden decrease in DNA quantity at 420 ADD (Figure 4.28 (gel 1) and Figure 4.29).

4.8.1 Body Fragments

Soft muscle tissue samples collected from pig body fragments (Neck, rear legs and forelegs) showed DNA present until 342 ADD (Figure 4.28 (gel 2) and Figure 4.30). Body fragments completely decomposed after 342 ADD and no soft muscle tissues were present.

4.8.2 Suspended Tissues

DNA quantification of pig suspended tissues showed that DNA was present until 490 ADD. The decrease in DNA quantity was observed at 342 ADD. (Figure 4.28 (gel 3) and 4.31).

0 ADD 79 ADD 210 ADD

342 ADD 420 ADD 490 ADD

Figure 4 24 Shown above is decomposition of pig whole carcases at different ADD during September 2010 field experiments**.**

0 ADD 79 ADD 210 ADD

342 ADD 420 ADD 490 ADD

Figure 4.25 Shown above is decomposition of pig body fragments at different ADD during September 2010 field experiments.

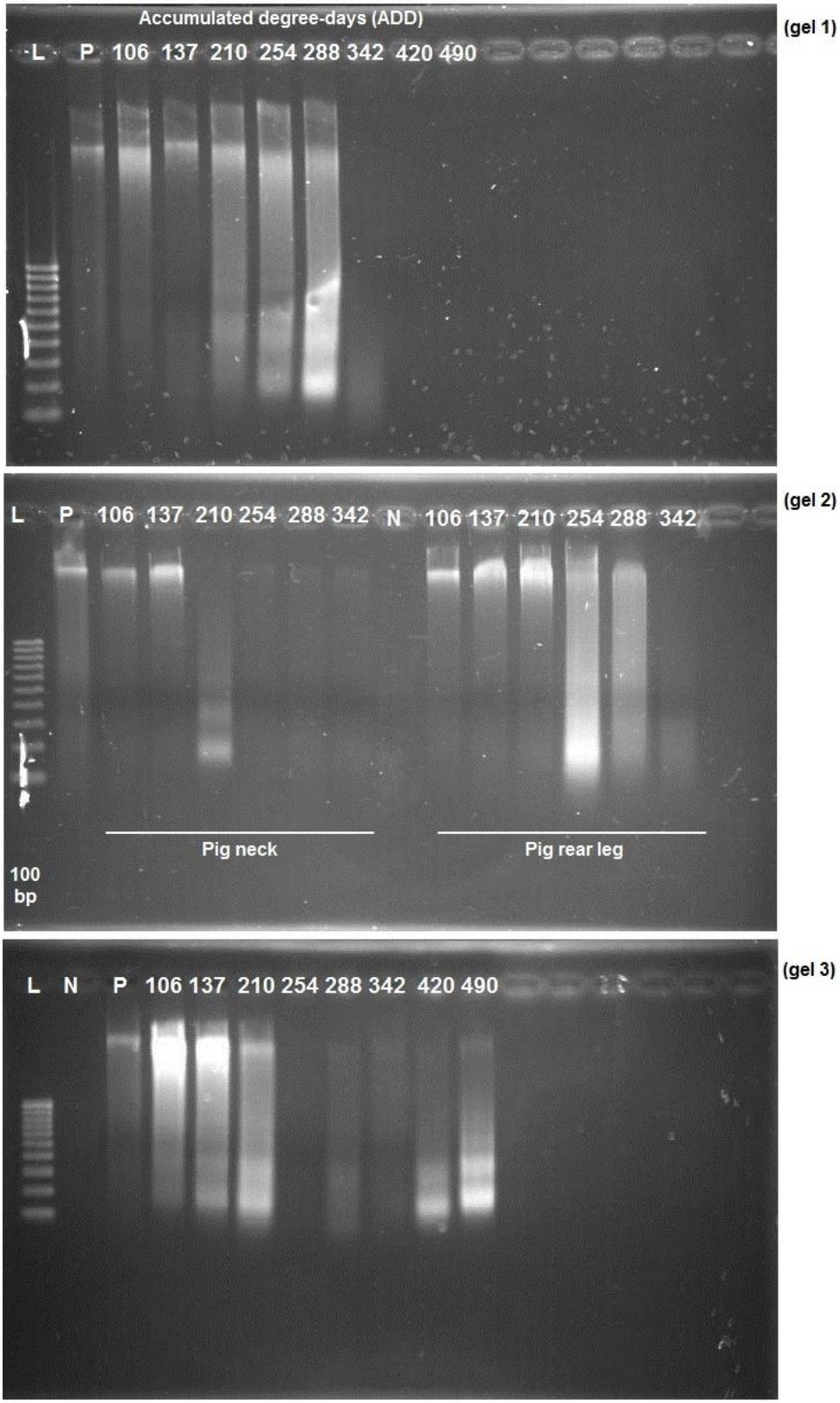

Figure 4.26 Shown above are examples of agarose gels of pig DNA samples collected at different ADD during September 2010 project, gel 1) whole carcases, gel 2) body fragments and gel 3) suspended soft muscle tissues. 100 bp DNA ladder (L) and positive control (P) are also shown. The small white spots appeared due to faulty gel tank.

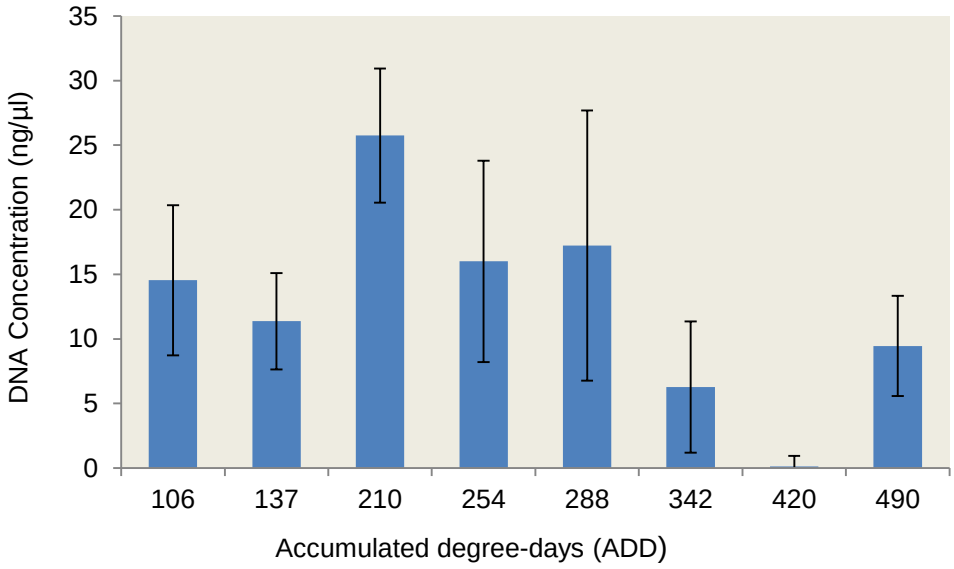

Figure 4.27 Shown above is the DNA quantification using PicoGreen for pig whole carcases in September 2010. The error bars indicate the standard error from three replicates. A total of 200 µl was extracted from each sample and 3 µl was used for quantification.

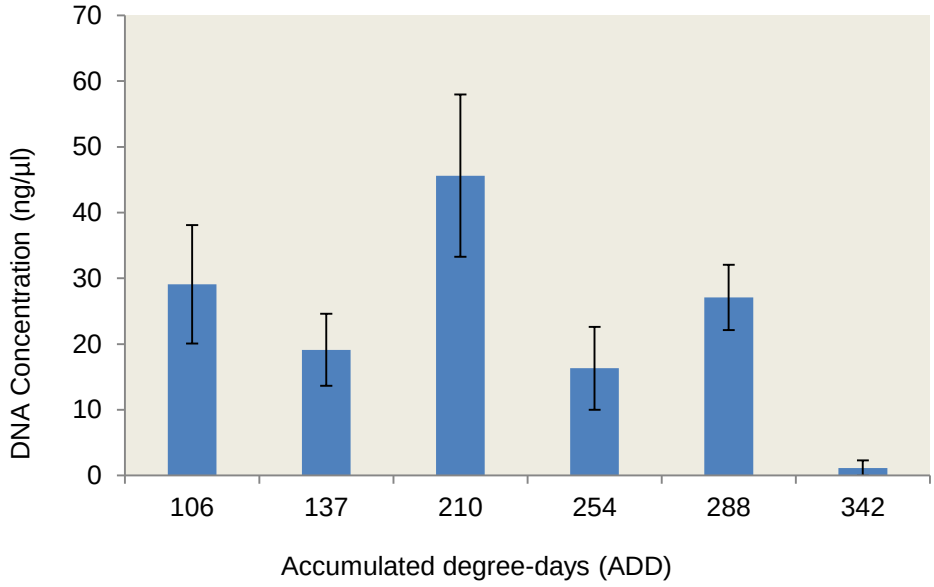

Figure 4.28 Shown above is the DNA quantification using PicoGreen for pig body fragments in September 2010. The error bars indicate the standard error from three replicates. A total of 200 µl was extracted from each sample and 3 µl was used for quantification.

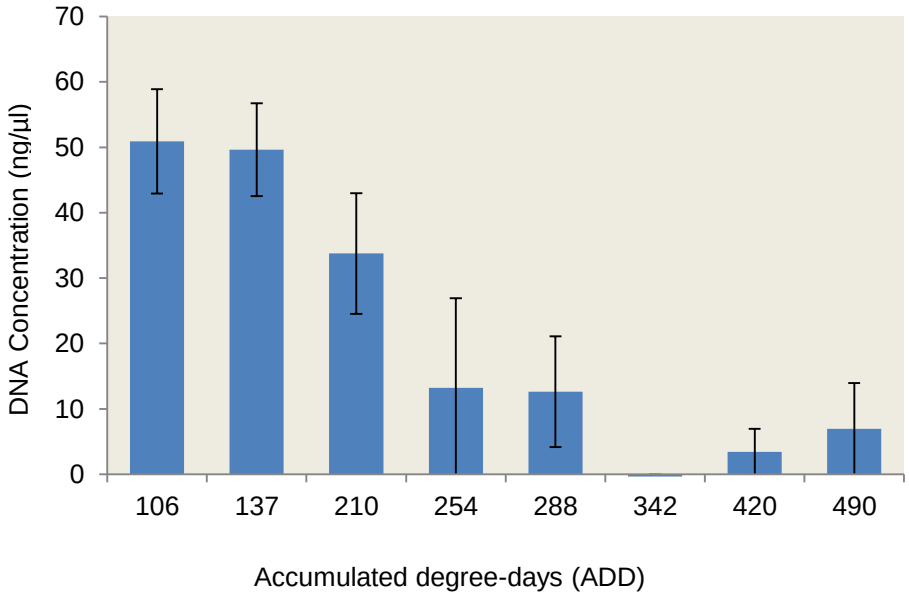

Figure 4.29 Shown above is the DNA quantification using PicoGreen pig suspended muscle tissues in September 2010. The error bars indicate the standard error from three replicates. A total of 200 µl was extracted from each sample and 3 µl was used for quantification.

4.8.3 4-plex Multiplex Amplification of Pig Whole Carcases

Multiplex amplification of pig whole carcases showed dropout in amplification of 305 bp and 384 bp at 420 ADD and 490 ADD. There was failure of amplification of 194 bp at 420 ADD and one sample amplified at 490 ADD. 70 bp produced amplification until 490 ADD (Figure 4.32 and Table 4.9).

Table 4.9 Shown below are the results of multiplex (4-plex) amplification of pig whole carcases tissues collected during September 2010.

Days	Accumulated degree-days (ADD)	Pig whole carcases			
		70 bp	194 bp	305 bp	384 bp
9	106	+++	+++	+++	+++
11	137	+++	+++	+++	+++
15	210	+++	+++	+++	+++
18	254	+++	+++	+++	+++
22	288	+++	+++	+++	+++
30	342	+++	+++	+++	+++
38	420	+++	---	**---**	**---**
44	490	+++	+--	**---**	**---**

‘‘+’’ denotes the presence and ‘‘-’’ represents the absence of PCR amplicons in triplicate samples during multiplex (4-plex) amplification.

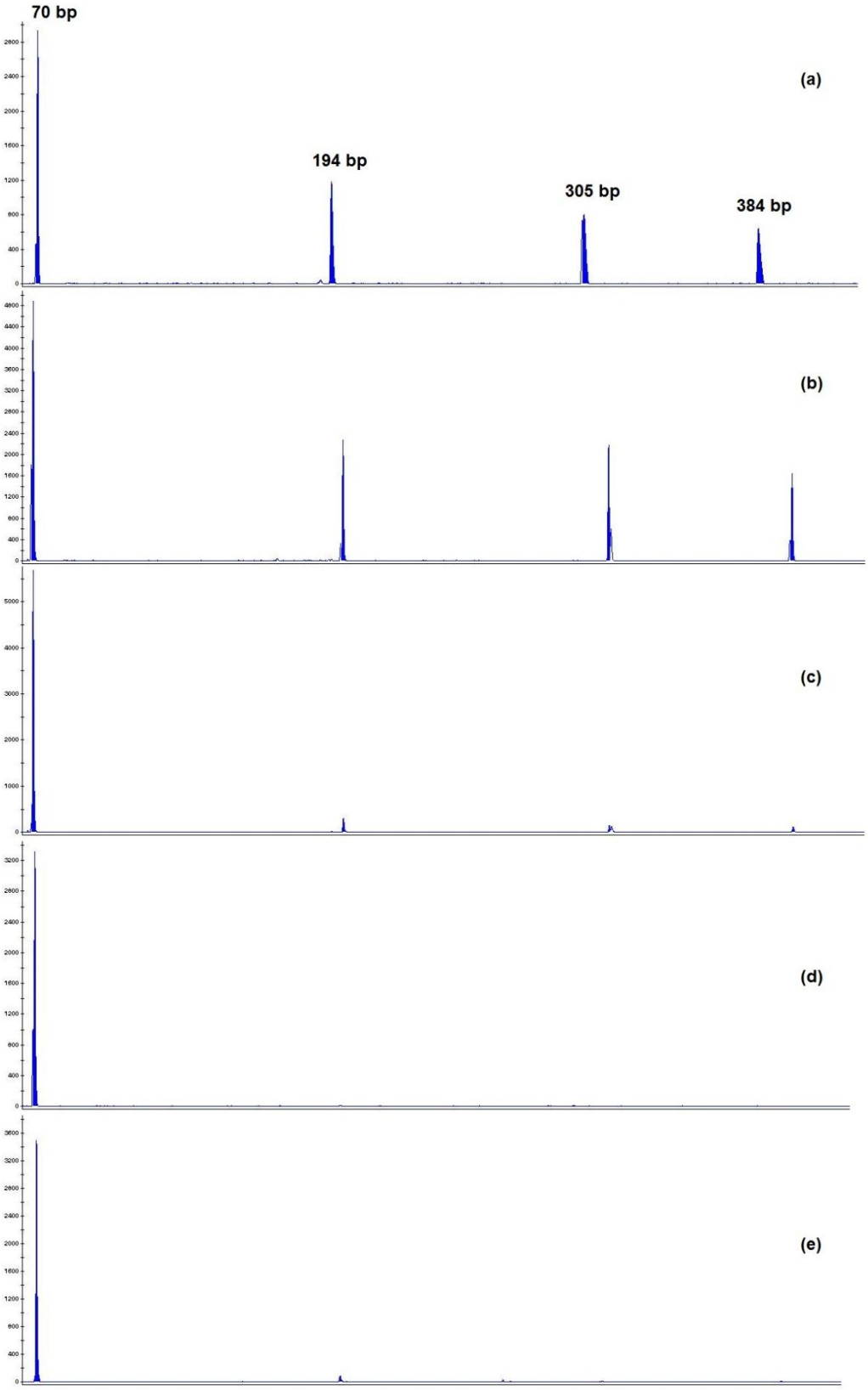

Figure 4.30 Shown above are examples of electropherograms of pig DNA obtained from whole carcases during September 2010 b) day 18 (254 ADD), c) day 30 (342 ADD), d) day 38 (420 ADD) and e) day 44 (490 ADD). Human positive control (a) also shown.

4.8.4 4-plex Multiplex Amplification of Pig Body Fragments

Pig body fragments showed full 4-plex multiplex amplification in all samples until 342 ADD except one sample which showed dropout of 384 bp (Figure 4.33 and Table 4.10).

Table 4.10 Shown below are the results of multiplex (4-plex) amplification of pig body fragments tissues collected during September 2010.

Days	Accumulated degree-days (ADD)	Pig body fragments			
		70 bp	194 bp	305 bp	384 bp
9	106	+++	+++	+++	+++
11	137	+++	+++	+++	+++
15	210	+++	+++	+++	+++
18	254	+++	+++	+++	+++
22	288	+++	+++	+++	+++
30	342	+++	+++	+++	++-

‘‘+’’ denotes the presence and ‘‘-’’ represents the absence of PCR amplicons in samples during 4-plex multiplex amplification.

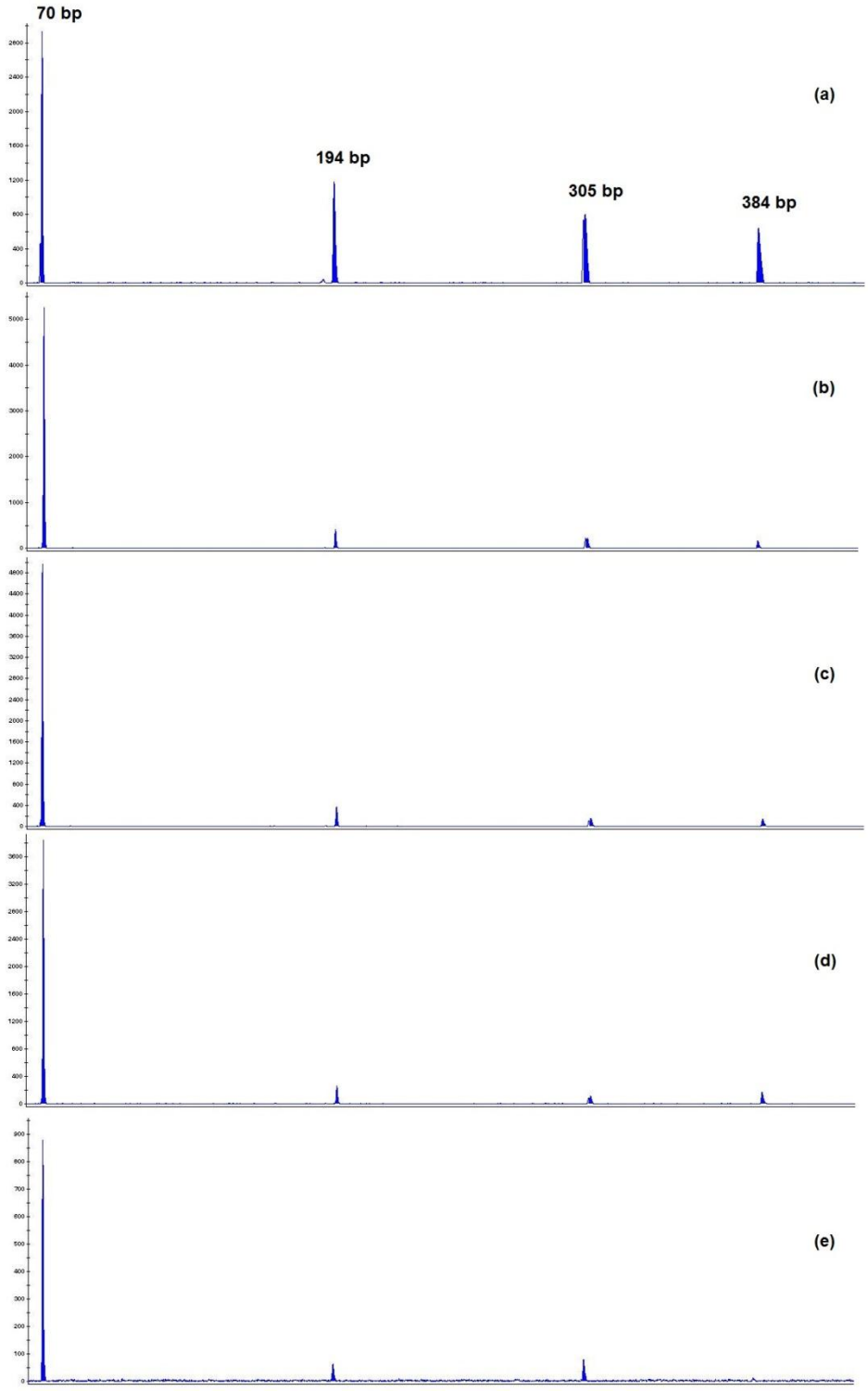

Figure 4.31 Shown above are examples of electropherograms of pig DNA obtained from body fragments during September 2010 at b) day 18 (254 ADD), c) day 22 (342 ADD), d and e) day 30 (342 ADD). Human positive control (a) also shown.

4.8.5 4-plex Multiplex Amplification of Pig Suspended Tissues

Dropout in amplification of 194 bp, 305 bp and 384 bp was observed at 254 ADD, 342 ADD, 420 ADD and 490 ADD. There was complete failure of amplification of 305 bp at 490 ADD. 70 bp amplified until 490 ADD (Figure 4.34 and Table 4.11).

Table 4.11 Shown below are the results of multiplex (4-plex) amplification of pig suspended tissues collected during September 2010.

Days	Accumulated degree-days (ADD)	Pig suspended tissues			
		70 bp	194 bp	305 bp	384 bp
9	106	+++	+++	+++	+++
11	137	+++	+++	+++	+++
15	210	+++	+++	+++	+++
18	254	+--	+--	+--	+--
22	288	+++	+++	+++	++-
30	342	+++	++-	+--	+--
38	420	+++	+--	+--	+--
44	490	+++	+++	---	++-

‘‘+’’ denotes the presence and ‘‘-’’ represents the absence of PCR amplicons in samples during multiplex (4-plex) amplification

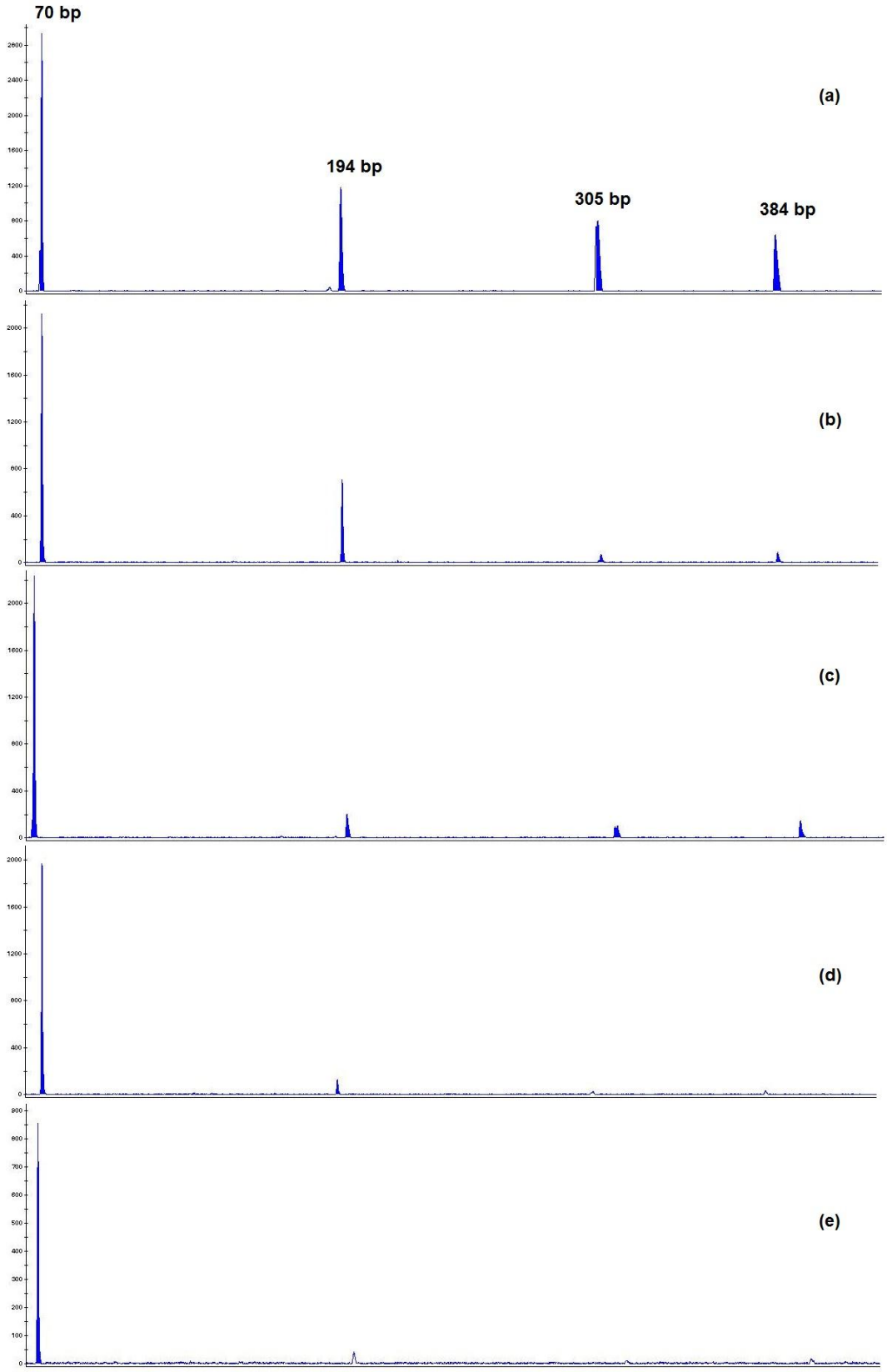

Figure 4.32 Shown above are examples of electropherograms of pig DNA obtained from suspended tissues during September 2010 at day 18 (254 ADD), c) day 38 (420 ADD), d and e) day 44 (490 ADD). Human positive control (a) also shown.

4.9 Species Variation between Rabbits and Pigs

Multiplex amplification of pig and rabbit muscle tissue samples collected at different time points showed that DNA was more persistent in pig whole carcases, body fragments and suspended tissues compared to the rabbit whole carcases, body fragments and suspended tissues. Multiplex amplification was obtained in pig whole carcases and body fragments until 490 ADD and 342 ADD respectively while rabbit whole carcases and body fragments showed multiplex amplification until 254 ADD and 288 ADD respectively. Drop out in amplification of larger amplicons was observed in rabbit and pig whole carcases after 137 ADD and 342 ADD respectively.

In case of suspended tissues, collection of samples was possible in both pig and rabbit until 490 ADD, but dropout in amplification of 194 bp, 305 bp and 384 bp occurred in rabbit after 210 ADD, while in case of pig, the amplification of larger amplicons was possible until 420 ADD.

4.10 Seasonal Variation

4.10.1 Rabbit Field Experiments

DNA degradation experiments in terms of ADD showed that DNA was more persistent in rabbit tissue samples collected from whole carcases, body fragments and suspended tissues during September 2010 followed by August 2009 . Rabbit whole carcases in June 2010 showed higher rate of DNA degradation compared to September 2010 and August 2009. In the September 2010 experiments, full multiplex amplification was obtained until 137 ADD (whole carcases), and 210 ADD (body fragments and suspended tissues), while in August 2009 , full multiplex amplification was obtained until 112 ADD (whole carcases and body fragments) and until 141 ADD (suspended tissues). In June 2010 experiments, full multiplex amplification was possible until 64 ADD.

4.10.2 Pig Field Experiments

Multiplex amplification results obtained from pig muscle tissue samples collected from field experiments showed that DNA was more persistent in pig whole carcases which were exposed to the field in February 2010 (until day 90 (603 ADD)), followed by September 2010 (until day 44 (490 ADD)) and May 2010 (until day 27 (338 ADD)).

During September 2010 body fragments produced full amplification until muscles were collected (342 ADD), while in case of whole carcases and suspended tissues, the amplification was possible until 490 ADD but there was complete failure of amplification of 305 bp and 384 bp in pig whole carcases after 342 ADD, while in suspended tissues, the amplification of 305 bp and 384 bp was possible until 420 ADD.

4.11 Statistical Analysis

Due to the inconsistency in DNA quantification results using PicoGreen®, statistical analysis was performed using only 4-plex multiplex amplification data. Statistical analysis was performed to determine the effect of ADD on the amplification success of 4-plex amplicons (70 bp, 194 bp, 305 bp and 384 bp) in pig and rabbit muscle tissues collected at different ADD from whole carcases, body fragments and suspended tissues. The generalised linear mixed model fit by the Laplace approximation was used to carry out Analysis of Deviance using R version 2.10.1. The analysis was carried out including all the factors such as species (pig and rabbit), days, ADD, amplicons and parts (whole carcasses, body fragments and suspended tissues) as random variables but due to the complexity of the data, ADD and individual amplicons were focused on. The results showed that the relationship between ADD and amplification success was not different for 305 bp and 384 bp (305 bp-384 bp: Z=0.96, p=0.76) but was different for pairwise comparison of every other PCR amplicons (Table 4.12).

Table 4.12 Shown below is the amplification success 4-plex multiplex amplicons in pig and rabbit muscles with increase in ADD. Data above diagonal are the probability of getting multiplex amplicons and below diagonal are the logits for the comparisons of different amplicons with increase in ADD.

Amplicons	Probability			
(bp)	70 bp	194 bp	305 bp	384 bp
70 bp	_	***	***	***
194 bp	4	_	0.003	0.006
305 bp	6.15	1.54	_	0.79
384 bp	5.92	1.48	0.96	_

*** represents highly significant difference (p<0.0001).

A comparison within 4-plex amplicons was also performed to determine the amplification success of multiplex amplicons in pig and rabbit muscles with increase in ADD. The results showed that at 0 ADD there was no difference in amplification success of the 4 amplicons but with increase in ADD the probability of getting 194 bp, 305 bp and 384 bp was 4, 6.15 and 5.92 times lower than 70 bp (70 bp-194 bp: $Z=4$, $p<0.001$; 70 bp-305 bp: $Z=6.15$, $p<0.001$; 70 bp-384 bp: $Z=5.92$, $p<0.001$). Similarly, the probability of getting 305 bp and 384 bp was 1.54 and 1.58 times less than 194 bp (194 bp-305 bp: $Z=1.58$, $p<0.001$; 194 bp-384 bp: $Z=1.48$, $p<0.001$). The probability of getting 384 bp was 0.96 times less than 305 bp (305 bp-384 bp: $Z=0.96$, $p=0.79$) with increase in ADD.

DNA persistence was also assessed in whole carcases, body fragments and suspended tissues. The results showed that there was no significant difference in DNA persistence between whole carcasses versus suspended tissues ($Z=0.57$, $p>0.05$) and whole carcases versus body fragments ($Z=1.71$, $p>0.05$), but there was a significant difference ($Z=2.31$, $p<0.05$) in DNA persistence in suspended tissues and body fragments with increase in ADD. Overall, the statistical analysis showed that amplification success of the three larger amplicons (194 bp, 305 bp and 384 bp) reduces with increase in ADD in pig and rabbit whole carcases, body fragments and suspended tissues. Similar patterns of 4-plex multiplex amplification in relation to ADD have been explained in tables 4.2-4.11. Data from the PicoGreen® based quantification studies was not used in these analyses due to the inconsistency in DNA quantities with ADD.

4.12 Discussion

This research was conducted to provide empirical evidence to supplement advice available to the forensic community for the collection of muscle tissue for forensic analysis. This type of collection is normally carried out to determine the identity of individuals following mass disasters, such as plane crashes or natural disasters (Budowle and Eisenberg, 2005). A number of samples can be collected from the body for the purpose of DNA identification. Mostly in forensic cases, whole and non-disrupted cadavers are examined at autopsy. In such circumstances, liquid blood samples, buccal swabs or blood spots on a filter paper can be collected. In situations where the blood or buccal swabs are not available, especially in the case of highly fragmented remains, alternative biological samples must be collected for analysis. Thus in fragmented bodies, the preferable tissue type will be muscle tissue rather than bone

and teeth (although DNA preservation in hard tissues (bone and teeth) is superior to soft tissues especially when putrification has occurred) because the time and cost associated with the analysis of hard tissues is considerably greater (Graham et al., 2008).

Current advice from the leading agency in this field (INTERPOL) is that muscle tissue should not be collected from decomposing bodies after 24 hours post-mortem interval (Prinz et al., 2007). Our data suggests that this is very conservative and DNA is viable for several days, at least in temperatures that are likely to be encountered in the UK and other temperate zones. If this information is converted into practice then the time spent to identify individuals, and returning them to their family could be significantly reduced due to that fact that processing of hard tissues (bone and tooth material) is time consuming compared to soft muscle tissues (Budowle and Eisenberg, 2005).

It is well known that DNA degradation is influenced by temperature (Lindahl, 1993). Generally higher temperatures result in an increase in the rate of DNA decay. Many studies described the increase in the rate of DNA degradation with increase in post-mortem interval (Ogata et al., 1990; Hoff-Olsen et al., 2001; Phengon et al., 2008; Gunawardane, 2009), but only few studies (Prince et al., 2001; Megyesi and Haskell 2005; Larkin et al., 2010) with very limited number of observations have described the effect of ADD on carcase decomposition and DNA persistence in soft tissues.

Insect activity and the microbial fermentation in the gut are important factors for decomposition of the remains thus reducing the possibility of collecting soft muscle tissues (Nedwell, 1984; Simmons and Moffatt 2010) therefore, in the present study; body fragments were separated from the gut to determine DNA persistence in whole bodies verses body fragments. Similarly, to avoid direct insect assess, tissues were suspended inside the polypropylene tubes covered by wire mesh that inhibited insect access but allowed air and water to enter.

In this study to determine the DNA persistence in relation to ADD, it was found that the rabbit whole carcases and fragmented tissues decomposed at faster rate compared to the pig. This is likely due to the proportionately greater volume of the dipteran larval mass on relatively smaller carcases, causing faster consumption. The larval mass also produces proportionately more heat distributed throughout a relatively smaller body mass (Slone et al., 2007; Adlam and Simmons, 2007). Simmons and Moffatt (2010) observed that ADD and insect activity influence the rate of decomposition more than

any other variable. The authors also reported that the decomposition progressed at much slower rate when insects were excluded compared to the remains where insects could access the body. Results from this study also suggest this since suspended tissues of both pig and rabbit were more persistent to decomposition compared to body fragments.

Pig body fragments decomposed faster than pig whole carcases and suspended tissues due to higher access of insect activity from the start of the experiments. Low ADD and no insect activity delay the decomposition process in muscle tissue in whole carcases and body fragments hence DNA persists for a longer period of time. In August 2009 project, decomposition of rabbit carcasses was faster due to high temperature, rainfall (humidity) and insect activity, and there were no soft muscle tissue samples available to collect after day 7 (112 ADD). Dry weather conditions (no rainfall), high temperature and maggot mass growth caused faster decomposition of muscles in rabbit carcasses in June 2010 and there were no soft tissues to collect after day 4. In contrast to these experiments, rabbit carcasses which were exposed to the environment at the end of September 2010, showed longer times for decomposition and hence it was possible to collect soft tissues for a longer time (254 ADD) for DNA analysis. Similarly, in case of pig, decomposition was faster in pig carcases which were placed in the field in May/June 2010 (338 ADD) compared to those in September 2010 (490 ADD) and February 2010 (603 ADD).

Low environmental temperatures and no insect activity helped slow down the decomposition process. For example, pig carcases in February 2010 were more persistent to decomposition and muscle tissues were available for collection until 90 days and DNA was persistent for a longer period. Similarly, in case of September 2010 pig carcases; decomposition was much slower compared to May/June as there was low temperature and less insect activity. During this study despite controlling for temperature within ADD calculations, results suggest that the seasonal variation seen was due to variation in maggot mass growth. As high temperature in summer favours maggot mass growth, therefore carcass decomposition increases in summer which ultimately effect on DNA persistence in muscles.

In summary, the experiments performed in this study showed that DNA degradation is highly influenced by increase in ADD along with many other factors such as type and size of species, rainfall (humidity), insect activity and the environmental temperature especially at the start of the experiment. DNA degradation was also seen to vary

between body fragments and suspended tissues. The results obtained from this study correlate with previous findings (Gunawardane 2009; Larkin et al., 2010).

The research work performed in this study suggested that muscle tissues if available should be collected for DNA profiling, since even if degraded, a profile can be obtained. The results also suggested that the isolation of tissues from insect activity as quickly as possible (even if immediate storage is not possible) may be beneficial for DNA persistence.

Due to high sensitivity, throughput and lower cost, PicoGreen® dsDNA quantification was used to quantify DNA. The inconsistency observed in the results with respect to DNA quantity with increasing ADD is likely due to the non-specificity of PicoGreen® dye as it binds to any dsDNA regardless of species. The increase in DNA quantity at later stages of decomposition was likely due to the bacterial and fungal growth on the decomposing tissues. This has also been described by Larkin et al., (2010). In some cases, amounts of DNA were also found to decrease with ADD, making any valid conclusions about DNA quantity and ADD difficult. In fact, there have been a number of studies that have found lack of consistency with PicoGreen®. Haque et al., (2003) reported that inconsistency in PicoGreen® quantification results might be due to the variability in laboratory sample handling steps or due to residual (unmodeled) factors. The author also reported the amount of variance attributed to the entire laboratory handling procedures was 4.3% and intra-assay (between replicates) coefficient of variance (CV) was 13.6%. Rengarajan et al., (2002) also evaluated the sensitivity and linearity of PicoGreen® quantification and demonstrated that the intra-assay CV of PicoGreen® was 8.3%. It was reported that the smaller volumes of DNA (2.0 µl -5.0 µl) used for PicoGreen® quantification also introduced more sample variability due to the sampling effect or increased failure to transfer smaller volumes (Ahn et al., 1996; Singer et al., 1997; Rengarajan et al., 2002; Haque et al., 2003).

4.13 Conclusion

Carcass decomposition and hence DNA persistence in soft muscle tissues is highly influenced by an increase in average daily temperature and insect activity. The rate of DNA degradation in muscle tissues increases with an increase in ADD. The type of species also affects DNA persistence. It was observed that DNA was more persistent in pig muscle tissue compared to rabbit muscle tissue. DNA degradation was faster in rabbit carcasses in June 2010 compared to those in August 2009 and September 2010, similarly, in pig, DNA was more persistent in whole carcases in February 2010 followed by September 2010 and May 2010. The seasonal variation in DNA persistence observed was most likely due to variability in maggot mass growth (increased maggot mass increased carcass decomposition and ultimately decrease DNA persistence). It was observed that with increase in ADD, there was a significant difference ($p<0.05$) in DNA persistence (using the 4-plex multiplex) between body fragments and suspended tissues but there was no difference in DNA persistence in whole carcases versus suspended and whole carcasses versus fragmented tissues in both species.

CHAPTER 5

DNA PRESERVATION

5.1 Introduction

Muscle tissue is an abundant source of DNA and when recovered from human remains can be used for identification purposes. However, successful analysis depends in part on optimal collection and preservation of biological material obtained from deceased individuals. Inefficient preservation methods can lead to degradation of intact DNA to such an extent that data is not always available for victim identification (Alonso et al., 2001; Prinz et al., 2007; Michauld and Foran, 2011).

Freezing at -20 °C is the primary technique recommended to preserve biological material following a mass disaster (FBI, 2007; NIJ, 2005), although freezing at -80 °C or in liquid nitrogen (-196 °C) are also used. Refrigeration at 4 °C also slows the action of endogenous nucleases and microbial growth (Matsuo et al., 1999; Murphy et al., 2002). In mass fatality incidents, immediate freezing of the samples may not be available under field conditions. The DNA Commission of the International Society for Forensic Genetics (ISFG) mentioned the placement of soft muscle tissue samples in preservative solutions as an alternative to cold storage, if preservation at room temperature is required (Prinz et al., 2007). The methods selected in this study have all been described previously (Srinivasan et al., 2002; Rodriguez et al., 2002; Graham et al., 2008; Michauld and Foran, 2011) but in most cases, using fresh samples.

In the present study, partially decomposed soft muscle tissue samples from pig after field exposure for 79 ADD and 210 ADD and from rabbit after 45 ADD and 79 ADD, were preserved using 10% buffered formalin, 96% ethanol and cell lysis solution (with and without 1% sodium azide) for one year at room temperature. Tissue samples weighing 1 g and 0.5 g were stored in 5 ml and 0.25 g samples were preserved in 1 ml of preservative solutions. Day zero samples were also preserved in same way to work as positive controls. After extraction DNA was assessed using agarose gel electrophoresis and 4-plex multiplex amplification followed by CE to determine the efficiency of these preservative solutions using partially decomposed muscle tissue samples.

5.2 Aims and Objectives

- To assess the efficiency of different solutions to preserve partially decomposed soft muscle tissue samples of pig and rabbit stored at room temperature for a period of one year using agarose electrophoresis, DNA quantification and 4-plex multiplex PCR.

5.3 Results

5.3.1 Samples

Muscle samples were collected in field conditions and preserved in four different solutions (96% ethanol, 10% buffered formalin, cell lysis solution with and without 1% sodium azide). At three time points (1, 6 and 12 months) 25-30 mg samples were collected from each original sample (1g, 0.5g and 0.25g preserved in 5 ml, 5 ml and 1 ml respectively) of muscle tissues of pig and rabbit. Appropriate volumes of cell lysis aliquots (with and without sodium azide) (125 µl for 1g, 250 µl for 0.5g and 100 µl for 0.25g samples) were also collected at 6 months and 1 year (Section 2.1).

DNA extraction was carried out using DNeasy® Blood and Tissue kit and agarose gel electrophoresis was performed to determine the presence of DNA (Section 2.2.1 and 2.2.2). Samples were extracted in triplicate. DNA quantification was performed using Quant-iT™ PicoGreen® dsDNA reagents (Invitrogen™, UK) on the TECAN GEnios Pro Microplate Reader (Section 2.7).

DNA quantification using PicoGreen generally quantifies double standard DNA regardless of the species type, therefore the DNA seen on the agarose gels and detected through quantification can come from bacterial sources as well as from the muscle tissue. 4-plex Multiplex amplification was subsequently performed to detect DNA from the muscle tissue. Based on PicoGreen DNA quantification data, 0.6 ng DNA template was used for the 4-plex multiplex amplification. In samples where PicoGreen quantification showed negative results 6.9 µl of DNA extract were used (Section 2.7.2.4).

5.3.2 Ethanol Preservation

5.3.2.1 DNA Quantification of Rabbit Tissues using Agarose Gel Electrophoresis and PicoGreen®

DNA quantification of soft muscle tissue samples of rabbit preserved in 96% ethanol showed that it is a suitable preservative to preserve control and partially decomposed samples for one year (Figure 5.1, 5.2 and 5.3). Ethanol preserved samples changed to white colour and did become friable, but this did not seem to affect DNA extraction. Ethanol preservation preserved good quality of DNA after one year of storage at room temperature.

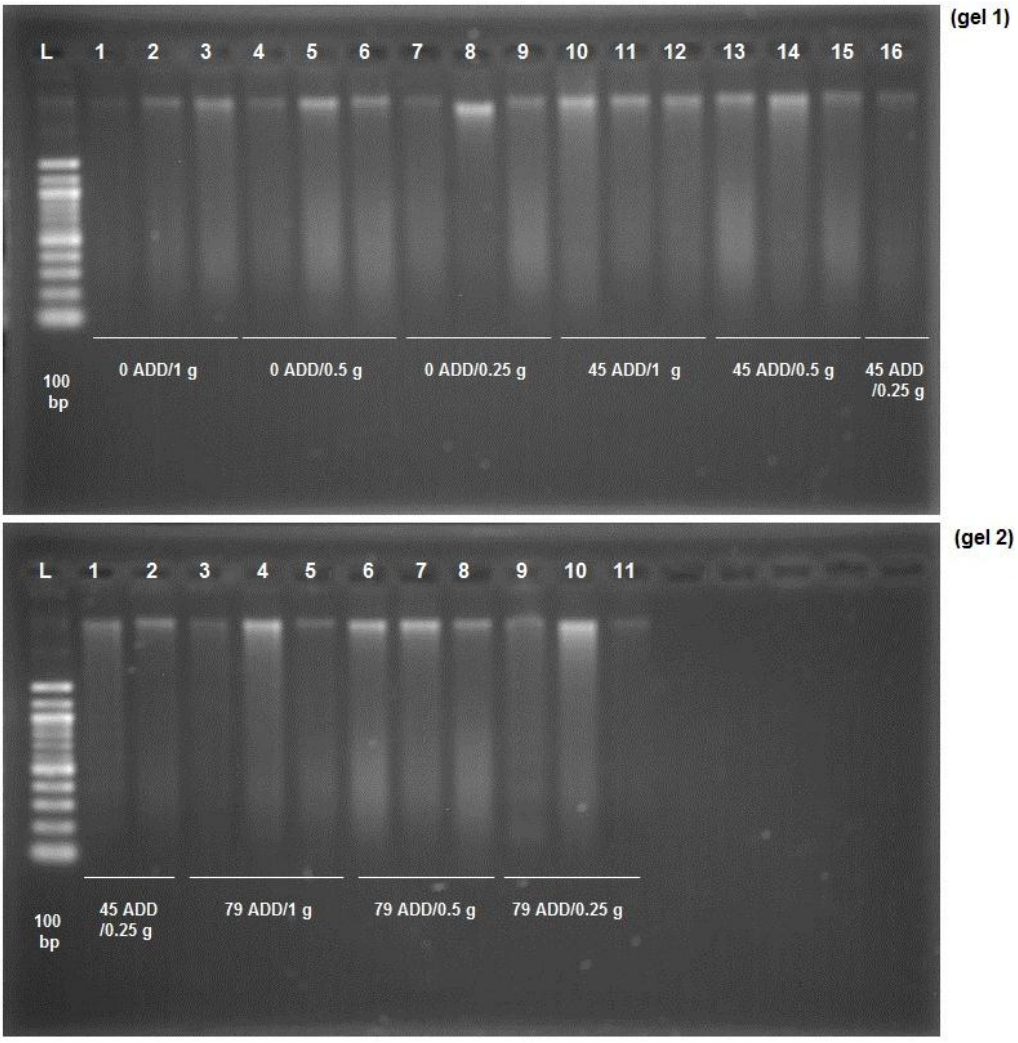

Figure 5.1 Agarose gels showing rabbit DNA obtained from ethanol preserved tissues. Soft muscle tissue samples were collected in triplicates from rabbit whole carcases at 0 ADD, 45 ADD and 79 ADD (gel 1 and 2) and preserved in 96% ethanol at room temperature for one year. The 100 bp DNA ladder (L) also shown.

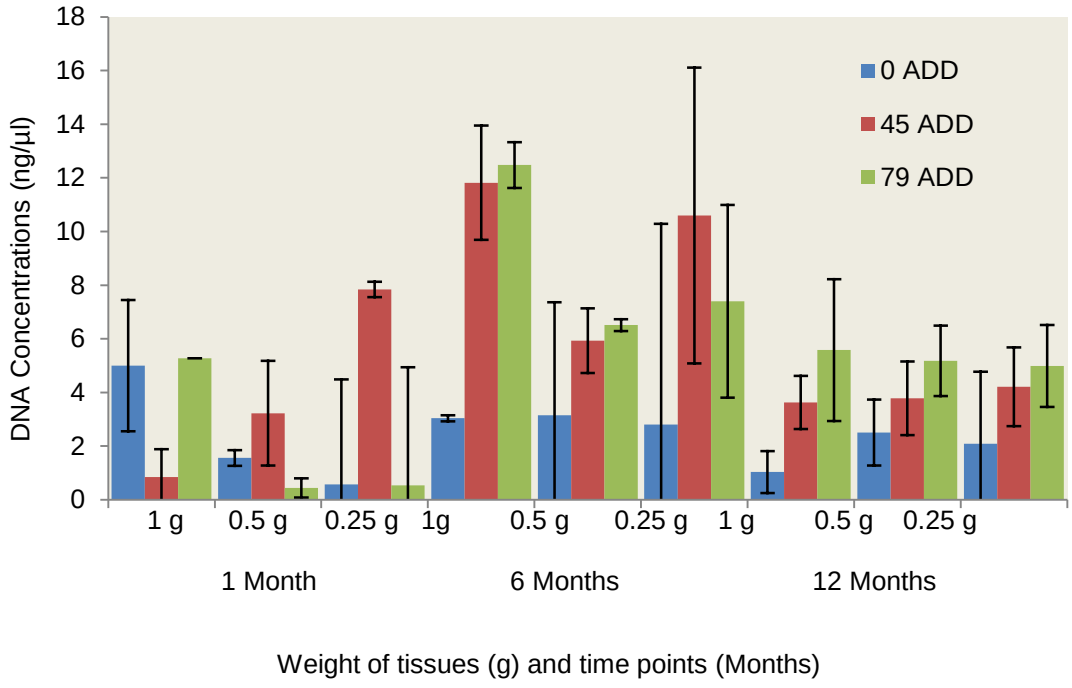

Figure 5.2 Shown above is an example of PicoGreen® DNA quantification of rabbit muscle tissue samples preserved in 96% ethanol at room temperature up to one year. The error bars indicate the standard error from three replicates. A total of 200 µl was extracted from each sample and 3 µl was used for quantification.

5.3.2.2 4-plex Multiplex Amplification

Rabbit ethanol preserved samples produced full 4-plex amplification in control and partially decomposed samples preserved for one year at room temperature (Figure 5.3 and Table 5.1)

Table 5.1 Shown below are the results of triplicate 4-plex multiplex PCR amplification of rabbit muscle tissue samples preserved in 96% ethanol at room temperature for one year.

Accumulated Degree-days (ADD)	Weight of tissues	Rabbit tissues preserved in 96% ethanol			
		70 bp	194 bp	305 bp	384 bp
0 ADD	1 g	+++	+++	+++	+++
	0.5 g	+++	+++	+++	+++
	0.25 g	+++	+++	+++	+++
45 ADD	1 g	+++	+++	+++	+++
	0.5 g	+++	+++	+++	+++
	0.25 g	+++	+++	+++	+++
79 ADD	1 g	+++	+++	+++	+++
	0.5 g	+++	+++	+++	+++
	0.25 g	+++	+++	+++	+++

‘‘+’’ denotes the presence of PCR amplicons in triplicate samples during 4-plex multiplex amplification.

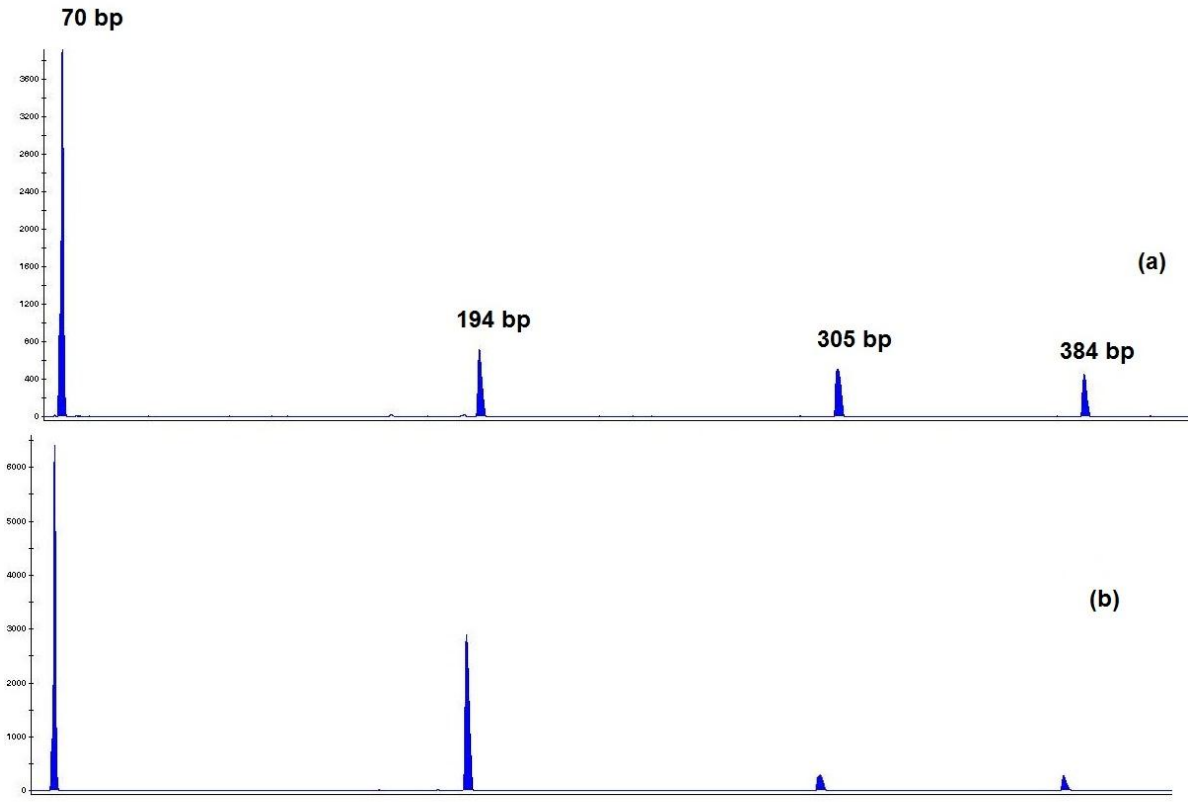

Figure 5.3 Shown above is an example of electropherograms of rabbit DNA samples obtained from soft muscle tissues preserved in 96% ethanol (b) for one year at room temperature. Day zero positive control (a) also shown.

5.3.2.3 Pig Tissues

Pig soft muscle tissues preserved in 96% ethanol showed DNA in all samples after one year of storage at room temperature (Figure 5.4, 5.5 and 5.6). Decolouration, shrinkage and toughness of soft tissues were also observed in pig samples.

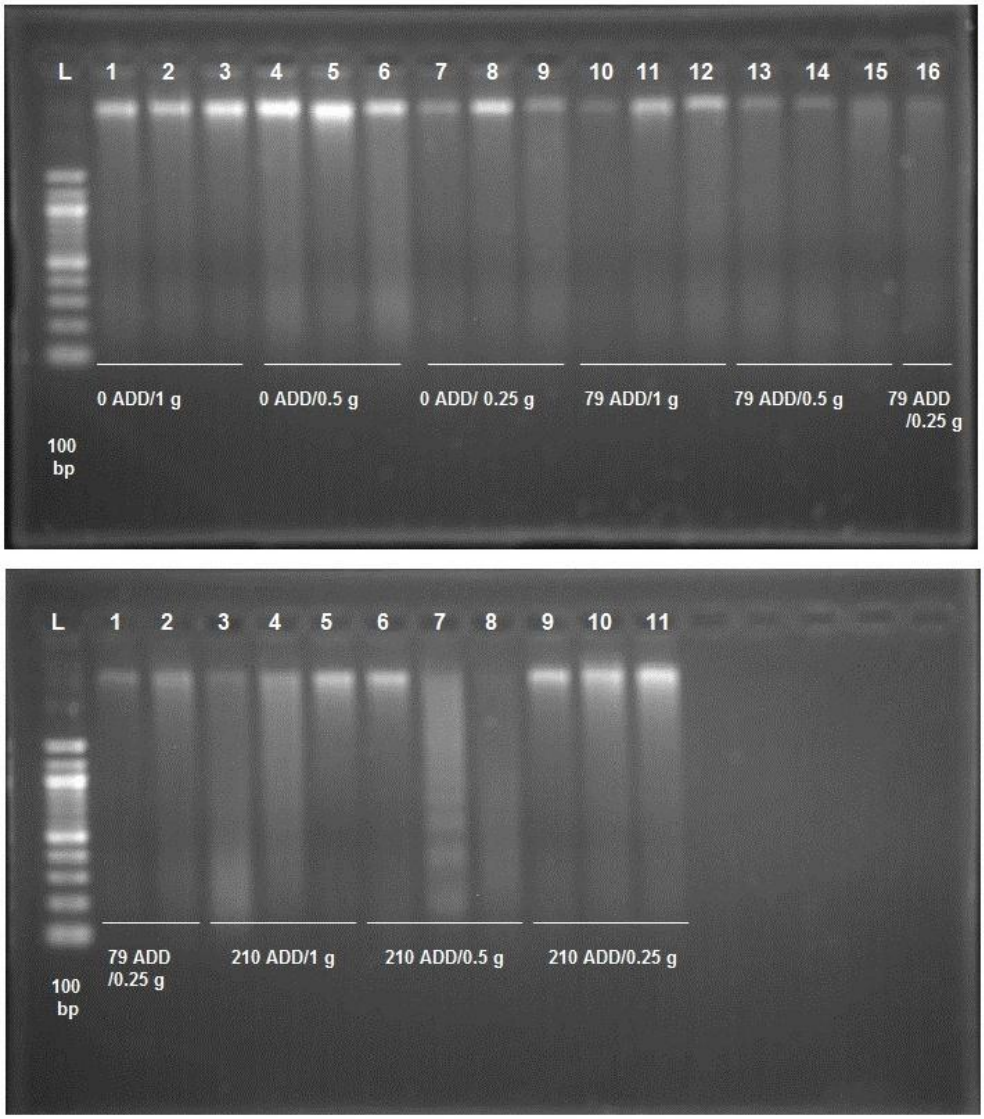

Figure 5.4 Shown above are agarose gels of pig DNA obtained from soft muscle tissue samples preserved in 96% ethanol (gel 1 and 2) at room temperature for one year. The 100 bp DNA ladder (L), weight of tissues (g) and ADD are also shown.

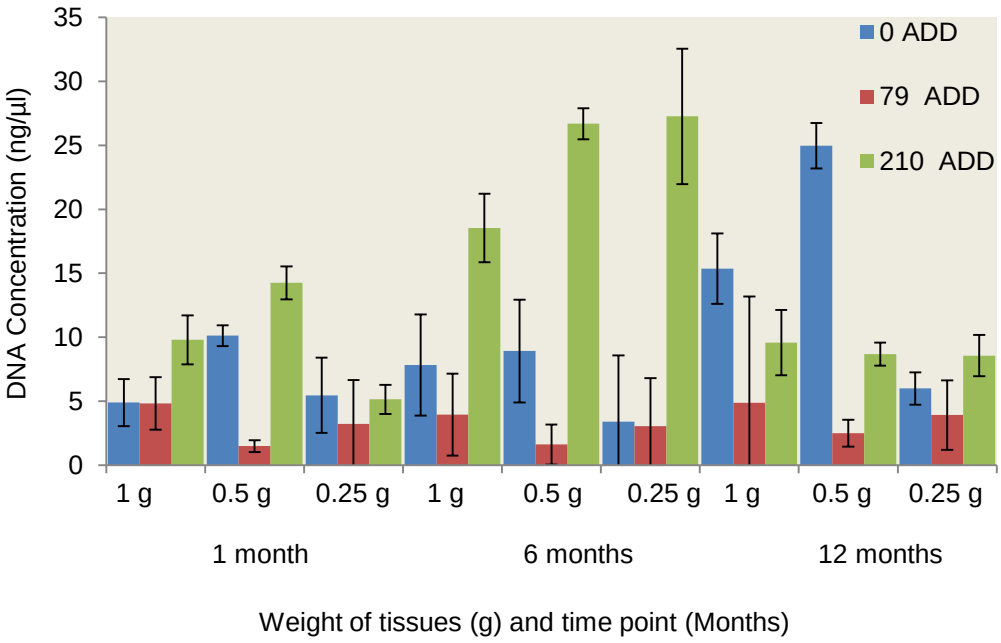

Figure 5.5 Shown above is an example PicoGreen® of DNA quantification of pig soft muscle tissue samples preserved in 96% ethanol at room temperature up to one year. The error bars indicate the standard error from three replicates. A total of 200 µl was extracted from each sample and 3 µl was used for quantification.

5.3.2.4 4-plex Multiplex Amplification

Full 4-plex amplification was obtained in fresh and partially decomposed samples of pig preserved in 96% ethanol for one year at room temperature (Table 5.2 and Figure 5.6 b), there was no allelic drop-out observed.

Table 5.2 Shown below are the results of 4-plex multiplex PCR amplification of pig muscle tissue samples preserved in 96 % ethanol at room temperature for one year.

Accumulated Degree-days (ADD)	Weight of tissues	pig tissues preserved in 96% ethanol			
		70 bp	194 bp	305 bp	384 bp
0 ADD	1 g	+++	+++	+++	+++
	0.5 g	+++	+++	+++	+++
	0.25 g	+++	+++	+++	+++
79 ADD	1 g	+++	+++	+++	+++
	0.5 g	+++	+++	+++	+++
	0.25 g	+++	+++	+++	+++
210 ADD	1 g	+++	+++	+++	+++
	0.5 g	+++	+++	+++	+++
	0.25 g	+++	+++	+++	+++

‘‘+’’ denotes the presence of PCR amplicons in triplicate samples during 4-plex multiplex amplification.

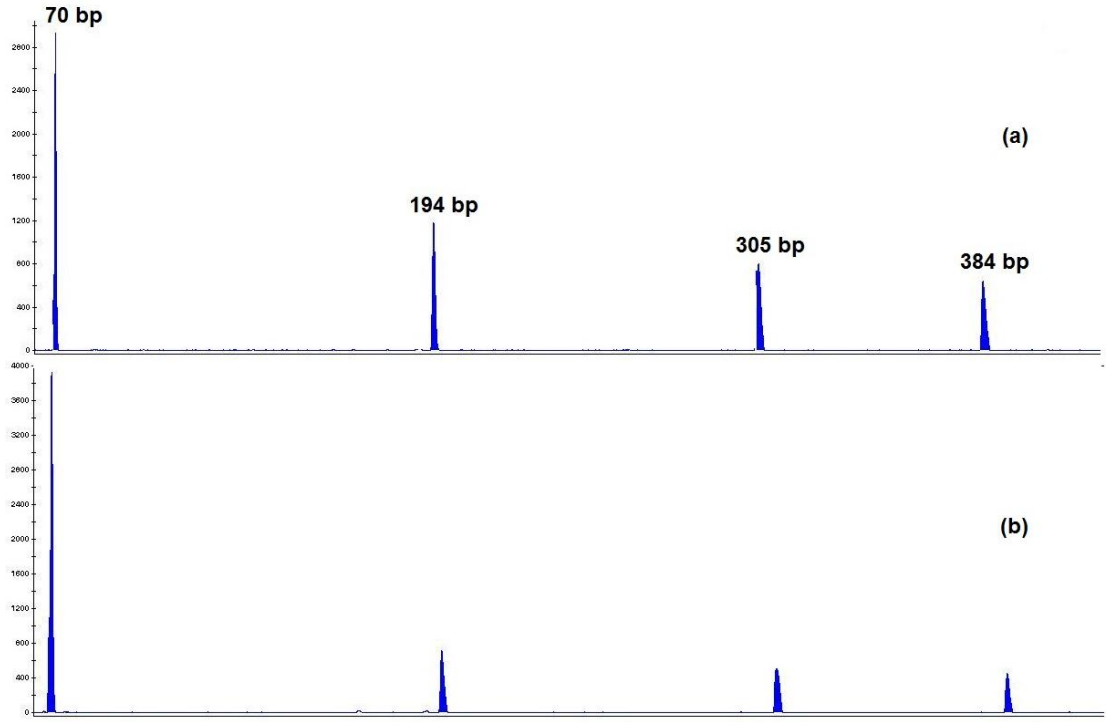

Figure 5.6 Shown above is an example of electropherograms of pig DNA samples obtained from soft muscle tissues preserved in 96% ethanol (b) for one year at room temperature. Day zero positive control (a) also shown.

5.3.3 Cell Lysis Solution

5.3.3.1 Rabbit Tissues

Commercially available cell lysis solution (Qiagen) was used to preserve fresh and partially decomposed soft muscle tissue samples of rabbit at room temperature for one year. DNA quantification of samples stored in cell lysis solution using PicoGreen® showed that DNA can be preserved successfully at room temperature for one year. There was good yield and good quality of DNA in control and partially decomposed samples except few samples preserved at 79 ADD (Figure 5.8 and 5.12). It was observed that soft muscle tissues are digested by the cell lysis solution; therefore, aliquots of cell lysis solution were also processed for DNA extraction. PicoGreen Quantification showed that high amount of DNA can be obtained by extracting cell lysis aliquots. Cell lysis aliquots gave higher quantity of DNA compared to tissues preserved in cell lysis solution (Figure 5.7 and 5.9).

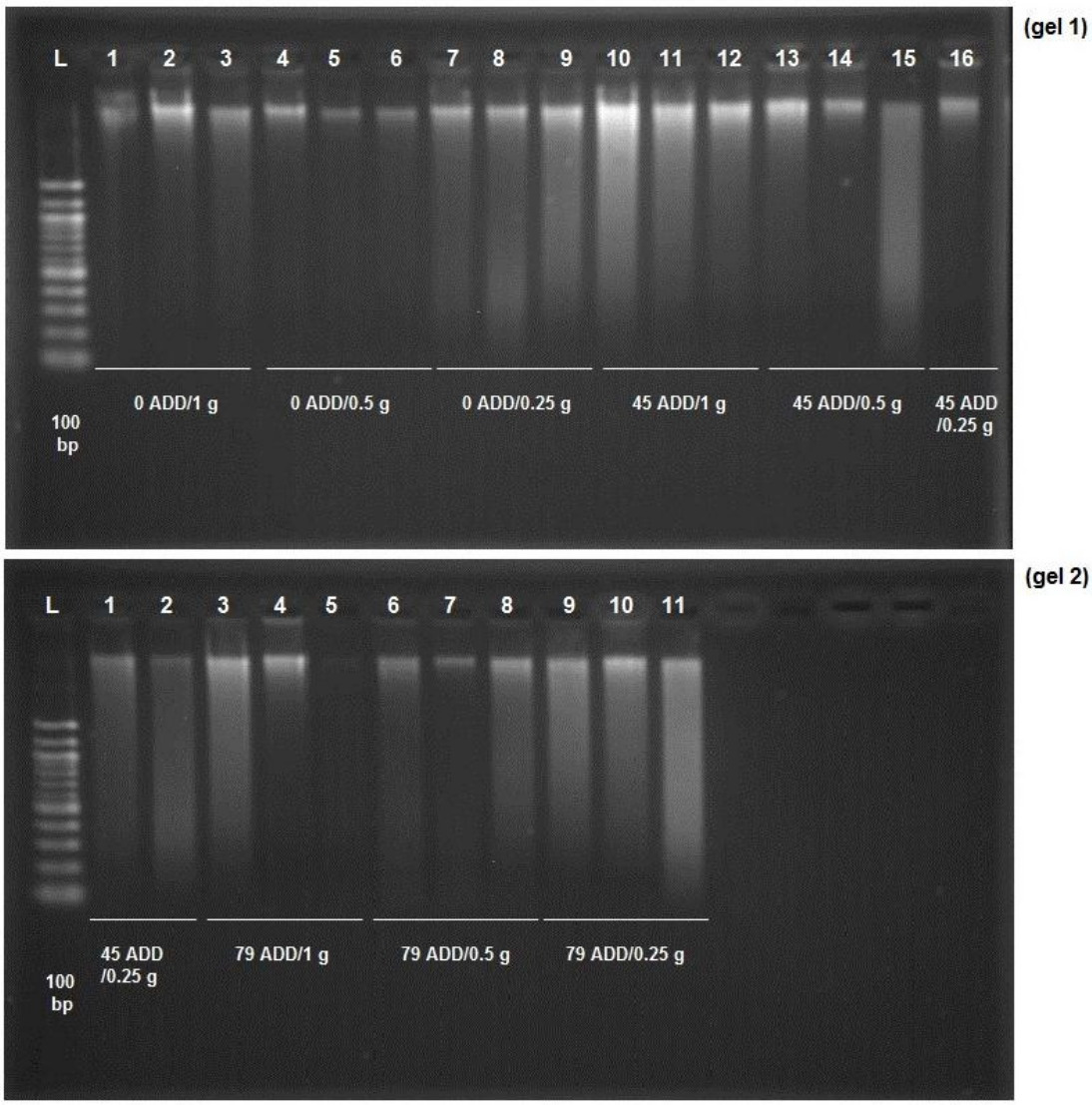

Figure 5.7 Shown above are agarose gels of rabbit DNA obtained from aliquots of cell lysis solution (gel 1 and 2) that was used to preserve soft muscle tissue samples at room temperature for one year. The 100 bp DNA ladder (L), weight of tissues (g) and ADD also shown.

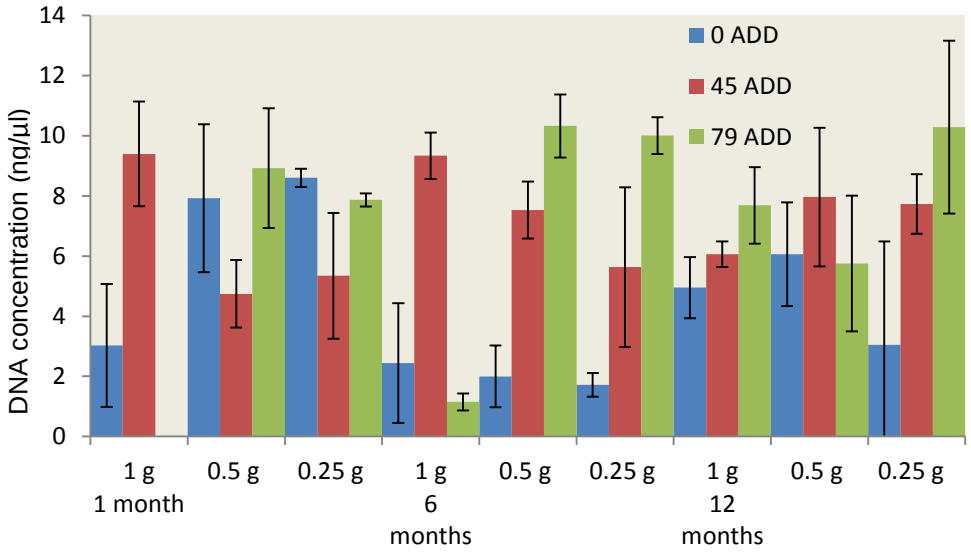

Figure 5.8 Shown above is an example of PicoGreen® DNA quantification of rabbit soft muscle tissue samples preserved in cell lysis solution at room temperature up to one year. The error bars indicate the standard error from three replicates. A total of 200 µl was extracted from each sample and 3 µl was used for quantification.

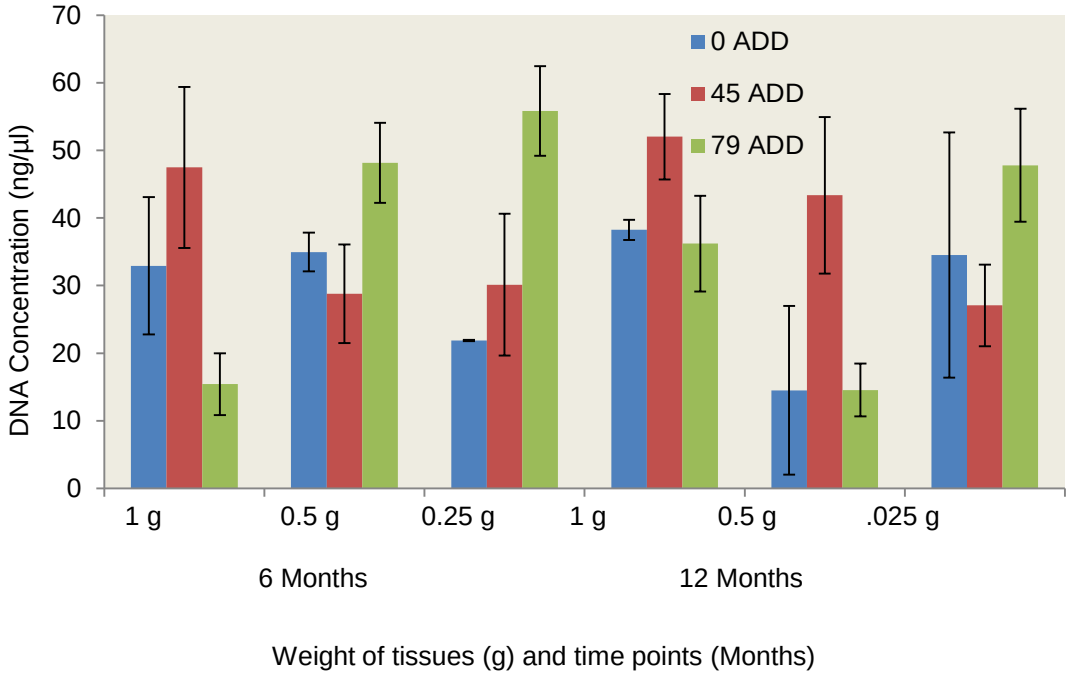

Figure 5.9 Shown above is an example of PicoGreen® DNA quantification of aliquots of rabbit cell lysis solution up to one year. The error bars indicate the standard error from three replicates. A total of 200 µl was extracted from each sample and 3 µl was used for quantification.

5.3.4 Cell Lysis Solution with 1% Sodium Azide

5.3.4.1 Rabbit Tissues

In this preservation methods 1% sodium azide was added in to cell lysis solution to stop bacterial or fungal growth in the solution for long term storage. DNA quantification of tissues preserved with the addition of 1% sodium azide showed that DNA can be preserved for one year in both partially decomposed and control samples, Although DNA yield was less in samples preserved for 12 months compared to 1 month and 6 months. Similarly, aliquots of solution containing sodium azide showed DNA to be preserved in cell lysis solutions with 1% sodium azide having more yield compared to the tissue (Figure 5.10, 5.11 and 5.12).

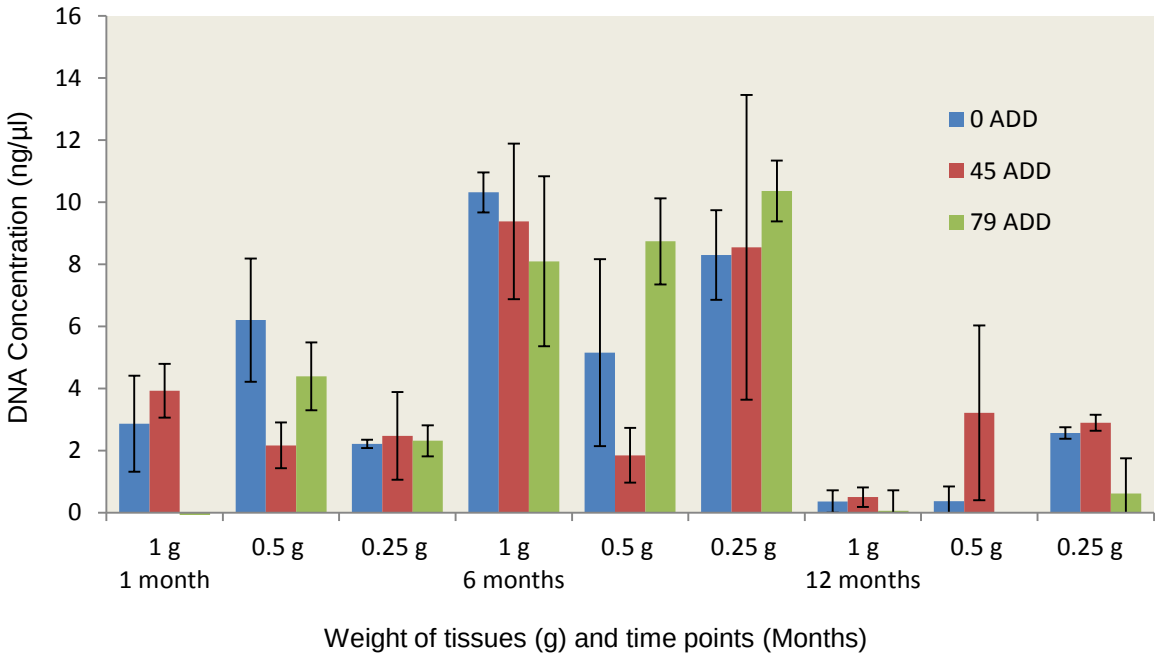

Figure 5.10 Shown above is PicoGreen® DNA quantification of rabbit soft muscle tissue samples preserved in cell lysis solution with 1% sodium azide at room temperature up to one year. The error bars indicate the standard error from three replicates. A total of 200 µl was extracted from each sample and 3 µl was used for quantification.

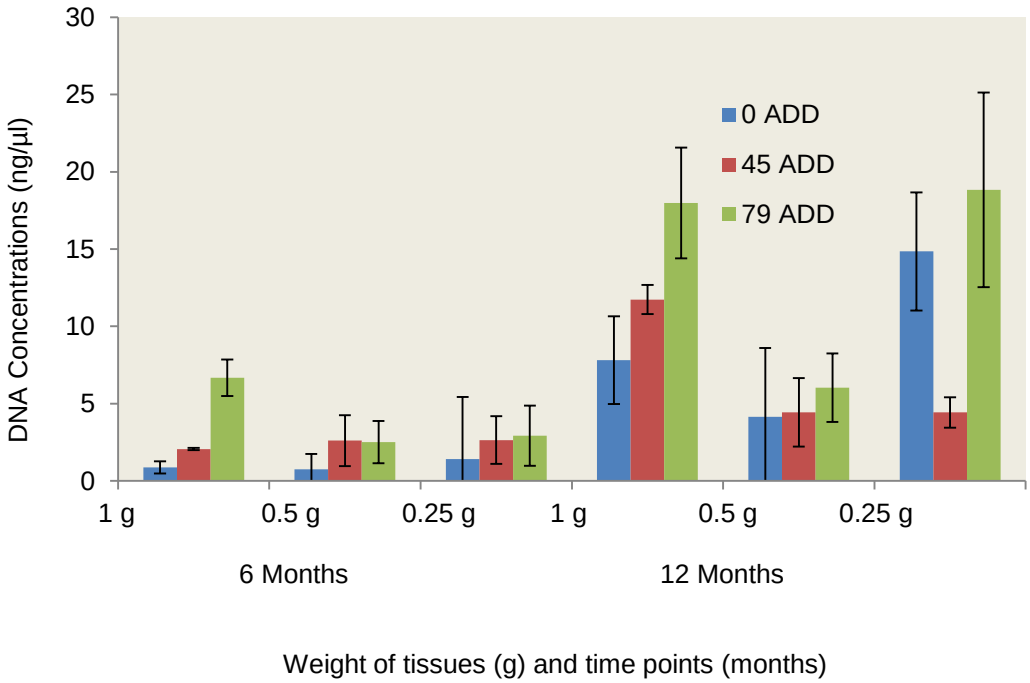

Figure 5.11 Shown above is PicoGreen® DNA quantification of aliquots of rabbit cell lysis solution with 1% sodium azide up to one year. The error bars indicate the standard error from three replicates. A total of 200 µl was extracted from each sample and 3 µl was used for quantification.

5.3.4.2 4-plex Multiplex Amplification

Rabbit tissue samples preserved in cell lysis solution with and without 1% sodium azide produced full 4-plex amplification in all day zero and degraded samples stored at room temperature for one year (Table 5.3 and 5.4, Figures 5.12b and 5.12d). Similarly, DNA extracted from aliquots of the preservative solutions also produced full profiles in all samples (Table 5.3, 5.4, Figures 5.12c and 5.12e). It was found that cell lysis solution (with and without 1% sodium azide) is suitable preservative to preserve DNA in degraded tissue samples and full 4-plex amplification can be obtained successfully after one year of storage at room temperature.

Table 5.3 Shown below are the results of the 4-plex multiplex PCR amplification of rabbit soft muscle tissue samples preserved in cell lysis solution at room temperature for one year.

Accumulated Degree-days (ADD)	Weight of tissues	Rabbit cell lysis solution (Tissues)				Rabbit cell lysis solution (aliquots)			
		70 bp	194 bp	305 bp	384 bp	70 bp	194 bp	305 bp	384 bp
0 ADD	1 g	+++	+++	+++	+++	+++	+++	+++	+++
	0.5 g	+++	+++	+++	+++	+++	+++	+++	+++
	0.25 g	+++	+++	+++	+++	+++	+++	+++	+++
45 ADD	1 g	+++	+++	+++	+++	+++	+++	+++	+++
	0.5 g	+++	+++	+++	+++	+++	+++	+++	+++
	0.25 g	+++	+++	+++	+++	+++	+++	+++	+++
79 ADD	1 g	+++	+++	+++	+++	+++	+++	+++	+++
	0.5 g	+++	+++	+++	+++	+++	+++	+++	+++
	0.25 g	+++	+++	+++	+++	+++	+++	+++	+++

‘‘+’’ denotes the presence of PCR amplicons in triplicate samples during 4-plex multiplex amplification.

Table 5.4 Shown below are the results of 4-plex multiplex (4-plex) PCR amplification of rabbit soft muscle tissue samples preserved in cell lysis solution with 1% sodium azide at room temperature for one year.

Accumulated Degree-days (ADD)	Weight of tissues	Rabbit cell lysis solution with 1% sodium azide (Tissues)				Rabbit cell lysis solution with 1% sodium azide (aliquots)			
		70 bp	194 bp	305 bp	384 bp	70 bp	194 bp	305 bp	384 bp
0 ADD	1 g	+++	+++	+++	+++	+++	+++	+++	+++
	0.5 g	+++	+++	+++	+++	+++	+++	+++	+++
	0.25 g	+++	+++	+++	+++	+++	+++	+++	+++
45 ADD	1 g	+++	+++	+++	+++	+++	+++	+++	+++
	0.5 g	+++	+++	+++	+++	+++	+++	+++	+++
	0.25 g	+++	+++	+++	+++	+++	+++	+++	+++
79 ADD	1 g	+++	+++	+++	+++	+++	+++	+++	+++
	0.5 g	+++	+++	+++	+++	+++	+++	+++	+++
	0.25 g	+++	+++	+++	+++	+++	+++	+++	+++

‘‘+’’ denotes the presence of PCR amplicons in triplicate samples in 4-plex multiplex amplification.

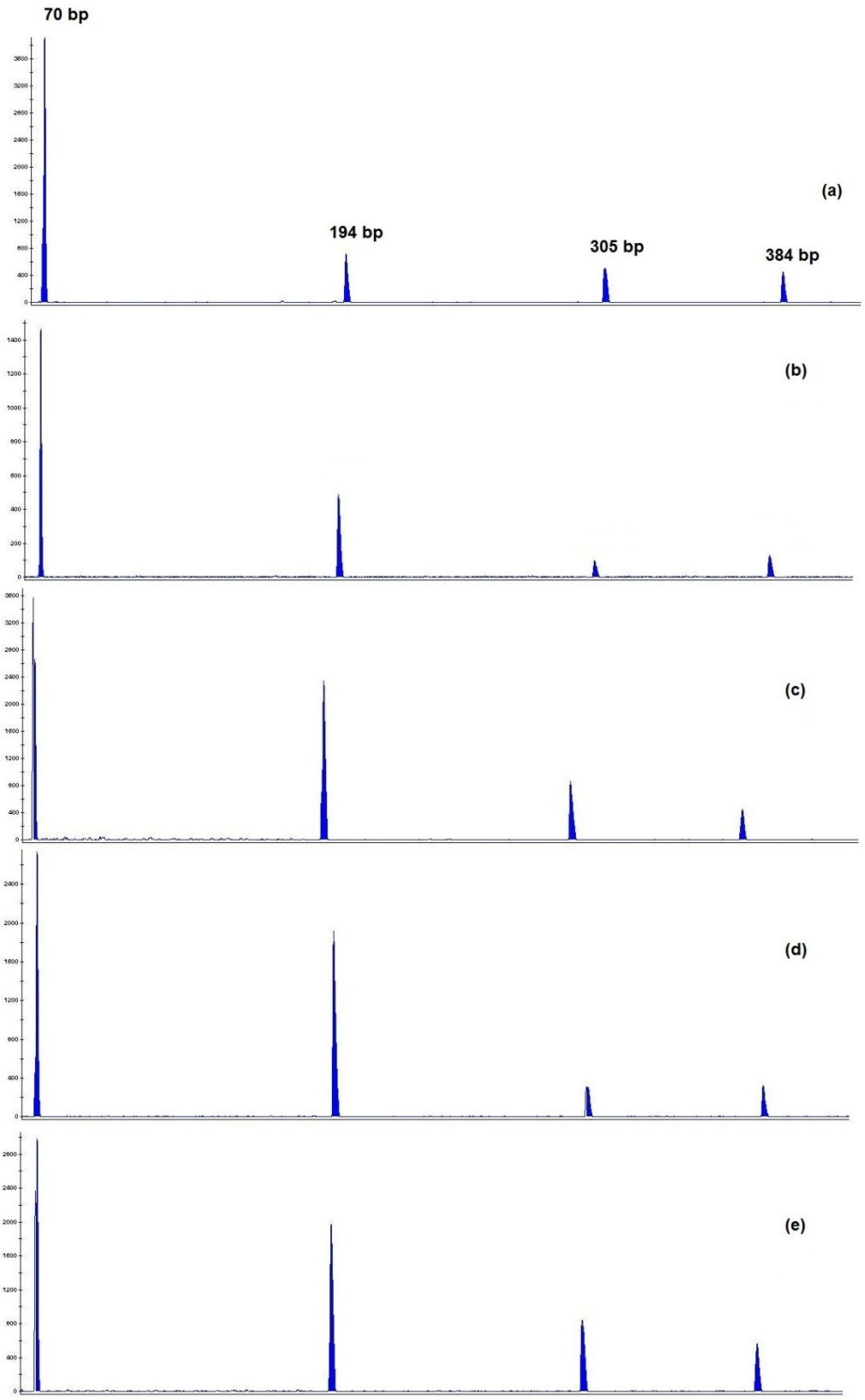

Figure 5.12 Shown above are examples of electropherograms of rabbit DNA samples obtained from soft muscle tissues preserved in cell lysis solution (with and without 1% sodium azide)**;** b) cell lysis solution (tissues), c) cell lysis solution (aliquots), d) cell lysis solution with 1% sodium azide (tissues), e) cell lysis solution with 1% sodium azide (aliquots) preserved at room temperature for one year. Day zero positive control (a) also shown.

5.3.4.3 Pig Tissues

DNA quantification results showed that cell lysis solutions can presrve DNA in fresh and partially decomposed soft mucsle tissue samples of pig for one year at room temperature, except partially decomposed 0.25 g preserved tissues. DNA quantification of the aliquots of cell lysis solutions were also performed to assess DNA preservation. DNA was present in all samples extracted from tissues after 6 and 12 months of storage at room temperature (Figure 5.13 and 5.14). and from aliquots (Figure 5. 15 and 5.16).

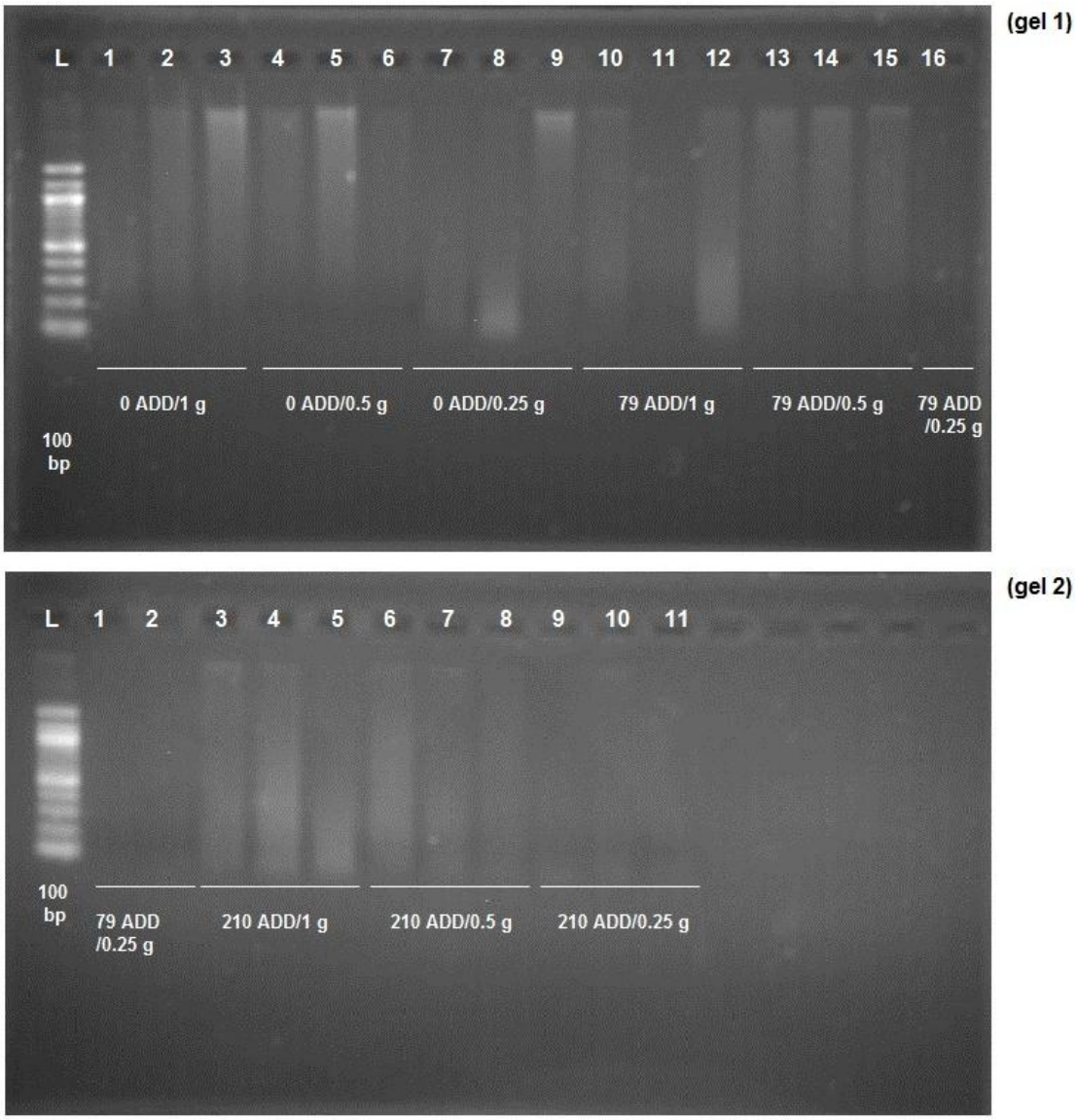

Figure 5.13 Shown above are agarose gels of pig DNA obtained from muscle tissue samples preserved in cell lysis solution (gel 1 and 2) at room temperature for one year. The 100 bp DNA ladder (L), weight of tissues (g) and ADD are also shown.

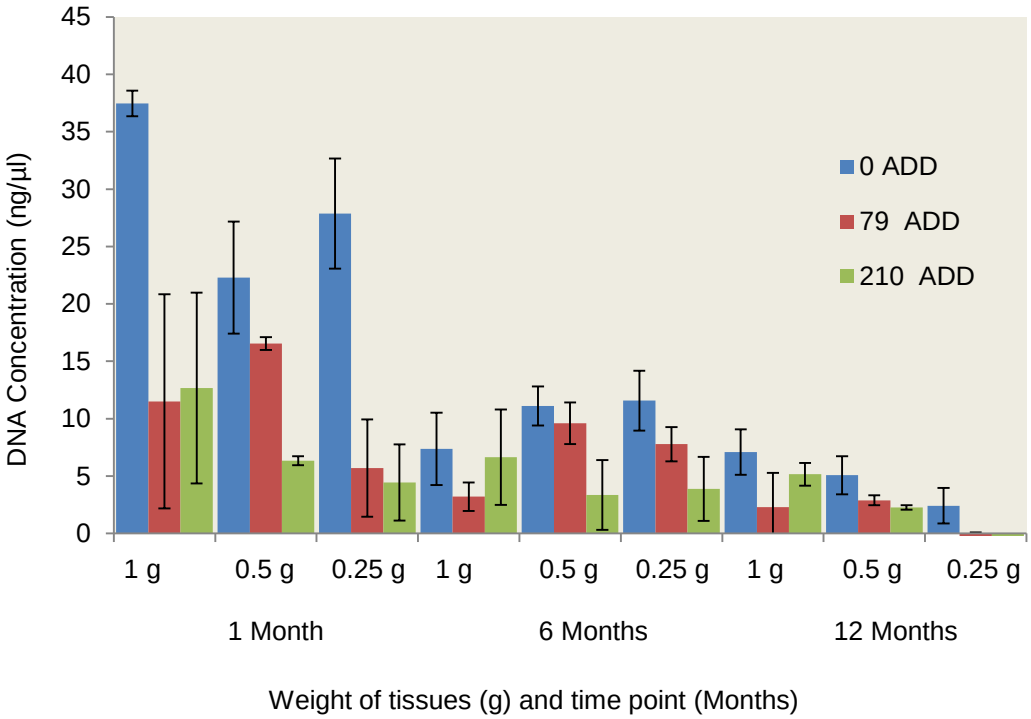

Figure 5.14 Shown above is an example of PicoGreen® DNA quantification of pig soft muscle tissue samples preserved in in cell lysis solution at room temperature up to one year. The error bars indicate the standard error from three replicates. A total of 200 µl was extracted from each sample and 3 µl was used for quantification.

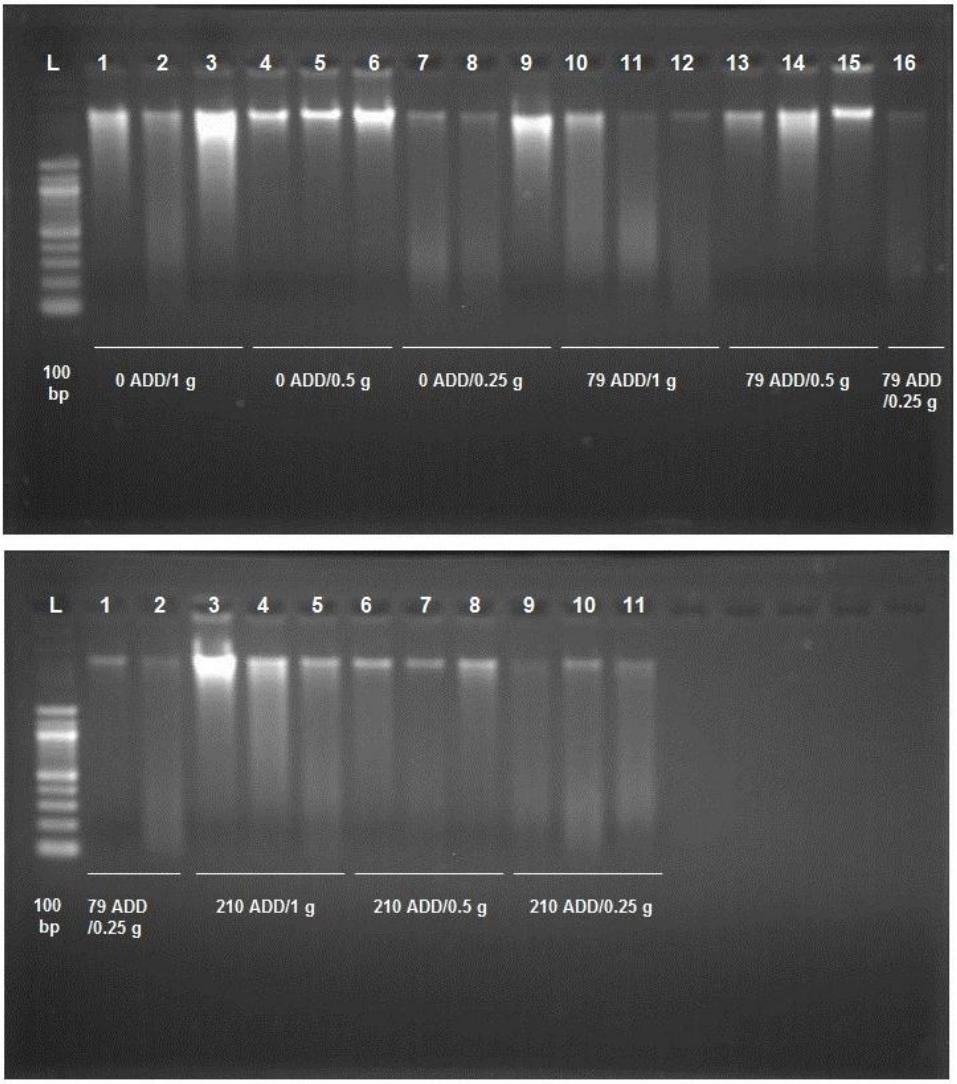

Figure 5.15 Shown above is agarose gel electrophoresis of pig DNA obtained from aliquots of cell lysis solution (gel 1 and 2) that was used to preserve soft muscle tissue samples at room temperature for one year. The 100 bp DNA ladder (L), weight of tissues (g) and ADD also shown.

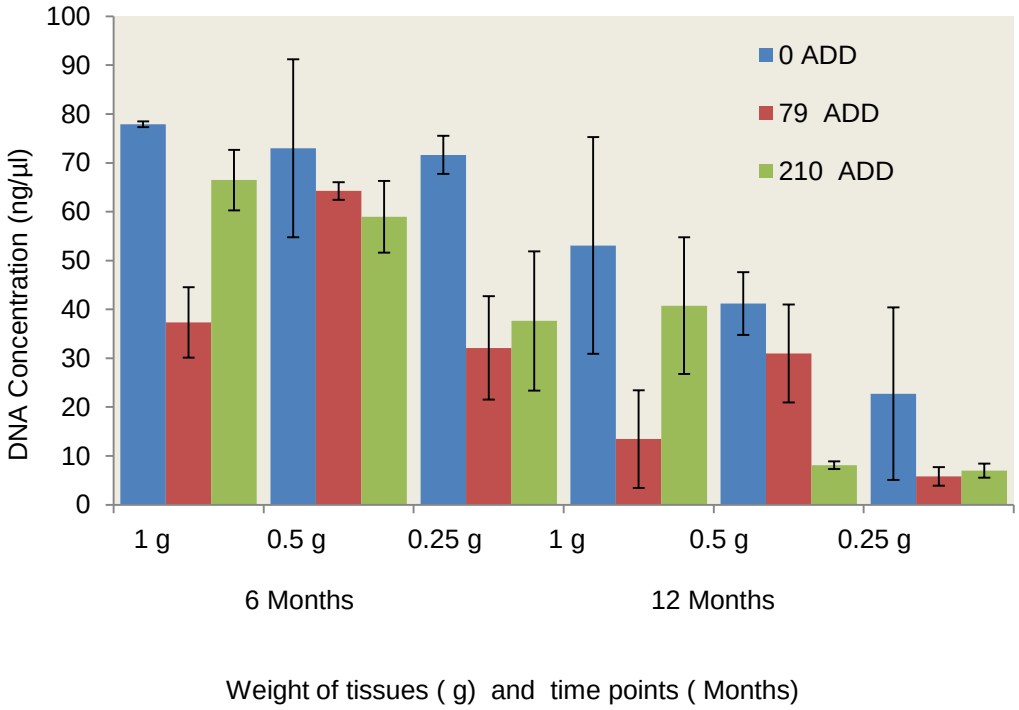

Figure 5.16 Shown above is an example of PicoGreen® DNA quantification of aliquots of pig cell lysis solution up to one year. The error bars indicate the standard error from three replicates. A total of 200 µl was extracted from each sample and 3 µl was used for quantification.

Preservation of pig tissues in cell lysis solution using 1% sodium azide yielded good quality of DNA. The DNA was preserved in partially decomposed samples and in control samples after one year of preservation at room temperature. (Figure 5.17, 5.18 and 5.20). Pig soft tissue samples were also digested in the solution after 6 months of preservation, therefore, aliquote of cell lysis solutions were also extracted to determine the DNA yeilds. It was found that high quality DNA can be obtained from the aliquots after one year of storage at room temperature and yields of DNA were higher compared to tissues (Figure 5.19, 5.20 and 5.21).

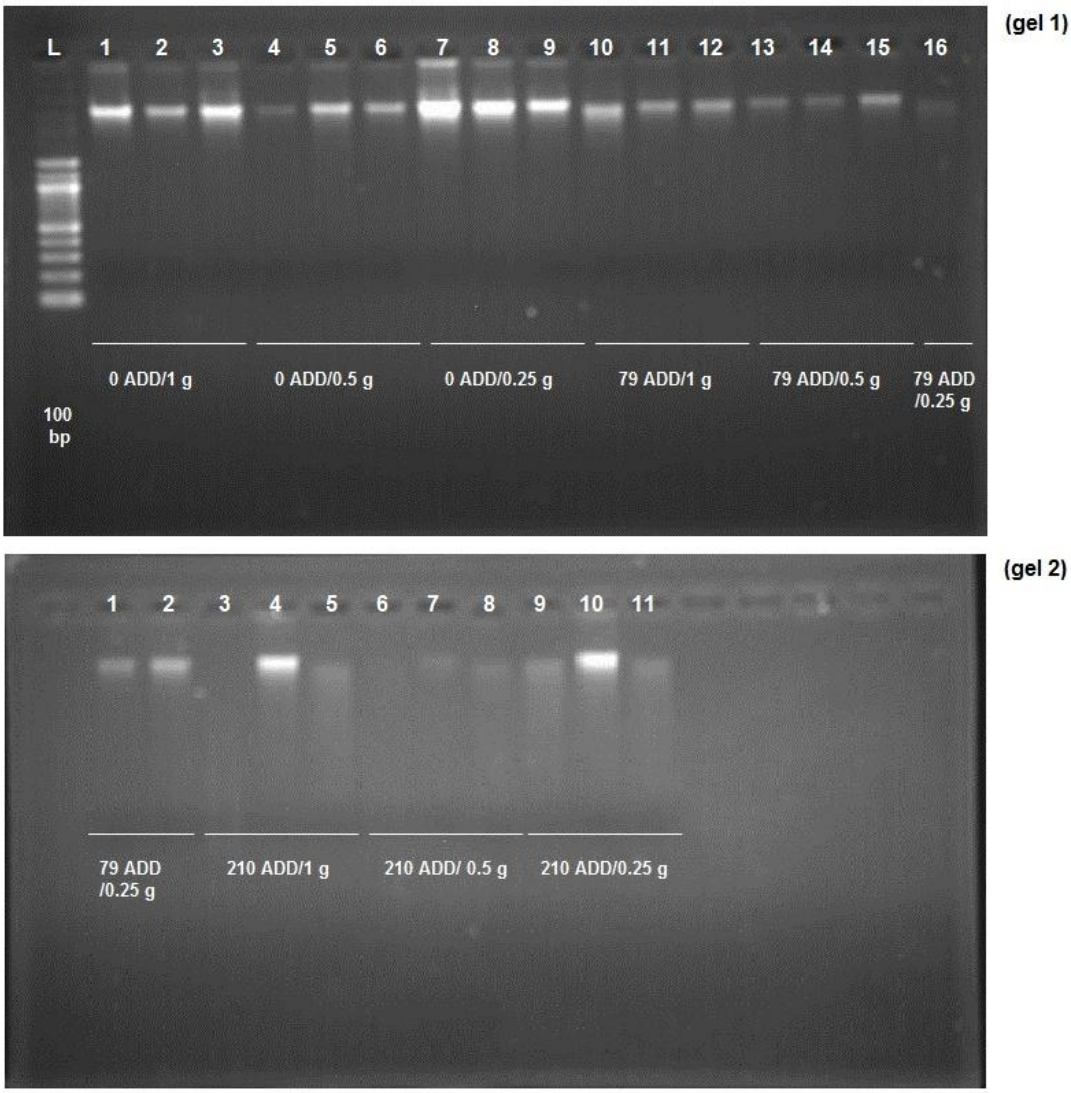

Figure 5.17 Shown above are agarose gels of pig DNA obtained from muscle tissue samples preserved in cell lysis solution with 1% sodium azide (gel 1 and 2) at room temperature for one year. The 100 bp DNA ladder (L), weight of tissues (g) and ADD are also shown.

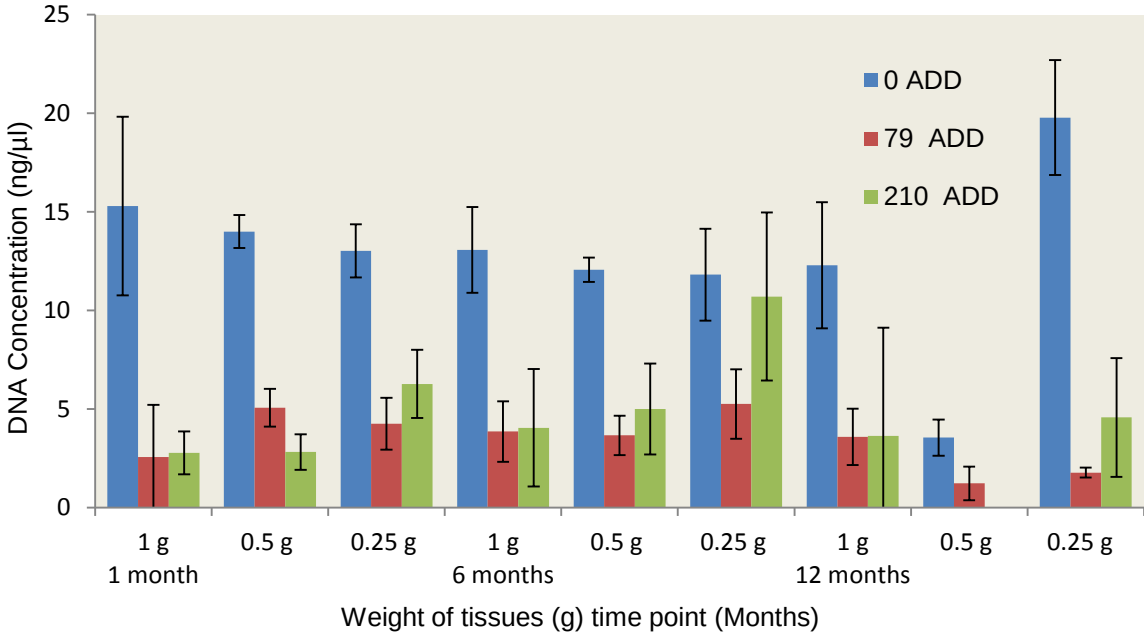

Figure 5.18 Shown above is PicoGreen® DNA quantification of pig soft muscle tissue samples preserved in in cell lysis solution with 1% sodium azide at room temperature up to one year. The error bars indicate the standard error from three replicates. A total of 200 µl was extracted from each sample and 3 µl was used for quantification.

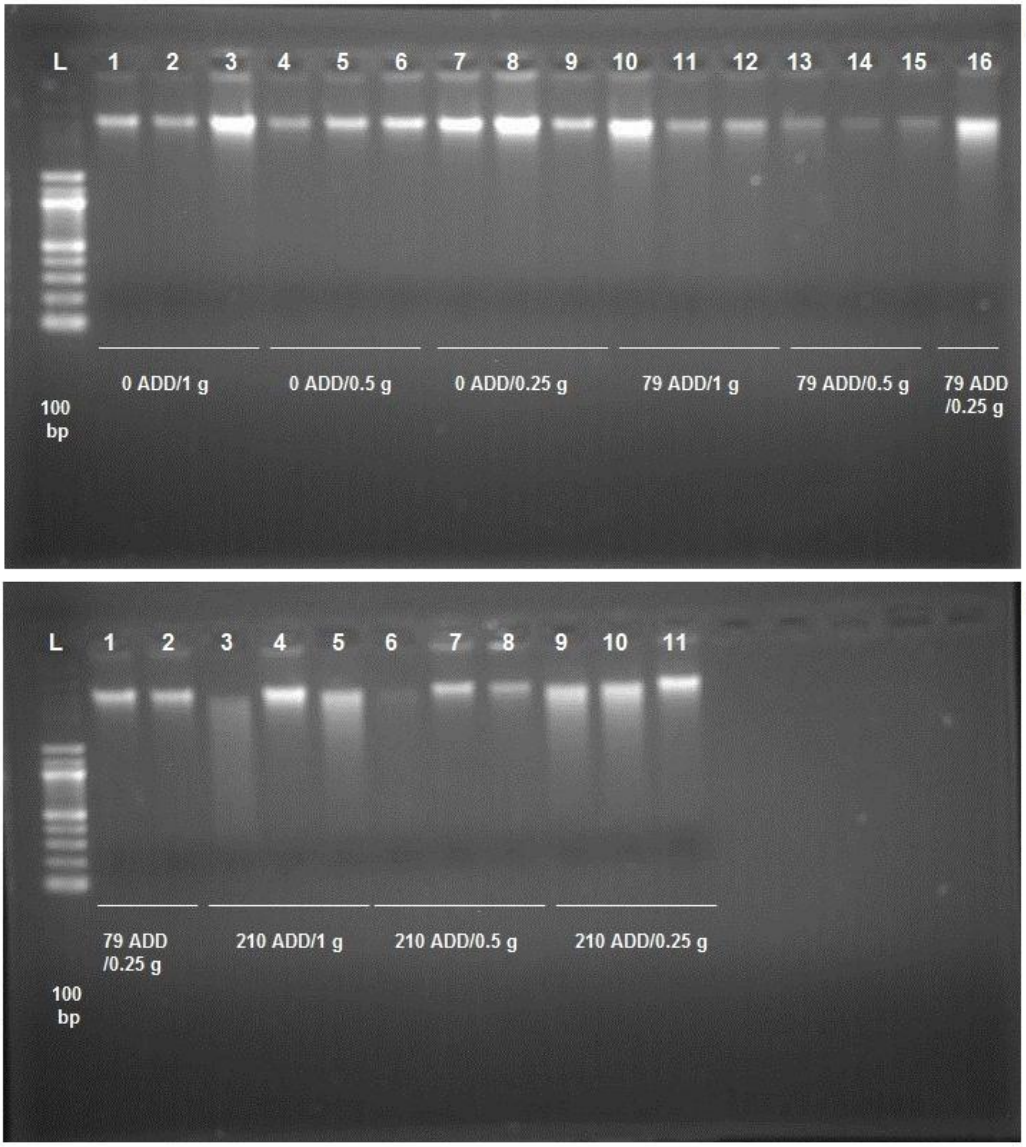

Figure 5.19 Shown above are agarose gels of pig DNA obtained from aliquots of cell lysis solution with 1% sodium azide (gel 1 and 2) that was used to preserve soft muscle tissue samples at room temperature for one year. The 100 bp DNA ladder (L) weight of tissues (g) and ADD also shown.

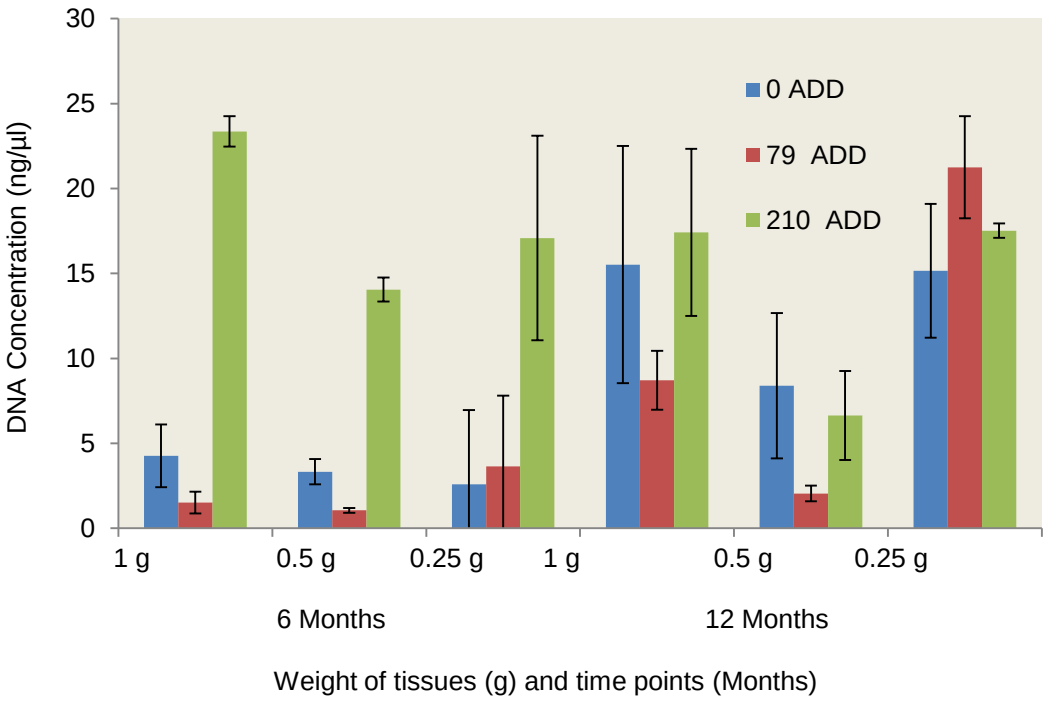

Figure 5.20 Shown above is PicoGreen® DNA quantification of aliquots of pig cell lysis solution with 1% sodium azide up to one year. The error bars indicate the standard error from three replicates. A total of 200 µl was extracted from each sample and 3 µl was used for quantification.

5.3.4.4 4-plex Multiplex Amplification

Pig samples preserved in cell lysis solution (with and without 1% sodium azide) produced full 4-plex amplification in all samples (Table 5.5 and 5.6, Figures 5.21b and 5.21d). Similarly, the aliquots of cell lysis solution also produced full 4-plex amplification (Tables 5.5 and 5.6, Figures 5.21c and 5.21e). The peak heights were higher in pig aliquots samples compared to the tissues. The results suggested that cell lysis solution (with and without 1% sodium azide) can be used to preserve soft tissues at room temperature for one year and full 4-plex amplification can be obtained successfully.

Table 5.5 Shown below are the results of 4-plex multiplex amplification of pig muscle tissue samples preserved in cell lysis solution at room temperature for one year.

Accumulated Degree-days (ADD)	Weight of tissues	pig cell lysis solution (Tissues)				pig cell lysis solution (aliquots)			
		70 bp	194 bp	305 bp	384 bp	70 bp	194 bp	305 bp	384 bp
0 ADD	1 g	+++	+++	+++	+++	+++	+++	+++	+++
	0.5 g	+++	+++	+++	+++	+++	+++	+++	+++
	0.25 g	+++	+++	+++	+++	+++	+++	+++	+++
79 ADD	1 g	+++	+++	+++	+++	+++	+++	+++	+++
	0.5 g	+++	+++	+++	+++	+++	+++	+++	+++
	0.25 g	+++	+++	+++	+++	+++	+++	+++	+++
210 ADD	1 g	+++	+++	+++	+++	+++	+++	+++	+++
	0.5 g	+++	+++	+++	+++	+++	+++	+++	+++
	0.25 g	+++	+++	+++	+++	+++	+++	+++	+++

‘‘+’’ denotes the presence of PCR amplicons in triplicate samples during 4-plex multiplex amplification.

Table 5.6 Shown below are the results of 4-plex multiplex amplification of pig muscle tissue samples preserved in cell lysis solution with 1% sodium azide at room temperature for one year.

Accumulated Degree-days (ADD)	Weight of tissues	Pig cell lysis solution with 1% sodium azide (Tissues)				Pig cell lysis solution with 1% sodium azide (aliquots)			
		70 bp	194 bp	305 bp	384 bp	70 bp	194 bp	305 bp	384 bp
0 ADD	1 g	+++	+++	+++	+++	+++	+++	+++	+++
	0.5 g	+++	+++	+++	+++	+++	+++	+++	+++
	0.25 g	+++	+++	+++	+++	+++	+++	+++	+++
79 ADD	1 g	+++	+++	+++	+++	+++	+++	+++	+++
	0.5 g	+++	+++	+++	+++	+++	+++	+++	+++
	0.25 g	+++	+++	+++	+++	+++	+++	+++	+++
210 ADD	1 g	+++	+++	+++	+++	+++	+++	+++	+++
	0.5 g	+++	+++	+++	+++	+++	+++	+++	+++
	0.25 g	+++	+++	+++	+++	+++	+++	+++	+++

‘‘+’’ denotes the presence of PCR amplicons in triplicate samples during 4-plex multiplex amplification.

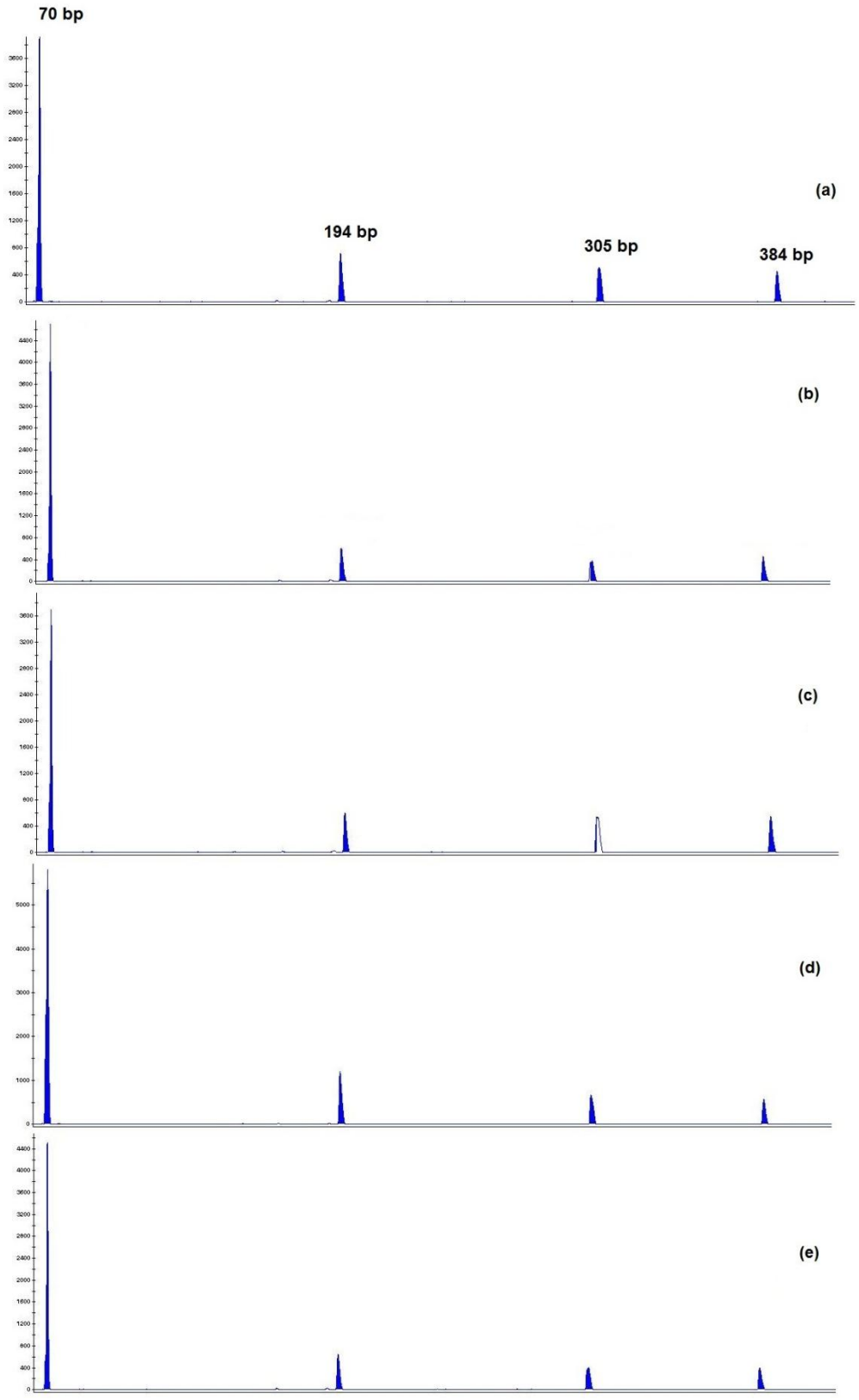

Figure 5.21 Shown above are examples of electropherograms of pig DNA samples obtained from soft muscle tissues preserved in cell lysis solution (with and without 1% sodium azide); b) cell lysis solution (tissues), c) cell lysis solution (aliquots), d) cell lysis solution with 1% sodium azide (tissues), e) cell lysis solution with 1% sodium azide (aliquots) preserved at room temperature for one year. Day zero positive control (a) also shown.

5.3.5 Formalin

5.3.5.1 Rabbit Tissues

Quantification of rabbit soft muscle tissues preserved in 10% buffered formalin, detected DNA in all control (0 ADD) and in some partially decomposed (79 ADD) samples after one month of storage at room temperature, whereas severe DNA degradation was found in all formalin preserved samples after 6 months and one year of storage (Figure 5.22, 5.23, 5.24 and 5.25). Rabbit formalin preserved tissues suggested that 10% buffered formalin is not suitable for long term storage of soft muscle tissue of rabbit at room temperature.

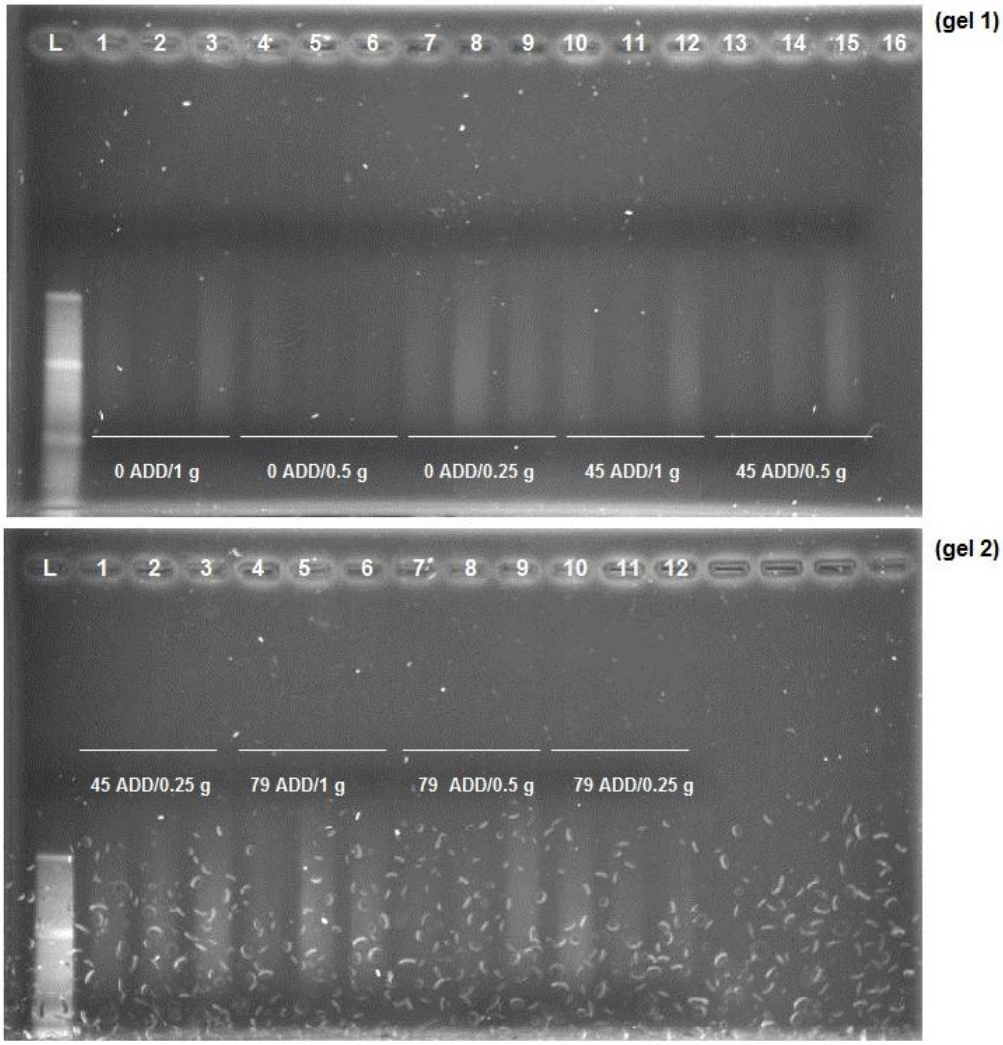

Figure 5.22 Shown above are agarose gels of rabbit DNA obtained from muscle tissue samples preserved in 10% buffered formalin (gel 1 and 2) at room temperature for 6 months. The hindIII DNA ladder (L), weight of tissues (g) and ADD are also shown. The small white spots appeared due to faulty gel tank.

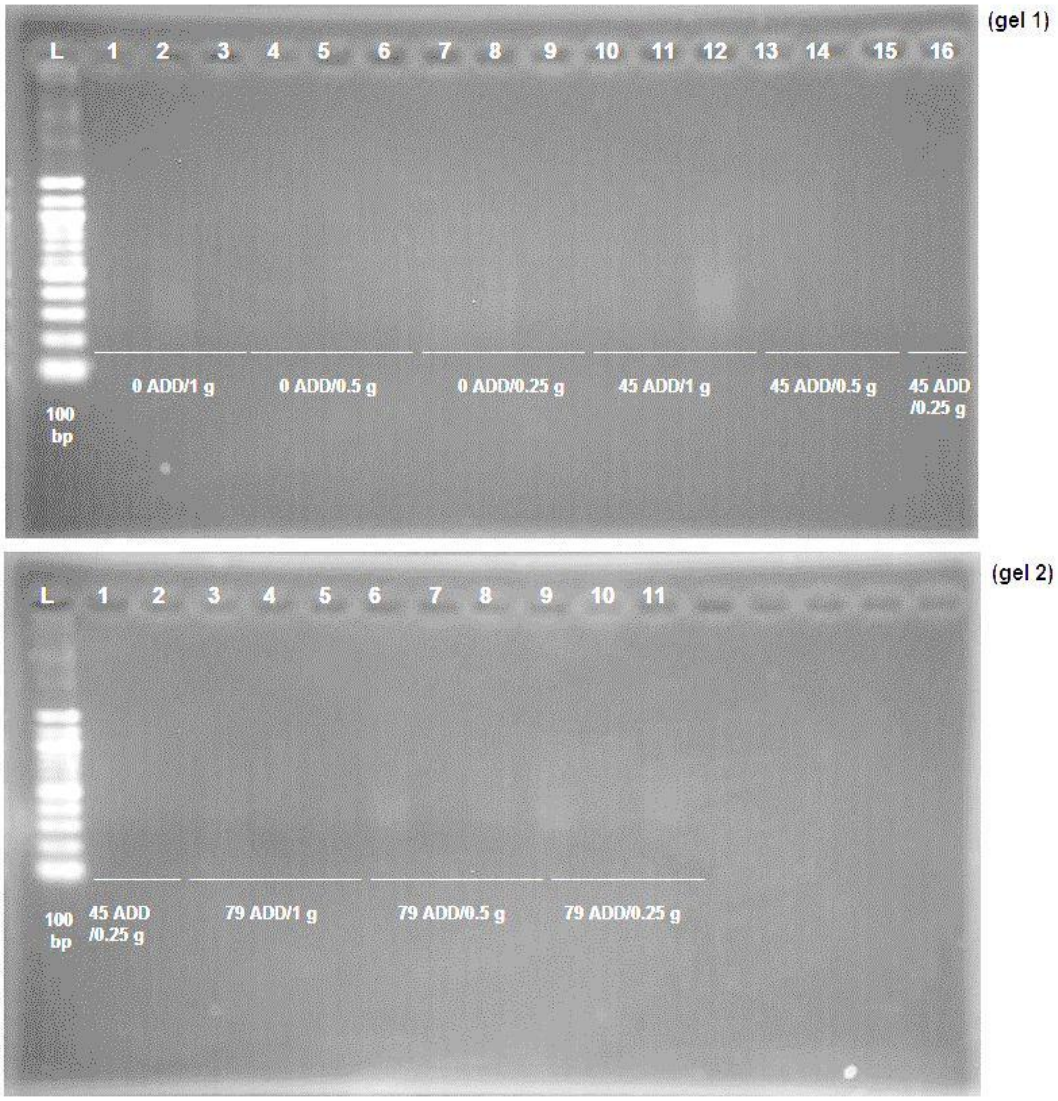

Figure 5.23 Shown above are agarose gels of rabbit DNA obtained from muscle tissue samples preserved in 10% buffered formalin (gel 1 and 2) at room temperature for one year. The 100 bp DNA ladder (L), weight of tissues (g) and ADD are also shown. This figure was inverted to improve resolution.

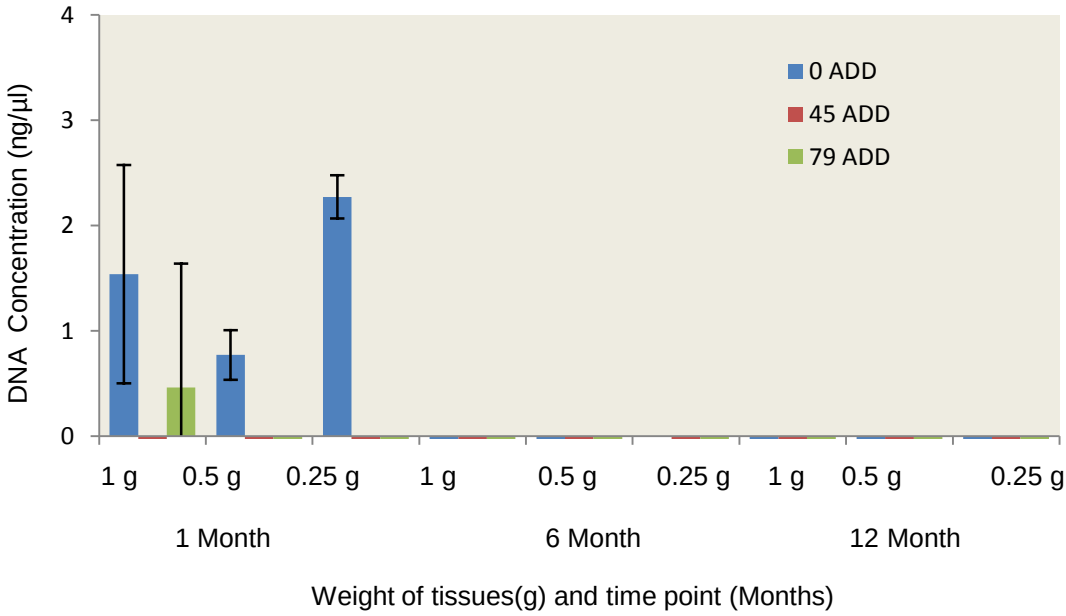

Figure 5.24 Shown above is an example of PicoGreen® DNA quantification of rabbit soft muscle tissue samples preserved in 10% buffered formalin at room temperature up to one year. The error bars indicate the standard error from three replicates. A total of 200 µl was extracted from each sample and 3 µl was used for quantification.

5.3.5.2 4-plex Multiplex Amplification

Rabbit soft muscle tissues samples showed complete failure of 194 bp, 305 bp and 384 bp amplicons after 6 months and 12 months of storage at room temperature (Table 5.7). The amplification of shorter amplicon (70 bp) was observed in all samples preserved for 6 months and in few samples stored for 12 months at room temperature (Figures 5.25b and 5.25c).

Table 5.7 Shown below are the results of 4-plex multiplex PCR amplification of rabbit soft muscle tissue samples preserved in 10% buffered formalin for 6 months and 12 months.

Accumulated Degree-days (ADD)	Weight of tissues	Rabbit 6 months formalin preserved tissues				Rabbit 12 months formalin preserved tissues			
		70 bp	194 bp	305 bp	384 bp	70 bp	194 bp	305 bp	384 bp
0 ADD	1 g	**+++**	---	---	---	**+**--	---	---	---
	0.5 g	**+++**	---	---	---	**+**--	---	---	---
	0.25 g	**+++**	---	---	---	**-**	---	---	---
45 ADD	1 g	**+++**	---	---	---	**+**--	---	---	---
	0.5 g	**+++**	---	---	---	---	---	---	---
	0.25 g	**+++**	---	---	---	---	---	---	---
79 ADD	1 g	**+++**	---	---	---	---	---	---	---
	0.5 g	**+++**	---	---	---	**+**--	---	---	---
	0.25 g	**+++**	---	---	---	**+--**	---	---	---

‘‘+’’ denotes the presence and ‘‘-’’ represents the absence of PCR amplicons in samples during 4-plex multiplex amplification.

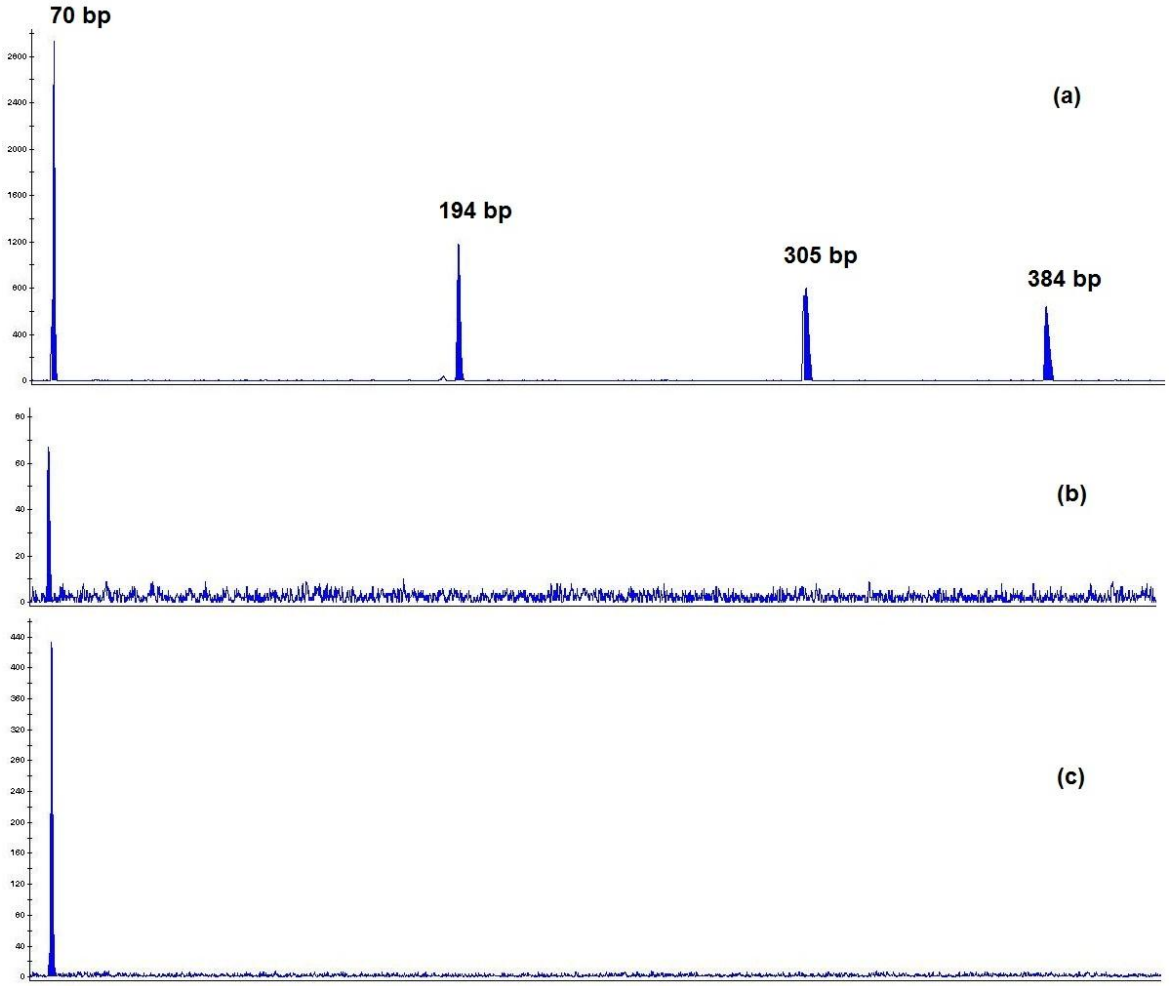

Figure 5.25 Shown above are example of electropherograms of rabbit DNA samples obtained from soft muscle tissues preserved in 10% buffered formalin for 6 months and one year (b and c) at room temperature. Day zero positive control (a) also shown.

5.3.5.3 Pig Tissues

Room temperature storage of pig soft muscle tissues in 10% buffered formalin showed DNA in all samples (0 ADD, 79 ADD and 210 ADD) after one month of preservation, while extensive DNA degradation was seen in all samples preserved for 6 months and one year, However, DNA was present in 0 ADD samples which were collected from 1g tissues preserved for 6 months and 0.25 g tissues preserved for one year (Figure 5.26, 5.27 and 5.28). Pig formalin preserved samples also took long time for digestion during DNA extraction process.

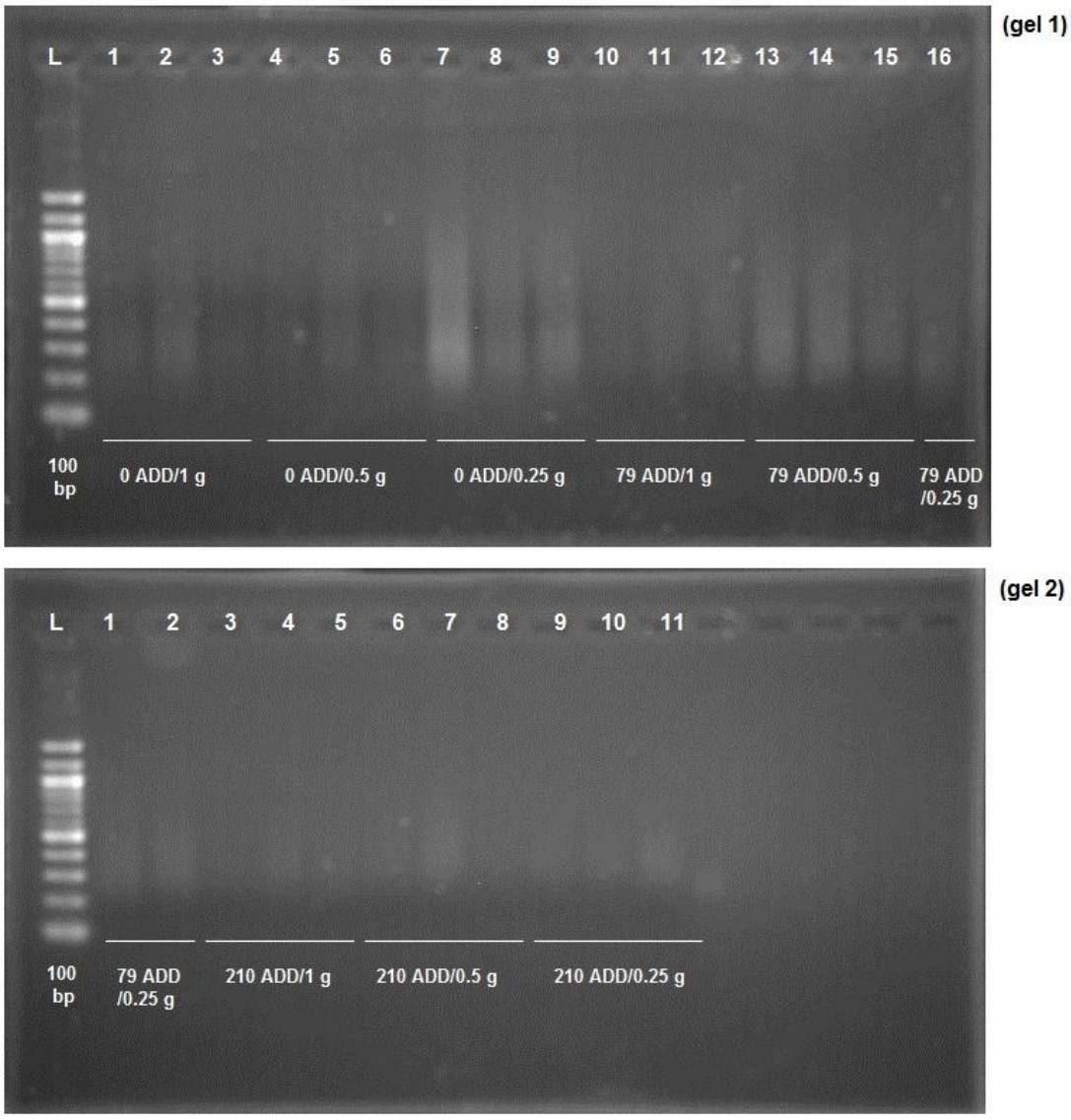

Figure 5.26 Shown above are agarose gels of pig DNA obtained from muscle tissue samples preserved in 10% buffered formalin (gel 1 and 2) at room temperature for one year. The 100 bp DNA ladder (L), weight of tissues (g) and ADD are also shown.

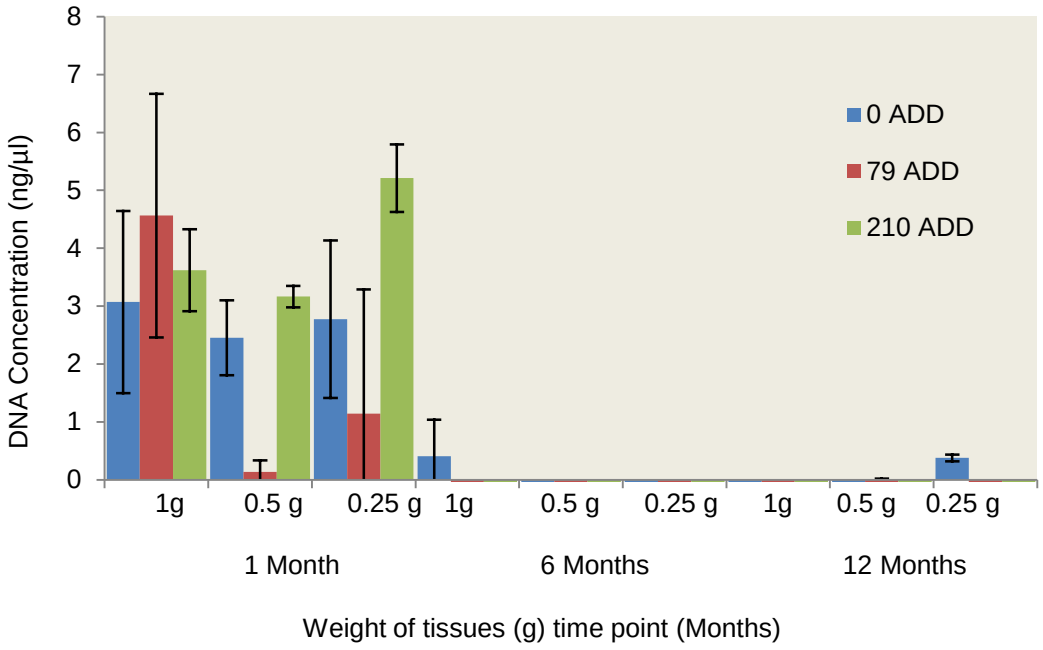

Figure 5.27 Shown above is an example of PicoGreen® DNA quantification of pig soft muscle tissue samples preserved in 10% buffered formalin at room temperature up to one year. The error bars indicate the standard error from three replicates. A total of 200 µl was extracted from each sample and 3 µl was used for quantification.

5.3.5.4 4-plex Multiplex Amplification

In pig samples preserved in 10% buffered formalin, there was complete failure of amplification for 194 bp, 305 bp and 384 bp amplicons after one year of storage at room temperature, however, the amplification of 70 bp was possible in all samples extracted from fresh and decomposing tissues (Table 5.8 and Figure 5.28b). It was observed that formalin is not suitable for long term (1 year) storage of soft muscle tissues especially when amplification of larger amplicons is required.

Table 5.8 Shown below are the results of 4-plex multiplex amplification of pig soft muscle tissue samples preserved in 10% buffered formalin at room temperature for one year.

Accumulated Degree-days (ADD)	Weight of tissues	Pig tissues preserved in formalin			
		70 bp	194 bp	305 bp	384 bp
0 ADD	1 g	**+++**	---	---	---
	0.5 g	**+++**	---	---	---
	0.25 g	**+++**	---	---	---
79 ADD	1 g	**+++**	---	---	---
	0.5 g	**+++**	---	---	---
	0.25 g	**+++**	---	---	---
210 ADD	1 g	**+++**	---	---	---
	0.5 g	**+++**	---	---	---
	0.25 g	**+++**	---	---	---

‘‘+’’ denotes the presence and ‘‘-’’ represents the absence of PCR amplicons in samples during 4-plex multiplex amplification.

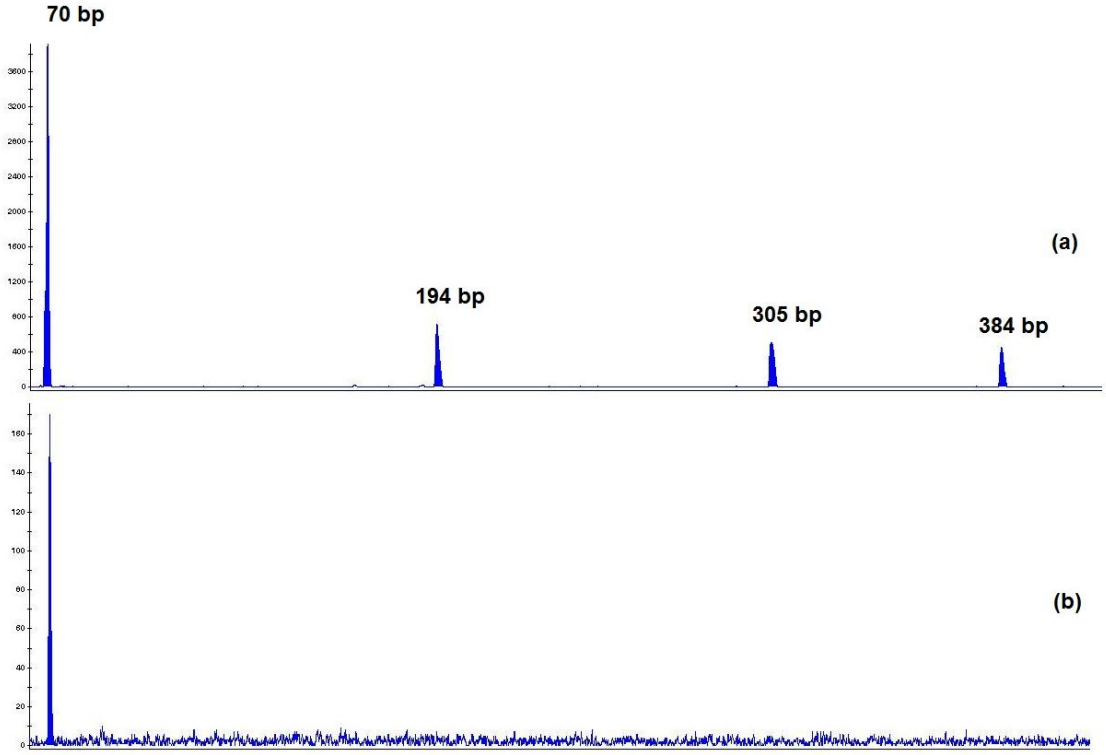

Figure 5.28 Shown above is an example of electropherograms of pig DNA samples obtained from soft muscle tissues preserved in 10% buffered formalin (b) for one year at room temperature. Day zero positive control (a) also shown.

5.3.6 Summary of Multiplex Amplification Results

All rabbit and pig soft muscle tissue samples preserved in 96% ethanol and cell lysis solution (with and without 1% sodium azide) produced full multiplex amplification after one year of storage at room temperature.

Drop-out of 194 bp, 305 bp and 384 bp was observed in all samples of rabbit and pig preserved in formalin at room temperature for one year. In pig, the amplification of 70 bp was obtained in all formalin preserved samples after one year of storage while in rabbit the amplification of 70 bp was possible only in few samples after one year of storage and in all samples of 6 month preservation at room temperature.

5.3.7 Dehydration (Oven Drying) of Muscle Tissues

Soft tissue samples (approximately 1 cm^3) were collected in triplicate at day zero from pig and rabbit whole carcases (Section 2.2.5), cut into small sized pieces (approximatly1 g) and incubated at 50 °C for 3:15 hours and then stored at 4 °C for one year. DNA extraction was carried out after one month, nine months and one year using DNeasy® Blood and Tissue kit according to the manufacturer's instructions and agarose gel electrophoresis was performed to determine the presence of DNA (Section 2.2.1 and 2.2.2). Samples were extracted in triplicate DNA quantification was performed using Quant-iT™ PicoGreen® dsDNA reagents (Invitrogen™, UK) according to the manufacturer's instructions on the TECAN GEnios Pro Microplate Reader (Section 2.7).

5.3.7.1 Rabbit Tissues

DNA was present until one year in all triplicate samples of rabbit tissues (Figure 5.29 and 5.30).

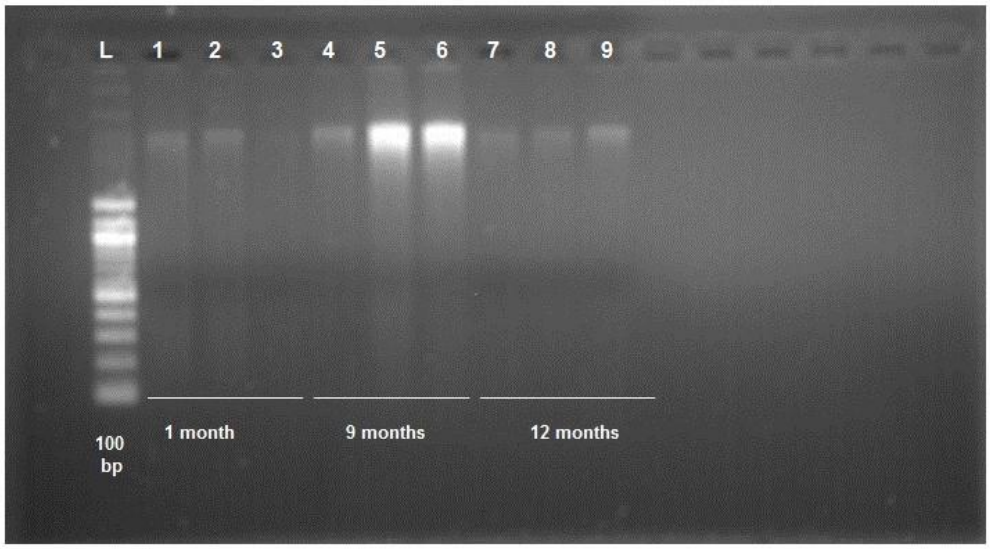

Figure 5.29 Shown above is agarose gel of rabbit DNA obtained from dehydrated muscle tissues stored at 4 °C and extracted after one month, nine months and one year. The 100 bp DNA ladder (L) also shown.

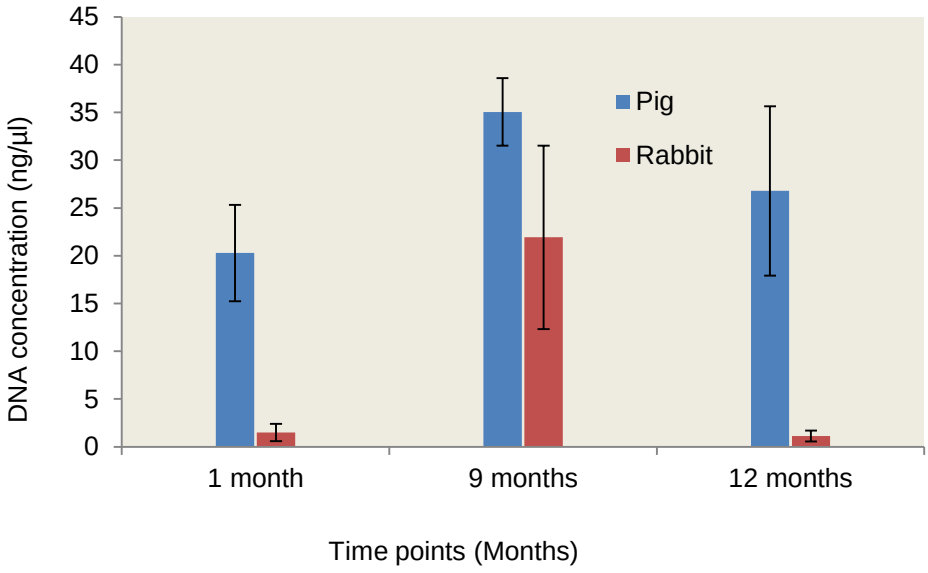

Figure 5.30 Shown above is an example of PicoGreen® DNA quantification of rabbit and pig dehydrated tissues after one month, six months and one year of storage at 4 °C. The error bars indicate the standard error from three replicates. A total of 200 µl was extracted from each sample and 3 µl was used for quantification.

5.3.7.2 4-plex Multiplex Amplification

Rabbit dehydrated samples showed full multiplex amplification after one year of storage at 4 °C (Table 5.9 and Figure 5.31).

Table 5.9 Shown below are the results of multiplex (4-plex) PCR amplification of rabbit dehydrated soft muscle tissue samples preserved at 4 °C for one year.

Time point (Months)	Rabbit heat dried tissues			
	70 bp	194 bp	305 bp	384 bp
1	+++	+++	+++	+++
9	+++	+++	+++	+++
12	+++	+++	+++	+++

‘‘+++’’ denotes the presence of PCR amplicons in triplicate samples during multiplex (4-plex) amplification.

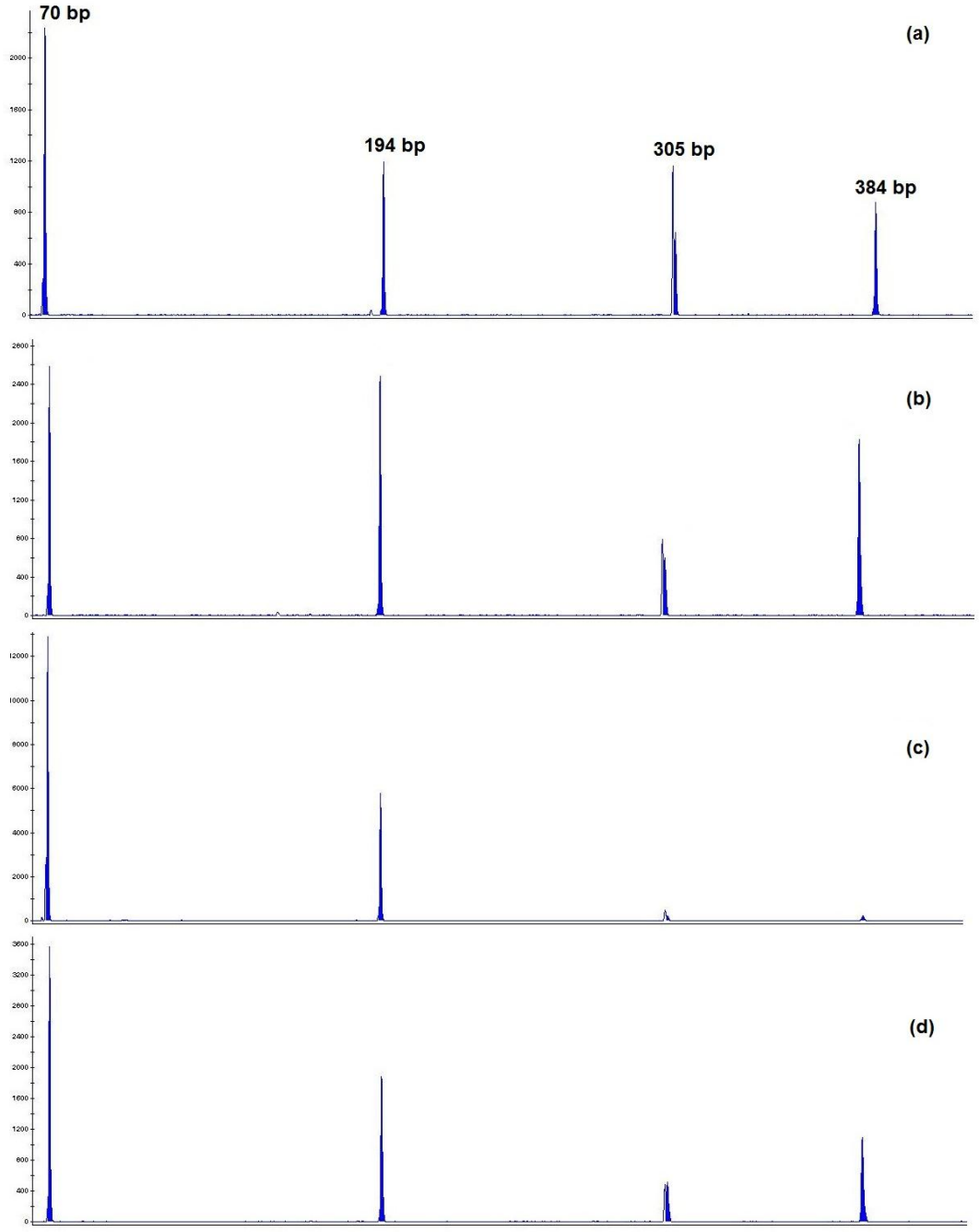

Figure 5.31 Shown above are examples of electropherogramms (b, c and d) of rabbit dehydrated samples (triplicate) after one year of storage at 4 °C. Human positive control (a) also shown.

5.3.7.3 Pig Tissues

In pig dehydrated samples DNA was also present until one year (Table 5.10 and Figure 5.32).

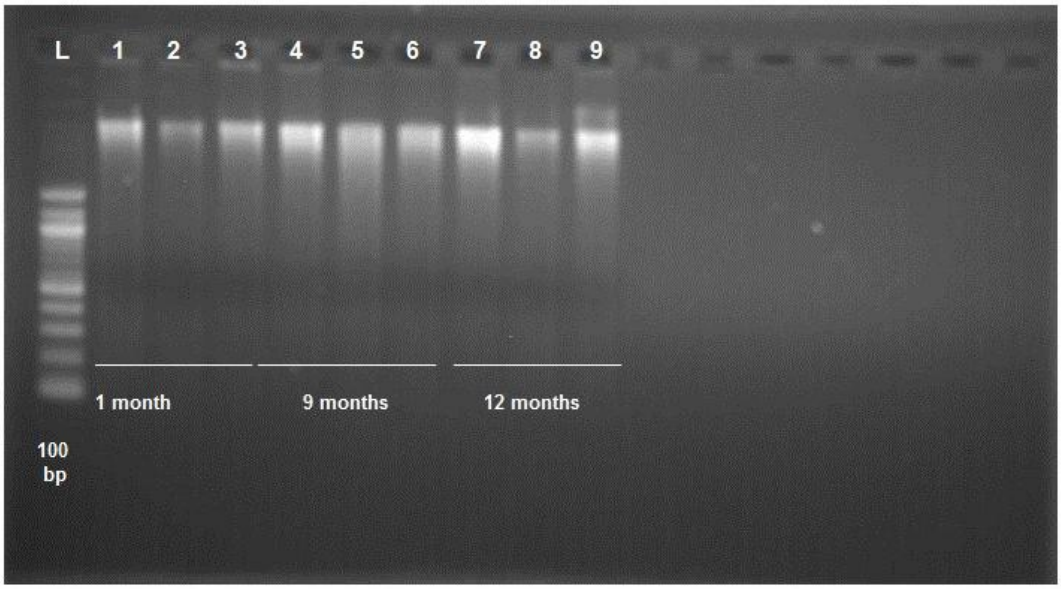

Figure 5.32 Shown above is agarose gel of pig DNA obtained from dehydrated muscle tissues stored at 4 °C and extracted after one month, nine months and one year. The 100 bp DNA ladder (L) also shown.

5.3.7.4 4-plex Multiplex Amplification of Pig Dehydrated Samples

Pig dehydrated soft muscle tissue samples produced full multiplex amplification after one year of storage at 4 °C (Table 5.10 and Figure 5.30).

Table 5.10 Shown below are the results of multiplex (4-plex) PCR amplification of pig dehydrated soft muscle tissue samples preserved at 4 °C for one year.

Time point (Months)	pig heat dried tissues			
	70 bp	194 bp	305 bp	384 bp
1	+++	+++	+++	+++
9	+++	+++	+++	+++
12	+++	+++	+++	+++

‘‘+’’ denotes the presence of PCR amplicons in triplicate samples during 4-plex multiplex amplification.

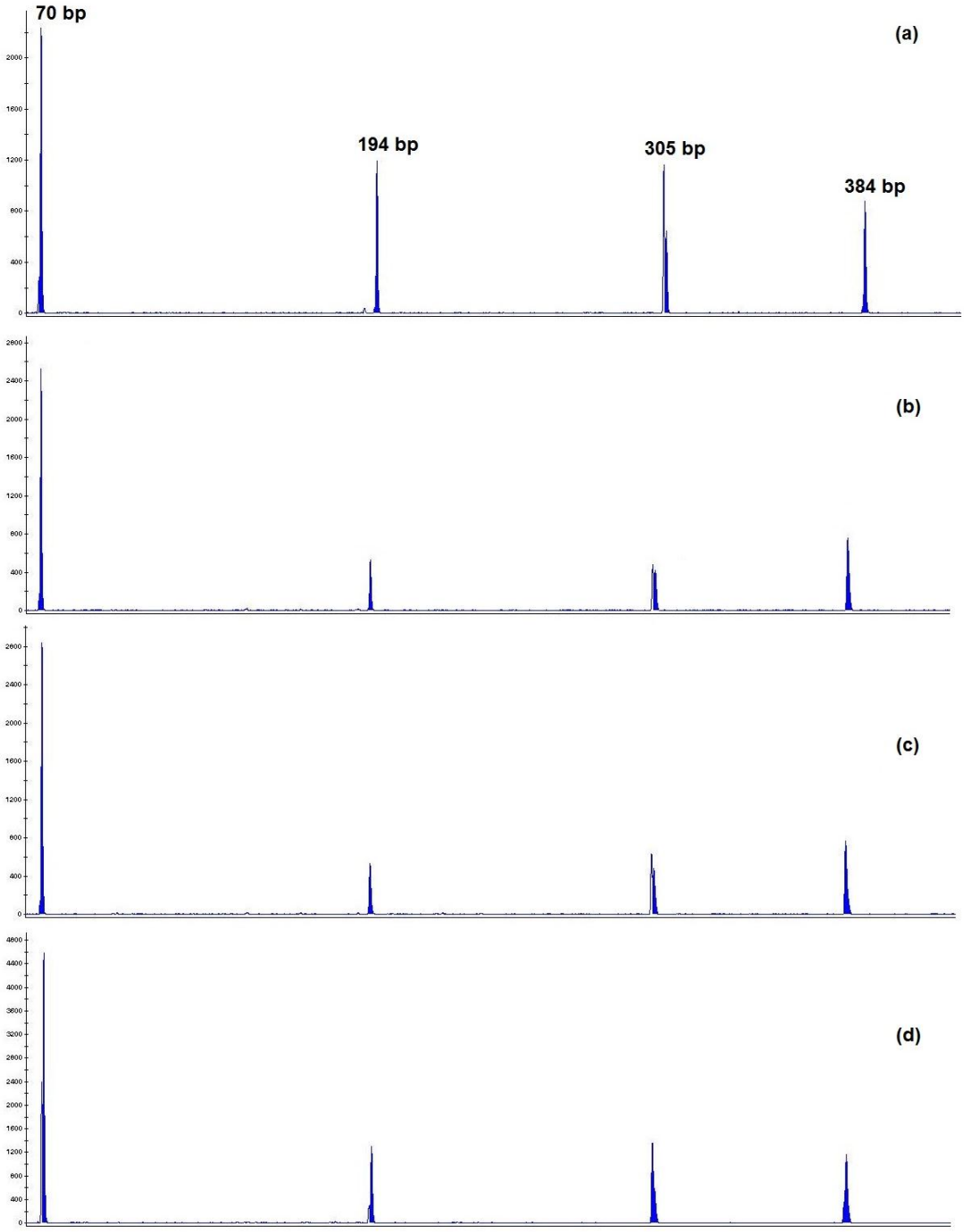

Figure 5.33 Shown above are examples of electropherogramms (b, c and d) of pig dehydrated samples (triplicate) after one year of storage at 4 °C. Human positive control (a) also shown.

5.4 Discussion

The goal of the experiments presented in this chapter was to determine the efficiency of different solutions to preserve partially decomposed soft muscle tissues that would increase the possibility of being able to generate a DNA profile at later time. Special considerations were given to the methods that are less expensive, need little preparation and do not require specialised equipment.

During autopsy of forensic cases, the majority of whole and non-disrupted cadavers will be examined. In such cases, depending on the laboratory practices, buccal swabs and liquid blood samples can be collected on filter paper for DNA analysis. In highly fragmented remains, buccal swabs or blood samples are not available, therefore; alternative biological samples must be collected (Prinz et al., 2007). In fragmented bodies, soft muscle tissues will be available in many cases. When bone or teeth are available these may be preferable for sampling as DNA preservation in bone and teeth is superior to soft tissues especially when putrification has occurred (Burger et al., 1999; Holland et al., 2003). However, the processing of hard tissues is time-consuming and labour-intensive, required: cleaning, de-fleshing, drying, cutting, grinding and de-calcification before DNA extraction. Comparatively, soft muscle tissues will need simple cutting and maceration before DNA extraction (Alonso et al., 2001).

Formalin is the most widely used fixative for specimen preservation, however, for molecular analysis the duration of fixation is the major factor affecting DNA preservation in formalin fixed tissues (Wandeler, et al., 2007; Lehmann and Kreipe, 2001). Long term preservation of tissues in formalin at room temperature causes severe DNA degradation. This is mainly because of progressive acidification of formalin into formic acid. Buffered formalin stabilizes the solution for a longer period of time than non-buffered formalin but cause DNA fragmentation (Koppelstaetter et al., 2005; Kunkle et al., 2006; Miething et al., 2006).

In the present study, DNA samples obtained from the pig and rabbit tissues preserved in 10% buffered formalin for one year were degraded and the larger amplicons could not be amplified (194 bp, 305 bp and 384 bp) after one year of storage at room temperature, however, amplification of the shorter fragment (70 bp) was possible in all pig samples. In case of rabbit, the 70 bp amplicon was amplified in few samples stored for one year and in all samples preserved for 6 months at room temperature. O'Leary et al., (1994)

and Francer and Kocher (1996) also found a very low success rate when amplifying larger PCR products (more than 300 bp) in formalin-fixed samples. In present study PicoGreen® quantification of formalin preserved samples suggested that DNA can still be obtained from fresh and partially decomposed tissues even after preservation in formalin for one month at room temperature.

Gillio-Tos et al., (2007) extracted DNA from 25-years old paraffin-embedded tissues and concluded that short sequences of DNA purified from small-sized tissues can be efficiently analysed, however, the analysis of longer DNA sequences (i.e. more than 300 bp) was not possible. Chakraborty et al., (2006) reported that successful DNA extraction was still possible from fish specimens fixed in buffered formalin at room temperature for one week.

Koshiba et al. (1993) found that formalin fixation caused extensive DNA degradation when formic acid formation was coupled with low pH and salt concentration at normal room temperature. Previous reports on DNA extraction from formalin fixed specimens have been described by many researchers **(**Shiozawa et al., 1992; Cano and Poinar, 1993; Shedlock et al., 1997; Chase et al., 1998) but in most cases success was not 100%. Moreover, the DNA fragments that could be sequenced and amplified were very small (100-200 bp) in most of the reports. Nucleic acid fragmentation is thought to occur due to the generation of DNA-protein cross-linkages in formalin solution (Feldman, 1973; Lehmann and Kreipe, 2001).

Ethanol is the most commonly used medium for tissue preservation in animal genetics with significant variations in the details of its applications. The storage of mammalian tissues in ethanol at room temperature has been described in many previous studies (Sibley and Ahliquist, 1981; Nietfeldt, 1989; Nagy, 2010). Alcohol preserves tissue samples without cross-linking the DNA (Srinivasan et al., 2002) which is important for subsequent extraction and analysis of DNA. Previous studies for preservation of marine invertebrates and human tissues (prostrate, kidney and liver) reported ethanol as an effective long-term tissue storage method that allows DNA recovery (Dawson and Jacobs, 1998; Gillespie et al., 2002; Kilpatrick, 2002). Ethanol preserves specimens by the inhibition of cellular enzymes and hence the quality of DNA remains intact or undergoes negligible degradation over a period of time (Penna et al., 2001).

Soft muscle tissue samples of pig and rabbit preserved in 96% ethanol gave consistent and better results compared to 10% buffered formalin. Full multiplex amplification was obtained in control (day zero) and partially decomposed samples of pig and rabbit after one year of storage at room temperature.

Our results for ethanol preservation are in contrast with the findings of Michaud and Foran (2011) in which author reported that ethanol was not suitable for long term storage (6 months) of partially decomposed pig soft muscle tissues and recommended use of ethanol only for short-term storage. Several researchers (Houde and Braun, 1988; Seutin et al., 1991) also demonstrated that tissues preserved in ethanol yielded primarily highly degraded DNA fragments and much of the degradation occurs during improper DNA extraction procedures of ethanol preserved tissues.

Allen-Hall and McNevin (2012) observed full AmpF*l* STR® Identifiler® profiles from human soft muscle tissue samples preserved in 70% ethanol at room temperature for 28 days. Kilpatrick (2002) recovered HMW DNA from ethanol preserved tissues for 2 years at room temperature (20 °C to 25 °C) in University of Vermont, Burlington, USA. Shiozawa et al., (1992) also reported that the DNA yields obtained from the ethanol preserved fish specimens were equal to those from frozen tissues and superior to formalin preservations. Similarly, Shedlock et al., 1997 found that the specimens preserved in ethanol give better yields of DNA compared to the formalin-fixed specimens and Gillespie et al., (2002) reported that DNA obtained from ethanol-fixed tissues consistently amplified more robustly compared to formalin-fixed tissues.

Commercially available cell lysis solutions have been used to preserve soft muscle tissue samples under field conditions. Fregeau et al., (2001) obtained high quality genetic profiles with DNA extracted from biopsies of human smooth muscles preserved in 1 ml of GenoFix up to one year at room temperature and for 3.5 years at -20 °C. LST buffer (a combination of potassium (K)and sodium (Na) salts with detergents and sodium azide as preservative) and commercially available Oragene™ DNA self-collection kit (DNA Genotek) were used by Graham et al., (2008) to preserve 5-100 mg of tissues for 12 months at room temperature. The author also suggested the use of these solutions to preserve muscle tissue samples under field condition for the purpose of disaster victim identification. The author found that Oragene™ was superior to LST buffer especially when compared with DNA yields of muscle tissues stored in 5 ml LST buffer, however, the quality of DNA obtained from tissues stored in LST buffer was

not significantly reduced compared to those recovered from samples stored in Oragene™.

Michaud and Foran, (2011) reported good quality DNA from pig tissues pig (0.2-0.5g) stored in solutions based in Dimethyl sulfoxide (DMSO), NaCl (Sodium chloride) and EDTA (Ethylenediaminetetra-acetic acid) at room temperature for 6 months.

RNA*later* (Ambion), a commercially available aqueous sulphate salt solution that fixes fresh tissues rapidly at room temperature was used previously, but the main disadvantage of this solution was that it interfered with DNA extractions during routine laboratory practices (Caputo et al., (2011).

Most of the preservative solutions described above used fresh tissues to preserve at room temperature, in the present study, commercially available cell lysis solution (Qiagen) was selected to preserve fresh and partially decomposed soft muscle tissues of pig and rabbit. The idea was to evaluate the efficiency of commercially available cell lysis solution to preserve DNA in partially decomposed tissues under field conditions such as normally occurs during mass disaster situations where immediate access to collect samples might not be possible.

Qiagen cell lysis solution (5 ml and 1 ml) with and without the addition of 1% sodium azide (antibacterial agent) was used to preserve pig and rabbit soft tissues (0.25 g – 1 g) collected after several days of decomposition; in pig day 7 (79 accumulated degree-days (ADD) and day 15 (210 ADD) and in rabbit day 3 (45 ADD) and day 7 (79 ADD). Fresh tissues (day zero) were used as controls.4-plex multiplex amplification was successful in all pig and rabbit samples stored in cell lysis solutions for one year.

DNA was also extracted from aliquots of cell lysis solutions as described previously by Graham et al., (2008) and 4-plex multiplex amplification was successful in all samples stored for one year. The results suggested that commercially available Qiagen cell lysis solution with or without 1% sodium azide is suitable for long term storage (12 months) of soft tissue samples at room temperature and full 4-plex amplification is possible from fresh and partially decomposed tissues. Tissue samples with size ranges of 0.25 g – 1 g can be selected for preservation. An additional advantage of using cell lysis buffer is that DNA extraction can be performed directly on aliquots of the solution, i.e. no extra processing of tissues is required. The results found in this study are in agreement with

the previous findings (Graham et al., 2008; Fregeau et al., 2001) for the use of commercially available cell lysis solutions to be used under field conditions.

Dehydration (Oven drying) of soft muscle tissues has also been used previously as successful preservation strategy (Michaud, 2006). Heat drying of tissues reduces the action of bacteria and cellular enzymes that cause DNA degradation. 70 °C is considered to be sufficient for sterilisation of food (USDA food safety guidelines). Allen-Hall and McNevin (2012) found full DNA profiles from soft muscle tissues dehydrated at 35 °C for 28 days. Dehydrated (oven dried) tissues used in this study produced full4-plex multiplex amplification in all pig and rabbit samples after one year of storage at 4 °C, suggesting that heat drying of tissues is also an effective way to preserve DNA in soft tissues for a longer period of time.

5.5 Conclusion

In conclusion, the studies presented in this chapter showed that 96% ethanol and cell lysis solution (with and without 1% sodium azide) are suitable for long term (1 year) storage of fresh and partially decomposed soft muscle tissues of pig and rabbit at room temperature. Tissue samples with size ranges of 0.25 g – 1 g can be selected for preservation. An additional advantage of using cell lysis buffer is that DNA extraction can be performed directly on aliquots of the solution, i.e. no extra processing of tissues is required. This system should therefore be considered as an additional method during Disaster victim identification (DVI) work to preserve fresh and partially decomposed samples.

Oven dried (dehydrated) soft muscle tissue samples of pig and rabbit produced full multiplex amplification after one year of storage at 4 °C, therefore, this approach can be used as an alternative method of soft muscle tissue preservation. However, this does require access to an oven.

10% buffered formalin is not suitable for long term storage of tissues at room temperature as it cause DNA degradation of larger fragments, However, amplification of the 70 bp product in rabbit was possible in few samples stored for one year and all samples preserved for one year, similarly, in pig, all samples amplified 70 bp amplicons after one year of storage at room temperature. PicoGreen® quantification of formalin preserved samples suggested that DNA can still be obtained from fresh and partially decomposed tissues even after preservation in formalin for one month at room temperature. Therefore, formalin can be used for short term (1 month) storage of tissues at room temperature.

CHAPTER 6

DNA DEGRADATION UNDER a CONTROLLED ENVIRONMENT

6.1 Introduction

In post-mortem tissues nucleases produced by host cells or released by microorganisms cause DNA to degrade into smaller fragments (Lindahl, 1993; Hofreiter et al., 2001). The rate of DNA degradation varies with light, humidity and temperature (Hochmeister, 1998; Pfeiffer et al., 1999; Bender et al., 2000; Cotton et al., 2000; Hoff-Olsen et al., 2001). High temperature and humidity cause bacterial and fungal growth that leads to further physical, chemical and biochemical degradation of HMW genomic DNA (Bar et al., 1988; Whitaker et al., 1995; Hofreiter et al., 2001). These highly degraded samples often results in partial DNA profiles with allele or complete locus dropout and loss of signal typically observed with larger-sized STR products. The amplification and profiling of shorter amplicons is more successful in degraded templates due to the fact that lower molecular weight loci are more likely to stay intact (Takahashi et al., 1997; Butler and McCord, 2003; Dixon et al., 2006).

In this study, the effect of temperature on DNA degradation was investigated. Post mortem soft muscle tissue samples of pig and rabbit were incubated at 27 °C, 37 °C and 47 °C for 21 days under controlled conditions in the laboratory. DNA extraction, quantification and 4-plex multiplex amplification was performed. DNA degradation was assessed between tissue samples incubated under controlled temperature verses tissue samples collected at different ADD from decomposing pig and rabbit at TRACES under natural environmental conditions.

6.2 Aims and Objectives

This chapter has the following aims and objectives.

- To determine the effect of different temperatures (27 °C, 37 °C and 47 °C) on DNA persistence in post-mortem soft muscle tissues of rabbit and pig.
- To determine the amplification success of 4-plex multiplex PCR to amplify 70 bp, 194 bp, 305 bp and 384 bp amplicons in pig and rabbit soft muscle tissues samples incubated at different temperature for 21 days.
- To determine DNA persistence in relation to ADD between samples incubated in the laboratory verses collected from decomposing pig and rabbit bodies in the field.

6.3 Results

6.3.1 Samples

Soft muscle tissue samples (1 cm^3) were collected from pig and rabbit carcases at day zero post-mortem (0 ADD). These samples were put inside 50 ml polypropylene tubes that were made air tight with a screw tap and incubated at 27 °C, 37 °C and 47 °C for 21 days (Section 2.1). Tissue samples (25-30 mg) were collected from each incubation temperature every second day and DNA extraction was carried out using DNeasy® Blood and Tissue kit and agarose gel electrophoresis performed to determine the presence of DNA (Sections 2.2.1 and 2.2.2). Samples were extracted in triplicate. DNA quantification of samples obtained from tissues incubated at different temperatures were quantified using Quant-iT™ PicoGreen® dsDNA reagents (Invitrogen™, UK) on the TECAN GEnios Pro Microplate Reader (Section 2.7).

6.3.2 Rabbit Tissues

6.3.2.1 Incubation at 27 °C

DNA quantification of rabbit soft muscle tissues incubated at 27 °C using PicoGreen® showed that DNA was present until day 21 (567 ADD). The DNA quantity was higher at day one (27 ADD) and then started decreasing with the passage of time but was still present until day 21 (Figure 6.1 (gel 1) and 6.2).

6.3.2.2 Incubation at 37 °C

Rabbit tissues incubated at 37 °C showed DNA until day 21 but day 7 (259 ADD) and day 9 (333 ADD) severe DNA degradation was seen (Figure 6.1 (gel 2) and 6.2).

6.3.2.3 Incubation at 47 °C

DNA quantification of samples incubated at 47 °C showed severe DNA degradation at day 1 (47 ADD) to day 17 (799 ADD). There was some DNA found at day 19 (893 ADD) and day 21 (987 ADD) (Figure 6.1 (gel 3) and 6.2) that could be bacterial.

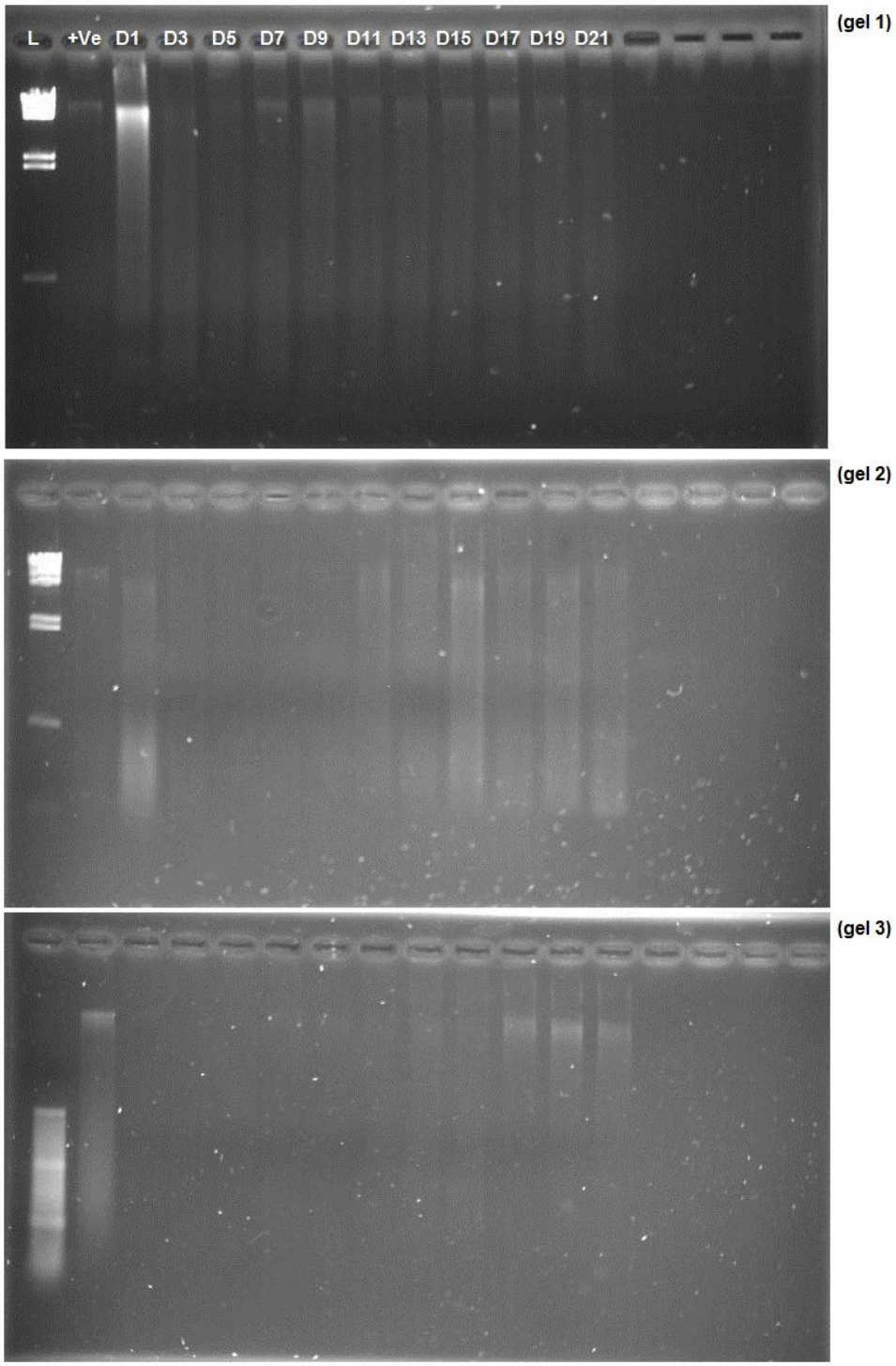

Figure 6.1 Shown above is agarose gels of rabbit soft muscle tissue samples incubated at 27 °C , 37 °C and 47 °C (gel 1, 2 and 3 respectively) up to 21 days (D1-D21). +Ve control (day zero) and HindIII ladder (L) also shown.

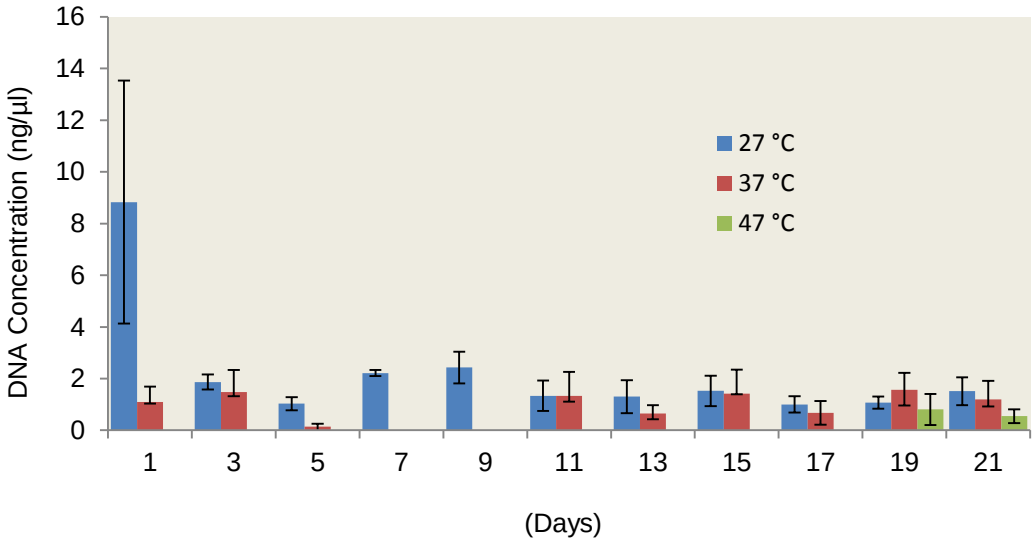

Figure 6.2 Shown above is DNA quantification using PicoGreen® for rabbit soft muscle tissue samples incubated at 27 °C, 37 °C and 47 °C up to 21 days. The error bars indicate the standard error from three replicates. A total of 200 µl was extracted from each sample and 3 µl was used for quantification.

6.3.3 4-plex Multiplex Amplification

4-plex multiplex PCR was performed to determine the amplification success of 70 bp, 194 bp, 305 bp and 384 bp amplicons in soft muscle tissue samples incubated at 27 °C, 37 °C and 47 °C for 21 days.

6.3.3.1 Incubation at 27 °C

Rabbit soft muscle tissue samples incubated at 27 °C showed full 4-plex multiplex profile at day 1 (27 ADD). Drop-out in amplification of 194 bp, 305 bp and 384 bp occurred at day 3 (81 ADD) and there was nearly complete failure of amplification after day five (Figure 6.3, Table 6.1). The amplification of 70 bp was consistent until day 17 (459 ADD). Tissue samples developed substantial odour by day seven and humidity was 88.5% to 100%.

Table 6.1 Shown below are the results of multiplex (4-plex) amplification of rabbit tissues incubated at 27 °C up to 21 days.

Days	Accumulated degree-days (ADD)	Rabbit soft tissues incubated at 27 °C			
		70 bp	194 bp	305 bp	384 bp
1	27	**+++**	**+++**	**+++**	**+++**
3	81	**+++**	**++-**	**+--**	**---**
5	135	**+++**	**++-**	**++-**	**++-**
7	189	**+++**	**+--**	**---**	**---**
9	243	**+++**	**+++**	**---**	**---**
11	297	**+++**	**+--**	**---**	**---**
13	351	**+++**	**++-**	**---**	**---**
15	405	**+++**	**---**	**---**	**---**
17	459	**+++**	**+--**	**+--**	**+--**
19	513	**++-**	**---**	**---**	**---**
21	567	**++-**	**++-**	---	---

‘‘+’’ denotes the presence and ‘‘-’’ represents the absence of PCR amplicons in samples during multiplex (4-plex) amplification.

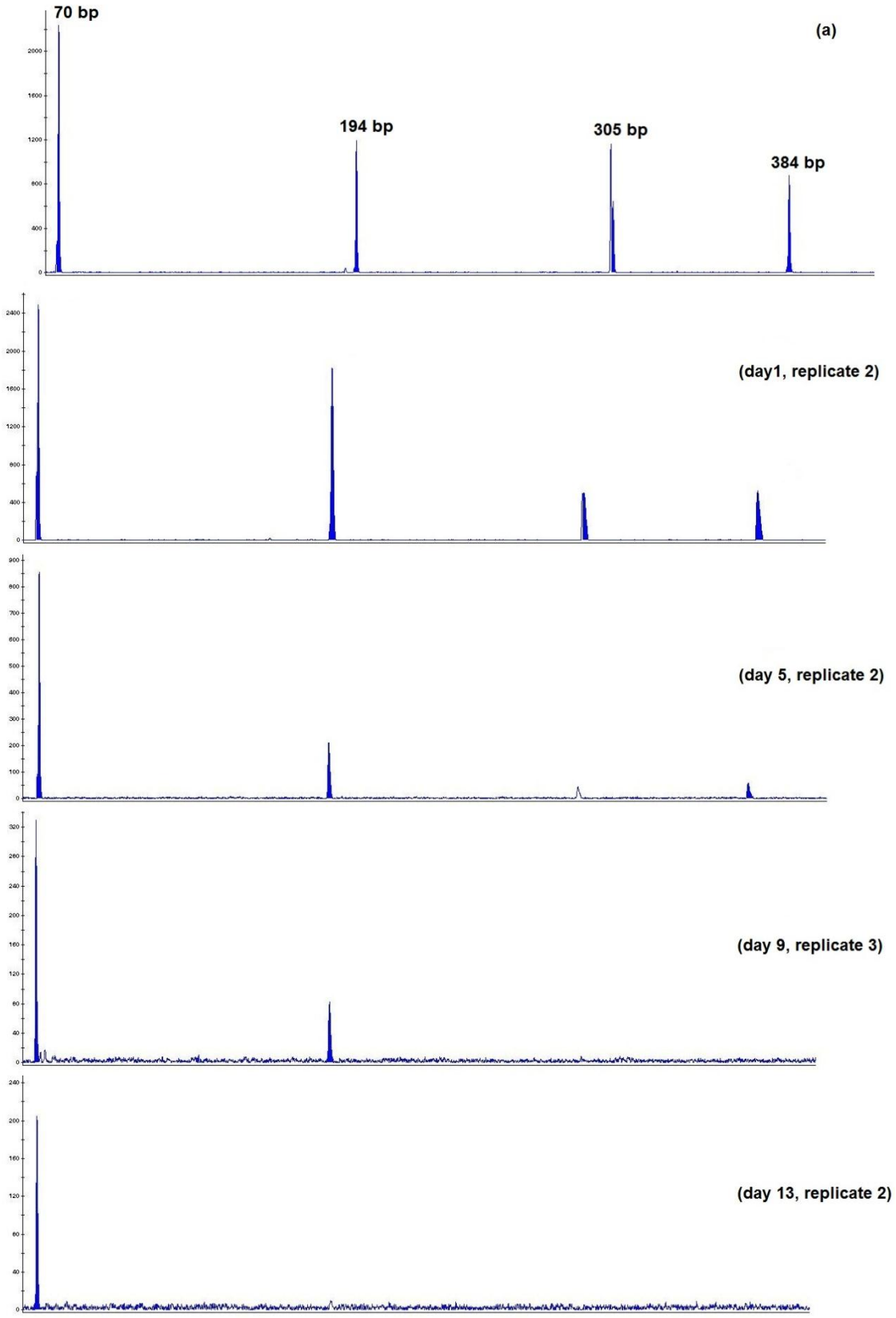

Figure 6.3 Shown above are examples of electropherograms of full and partial multiplex profile obtained from rabbit soft muscle tissues incubated at 27 °C for up to 21 days (567 ADD). Human positive control (a) also shown.

6.3.3.2 Incubation at 37 °C

Multiplex amplification of rabbit tissues incubated at 37 °C showed full 4-plex multiplex profile until day 3 (111 ADD). There was complete dropout in amplification of larger amplicons after day 5 until day 21 except day 13 and 15. The amplification of the 70 bp product was possible until day 21(777 ADD) but on days 7 and 9 no 70 bp product was amplified (Figure 6.4, Table 6.2). The humidity was 65% to 73.5% during incubation period.

Table 6.2 Shown below are the results of 4-plex multiplex amplification of rabbit tissues incubated at 37 °C for 21 days.

Days	Accumulated degree-days (ADD)	Rabbit soft tissues incubated at 37 °C			
		70 bp	194 bp	305 bp	384 bp
1	37	+++	+++	+++	+++
3	111	+++	+++	+++	+++
5	185	+++	++-	++-	++-
7	259	---	---	---	---
9	333	---	---	---	---
11	407	+--	+--	---	---
13	481	+++	+++	+++	+++
15	555	+++	+++	+--	+--
17	629	+++	++-	---	---
19	703	+++	+--	---	---
21	777	+++	---	---	---

‘‘+’’ denotes the presence and ‘‘-’’ represents the absence of PCR amplicons in samples during multiplex (4-plex) amplification.

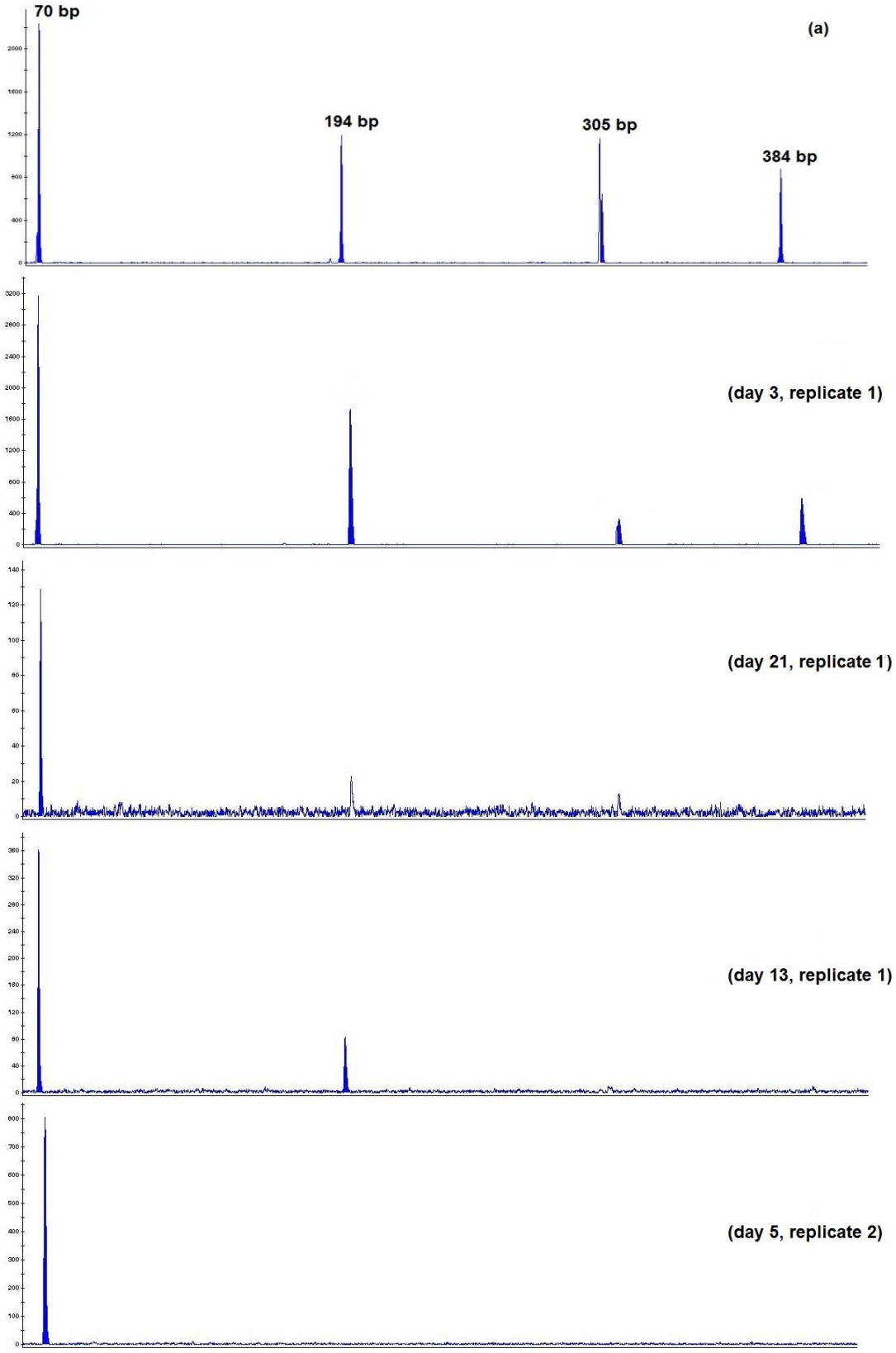

Figure 6.4 Shown above are examples of electropherograms of full and partial multiplex profile obtained from rabbit soft muscle tissues incubated at 37 °C for up to 21 days (777 ADD). Human positive control (a) also shown.

6.3.3.3 Incubation at 47 °C

Multiplex amplification of rabbit tissue samples incubated at 47 °C showed full 4-plex profile only until day one (47 ADD). There was complete failure of amplification of larger amplicons (305 and 384 bp) after day one. 194 bp was amplified in in one samples of each triplicate at day three, five, seven and 15. There was dropout in 70 bp starting at on day 5 (Figure 6.5 and Table 6.3). The humidity was 98% to 100% during the incubation period.

Table 6.3 Shown below are the results of 4-plex multiplex amplification for rabbit soft muscle tissues incubated at 47 °C for 21 days.

Days	Accumulated degree-days (ADD)	Rabbit soft tissues incubated at 47 °C			
		70 bp	194 bp	305 bp	384 bp
1	47	**+++**	**+++**	**+++**	**+++**
3	141	**+++**	**+--**	**---**	**---**
5	235	**+--**	**+--**	---	---
7	329	**++-**	**+--**	**---**	**---**
9	423	**++-**	**---**	**---**	**---**
11	517	**+--**	**---**	**---**	**---**
13	611	**+++**	**---**	**---**	**---**
15	705	**+--**	**+--**	**---**	**---**
17	799	**---**	**---**	**---**	**---**
19	893	**+---**	**---**	**---**	**---**
21	987	**---**	**---**	**---**	**---**

‘‘+’’ denotes the presence and ‘‘-’’ represents the absence of PCR amplicons in samples during multiplex (4-plex) amplification.

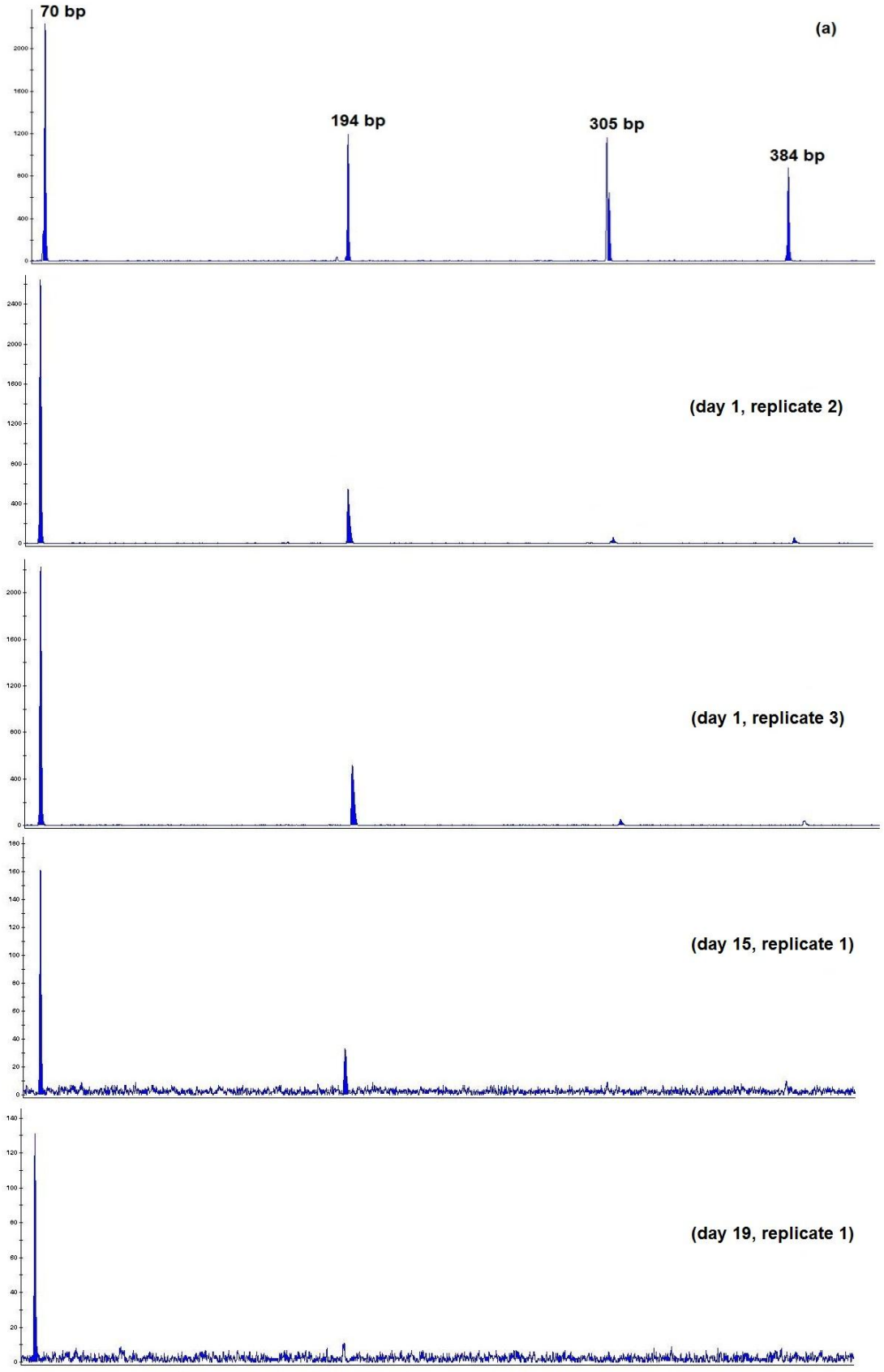

Figure 6.5 Shown above are examples of electropherograms of full and partial multiplex profile obtained from rabbit soft muscle tissues incubated at 47 °C up to 21 days (987ADD). Human positive control (a) also shown.

6.3.4 Pig Tissues

6.3.4.1 Incubation at 27 °C

DNA quantification of pig tissue samples incubated at 27 °C showed DNA at day one (27 ADD) and day three, there was severe DNA degradation observed in all samples from day five to day 21 (Figure 6.6 (gel 1) and 6.7). The humidity was 84% - 100% during incubation period.

6.3.4.2 Incubation at 37 °C

DNA quantification of tissue samples incubated at 37 °C showed DNA at day one (37 ADD) and persistence until day 21 (Figure 6.6 (gel 2) and 6.7). The humidity was 64.5% to 79% during incubation period.

6.3.4.3 Incubation at 47 °C

In DNA samples obtained from tissue samples incubated at 47 °C, there was DNA degradation after day one and persistence until day 21 (987 ADD) (Figure 6.6 (gel 3) and 6.7). The humidity was 79% - 100% during incubation period.

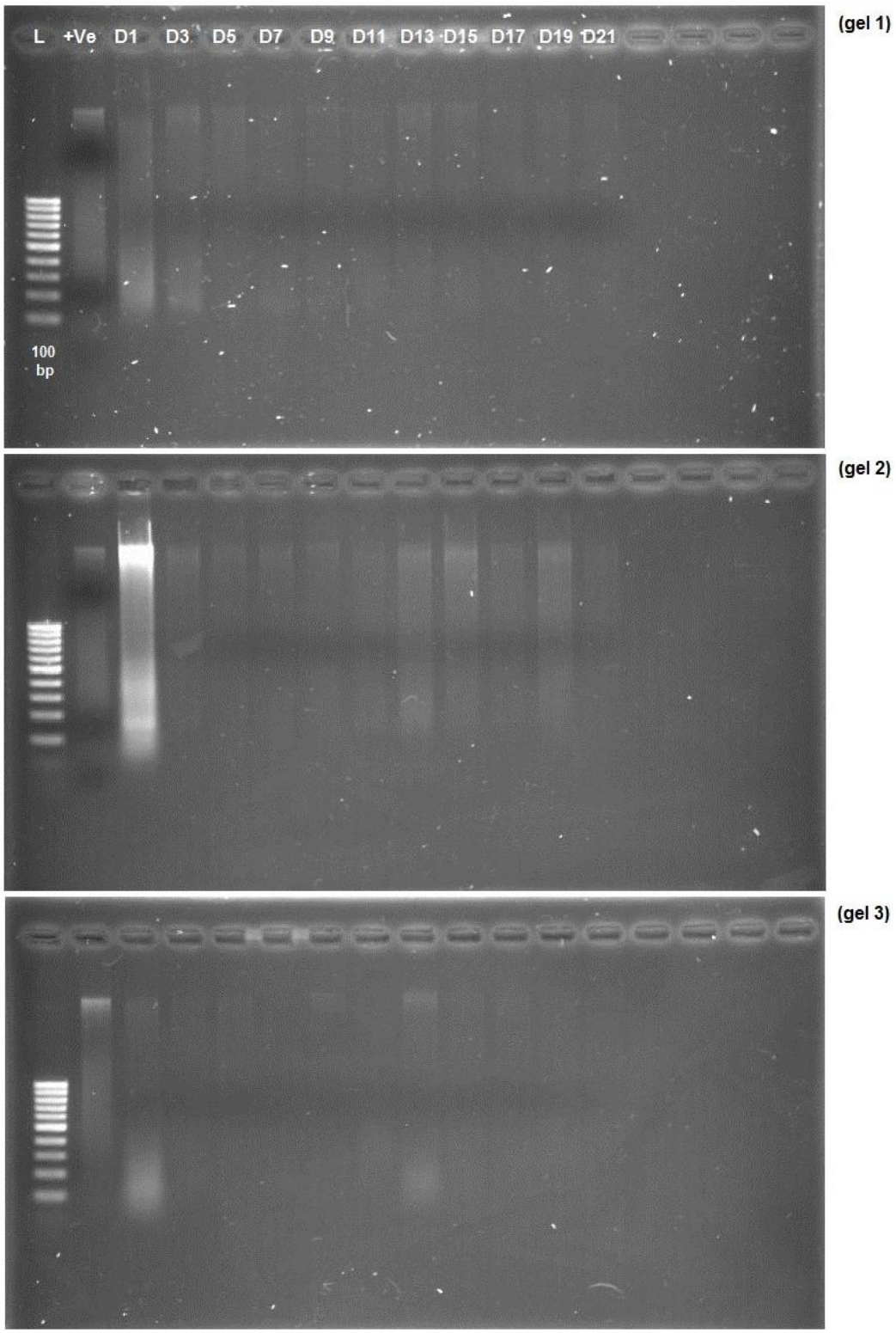

Figure 6.6 Shown above is agarose gel electrophoresis of pig soft muscle tissue samples incubated at 27 °C , 37 °C and 47 °C (gel 1, 2 and 3) up to 21 days (D1-D21). +Ve control (day zero) and 100 bp DNA ladder (L) also shown. The small white spots appeared due to faulty gel tank.

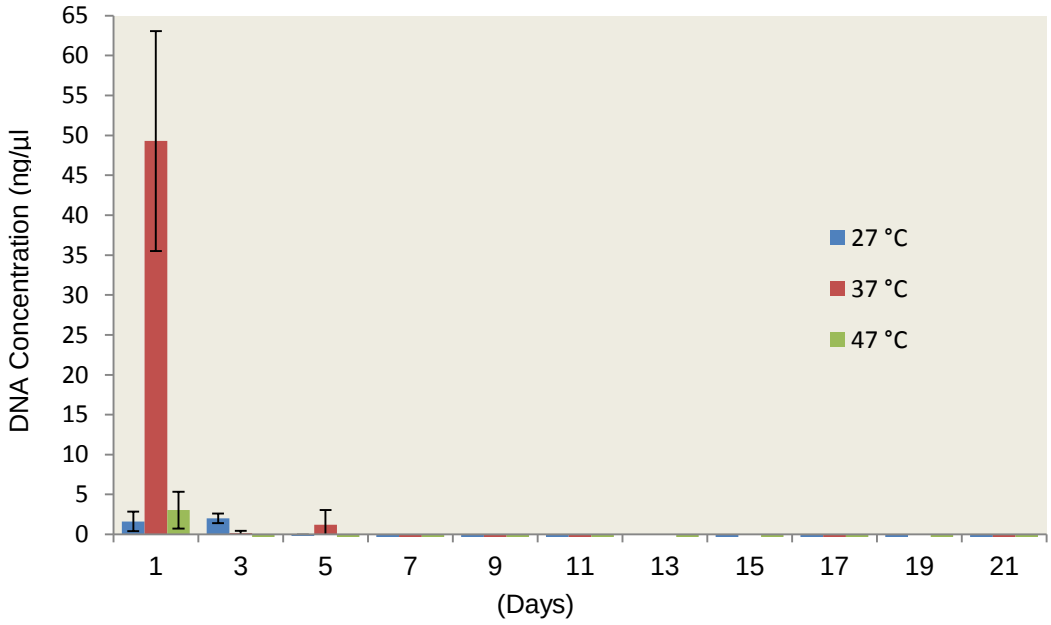

Figure 6.7 Shown above is DNA quantification using PicoGreen for pig soft muscle tissue samples incubated at 27 °C, 37 °C and 47 °C up to 21 days. The error bars indicate the standard error from three replicates. A total of 200 µl was extracted from each sample and 3 µl was used for quantification.

6.3.5 4-plex Multiplex Amplification

6.3.5.1 Incubation at 27 °C

Pig tissues samples incubated at 27 °C showed full 4-plex profile only on day one (27 ADD). There was complete failure of amplification for larger amplicons after day 5 except one sample amplified at day 19 (513 ADD).. The 70 bp was present until day 21 with exception of few samples in triplicate set which did not work after day 15 (Figure 6.8 and Table 6.4). During amplification some samples appeared with peak heights less than the threshold (50 RFU), therefore, there was no sizing data for them and these samples were treated as undetected.

Table 6.4 Shown below are the results of 4-plex multiplex amplification of pig tissues incubated at 27 °C up to 21 days.

Days	Accumulated degree-days (ADD)	pig soft tissues incubated at 27 °C			
		70 bp	194 bp	305 bp	384 bp
1	27	**+++**	**+++**	**+++**	**+++**
3	81	**++-**	**++-**	**++-**	**++-**
5	135	**+++**	**++-**	**++-**	**++-**
7	189	**+++**	**+++**	**---**	**---**
9	243	**+++**	**+--**	**---**	**---**
11	297	**+++**	**---**	**---**	**---**
13	351	**+++**	**+--**	**---**	**---**
15	405	**+++**	**+--**	**---**	**---**
17	459	**++-**	**+--**	**---**	**---**
19	513	**++-**	**+--**	**+--**	**+--**
21	567	**++-**	**---**	**---**	---

‘‘+’’ denotes the presence and ‘‘-’’ represents the absence of PCR amplicons in samples during multiplex (4-plex) amplification.

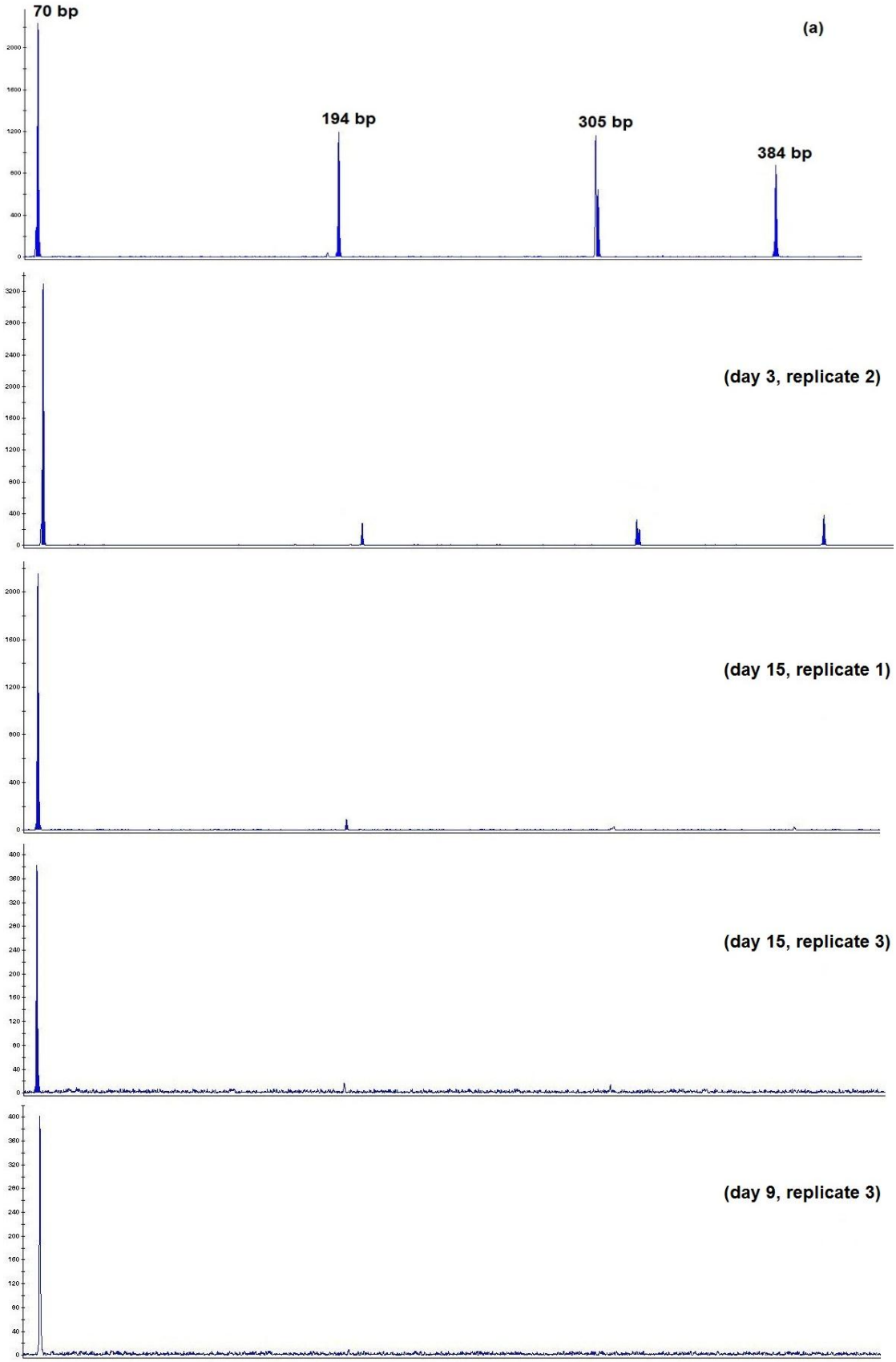

Figure 6.8 Shown above are examples of electropherograms of full and partial multiplex profile obtained from pig soft muscle tissues incubated at 27 °C up to 21 days (567 ADD). Human positive control (a) also shown.

6.3.5.2 Incubation at 37 °C

There was full 4-plex multiplex profile in all samples incubated at 37 °C until day three (111 ADD). There was complete failure of amplification for 305 and 384 bp after day three except one sample amplified for 384 bp at day 11.The amplification of 70 bp was consistent until day 11 (407 ADD) (Figure 6.9 and Table 6.5).

Table 6.5 Shown below are the results of 4-plex multiplex amplification of pig tissues incubated at 37 °C up to 21 days.

Days	Accumulated degree-days (ADD)	pig soft tissues incubated at 37 °C			
		70 bp	194 bp	305 bp	384 bp
1	37	**+++**	**+++**	**+++**	**+++**
3	111	**+++**	**+++**	**+++**	**+++**
5	185	**+++**	**+--**	**---**	**---**
7	259	**+++**	**++-**	**---**	---
9	333	**+++**	---	---	---
11	407	**+++**	**+--**	---	**+--**
13	481	**++-**	**+--**	---	---
15	555	**+--**			
17	629	**+++**		**---**	**---**
19	703	**++-**	---	---	---
21	777	**+--**	**---**	---	---

‘‘+’’ denotes the presence and ‘‘-’’ represents the absence of PCR amplicons in samples during multiplex (4-plex) amplification.

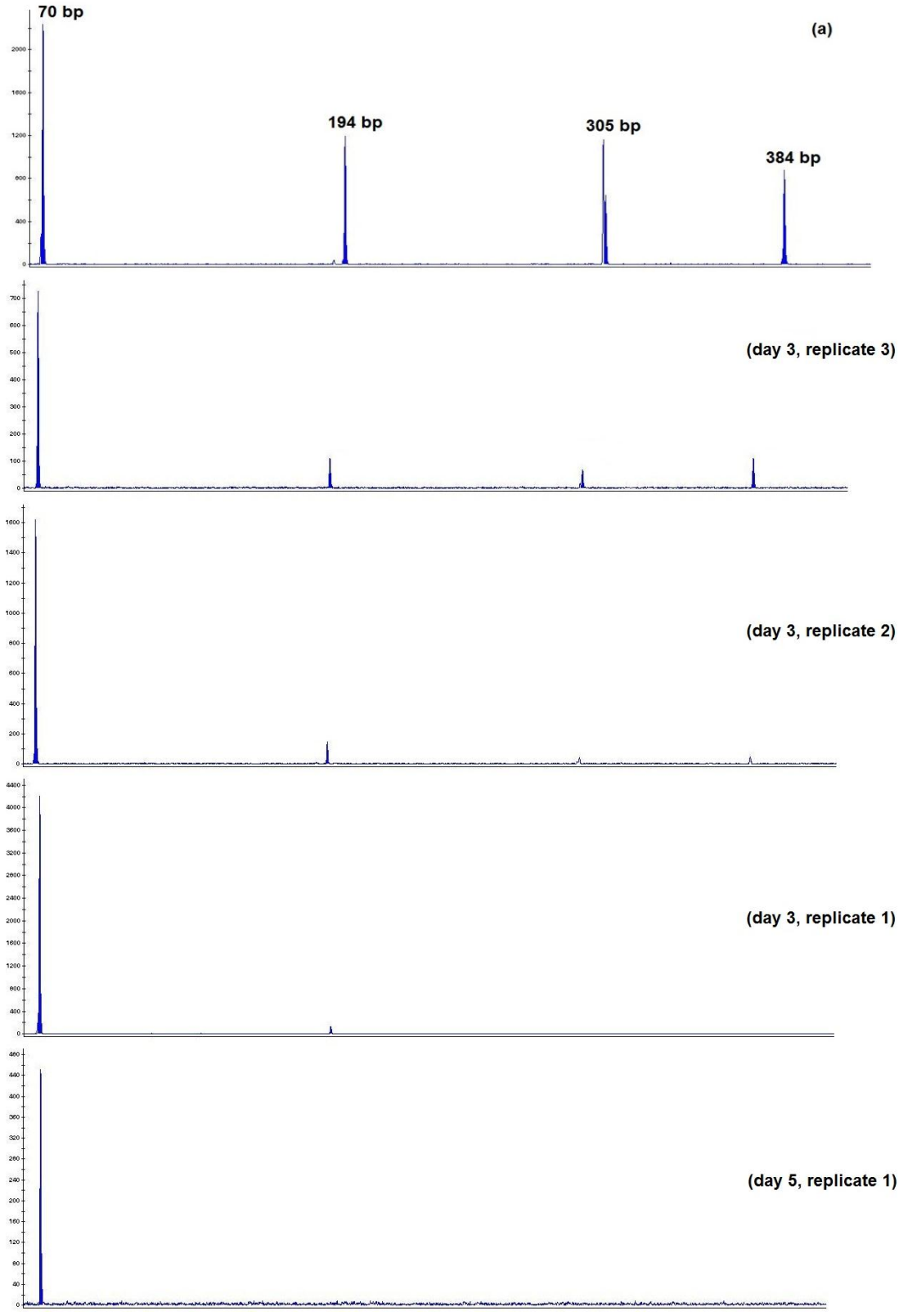

Figure 6.9 Shown above are examples of electropherograms of full and partial multiplex profile obtained from pig soft muscle tissues incubated at 37 °C up to 21 days (777 ADD). Human positive control (a) also shown.

6.3.5.3 Incubation at 47 °C

In muscle tissues incubated at 47 °C, there was complete failure of amplification of 194 bp, 305 bp and 384 bp after day three (141 ADD) in all triplicate samples (Table 6.6). Amplifications of 70 bp was consistent until day 3 (141 ADD) and intermittent until day 21 (987 ADD) (Figure 6.10).

Table 6.6 Shown below are the results of 4-plex multiplex amplification for pig muscle tissues incubated at 47 °C up to 21 days.

Days	Accumulated degree-days (ADD)	Pig soft tissues incubated at 47 °C			
		70 bp	194 bp	305 bp	384 bp
1	47	**+++**	**+++**	**+++**	**+++**
3	141	**+++**	**+++**	**+++**	**+++**
5	235	**++-**	---	---	---
7	329	**+--**	---	---	---
9	423	**++-**	---	---	---
11	517	**+--**	---	---	---
13	611	**+--**	---	---	---
15	705	**++-**	---	---	---
17	799	**+--**	---	---	---
19	893	**+--**	---	---	---
21	987	**++-**	---	---	---

‘‘+’’ denotes the presence and ‘‘-’’ represents the absence of PCR amplicons in samples during multiplex (4-plex) amplification.

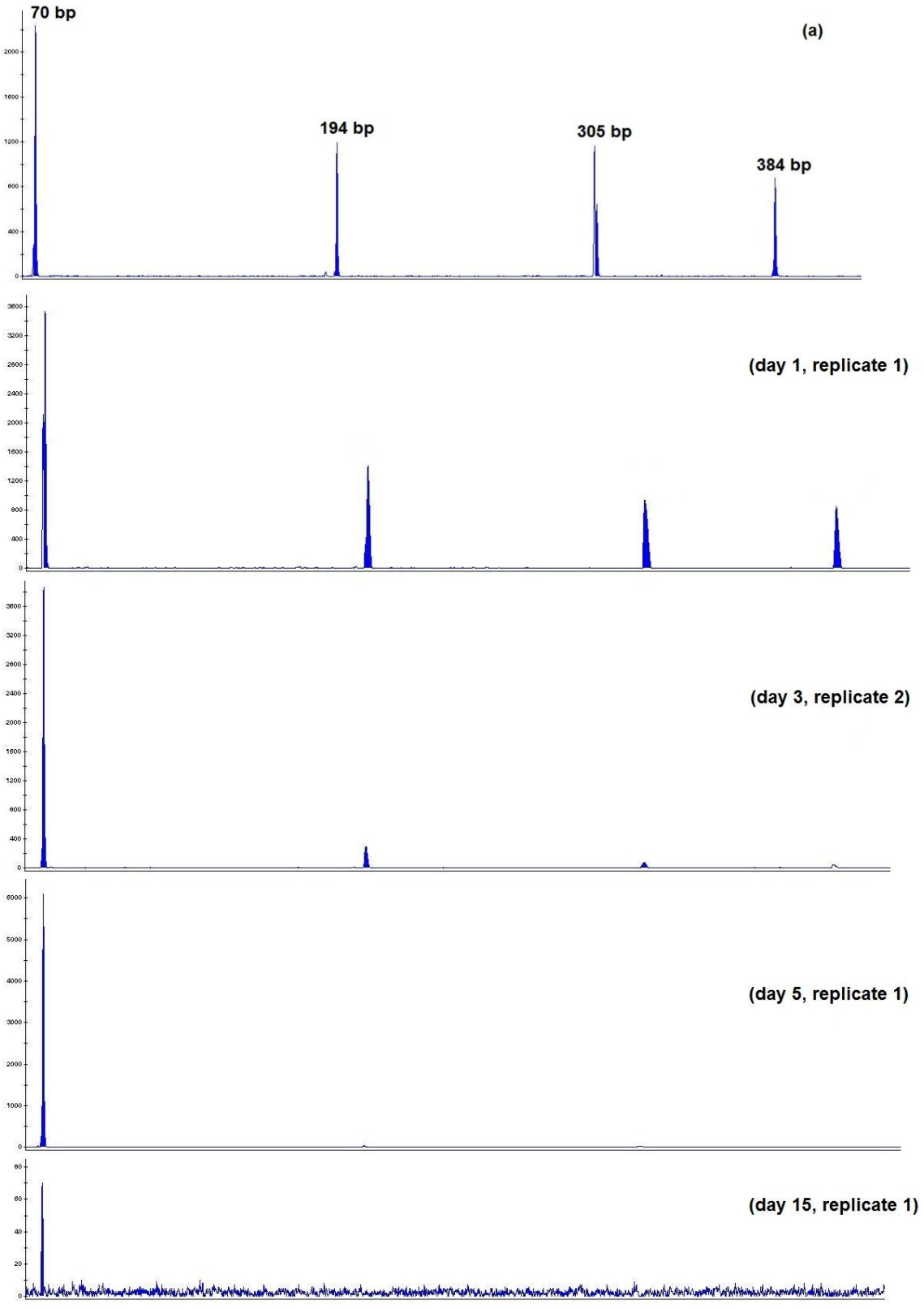

Figure 6.10 Shown above are examples of electropherograms of full and partial multiplex profile obtained from pig soft muscle tissues incubated at 47 °C up to 21 days (987 ADD). Human positive control (a) also shown…

6.3.6 Comparative Analysis between Pig and Rabbit Tissues

The amplification of the 70 bp product was obtained in all samples of pig incubated at 27 °C, 37 °C and 47 °C for 21 days although not in all samples of triplicates. Amplification of larger amplicons (305 bp and 384 bp) was possible until day five (27 °C) and day three (37 °C and 47 °C) but again not in all samples of triplicates. Drop-out in amplification of 194 bp was observed after day 3 (37 °C and 47 °C) and day 1 (27 °C).

In case of rabbit, the drop-out in amplification of 305 bp and 384 bp was observed after day 1 (27 °C and 47 °C) and day 5 (37 °C). The amplification was 70 bp was possible in most of the samples incubated at different temperatures. The drop-out in amplification of 194 bp was observed after day 1 (27 °C and 47 °C) and day 3 (37 °C).

6.4 Statistical Analysis

ANOVA (analysis of variance) was performed on DNA quantity obtained from pig and rabbit incubation experiments using statistical package R version 2.10.1. The significance level (alpha value) was 0.05. The ANOVA was performed to determine the effect of species, ADD and temperature on DNA persistence in soft muscle tissue of pig and rabbit. The results showed that DNA persistence in soft muscle tissues is highly influenced by ADD ($F_{1,186}$=6.11, p=0.014), species ($F_{1,186}$=39.32, p<0.0001) and temperature ($F_{2,186}$=24.32, p<0.0001).

6.5 Discussion

The main purpose of experiments performed in this chapter was to identify the effect of various heat treatments (27 °C, 37 °C and 47 °C) on DNA persistence in soft muscle tissues of rabbit and pig for a specific time point (21 days). 4-plex multiplex amplification was performed to determine the DNA persistent in soft tissues. These experiments were performed keeping in view mass disaster situations where bodies can be exposed to different environmental temperatures.

Overall, the results showed that the amplification of 70 bp was possible until day 21 in all samples of pig and rabbit incubated at 27 °C, 37 °C and 47 °C with the exception of few samples. Utsuno and Minaguchi (2004) described that the amplification efficiency increases as PCR target size reduces. Previous researchers (Takahashi et al., 1997; Butler and McCord, 2003) also explained that the higher chance of amplification of shorter fragments in degraded DNA samples is due to the likelihood that the longer fragments will be degraded with time. In this study amplification of larger amplicons was possible until day five maximum especially at 27 °C. Itani et al., (2011) reported that the quantity of amplified DNA in skeletal muscles of dead rats kept at 20 °C was reduced from 10 ng to 1 ng in 2-4 weeks. Dixon et al., (2006) incubated blood and saliva samples at 37 °C with 100% humidity and total DNA degradation in the incubated samples was achieved within a short time period of 12-16 weeks.

Lahiri and Schnabel (1993) reported that the blood samples incubated at 37 °C for 24 hours resulted in the same amount and quality of DNA as obtained from frozen samples (-70 °C). Qiagen (2008) collected HMW DNA from human blood samples stored at 22 °C for three days and DNA degradation was observed after one week.

In this study, controlled incubation temperatures were converted to ADD and multiplex amplification results were compared between tissue samples incubated in the laboratory verses soft tissues collected from decomposing pig and rabbit carcasses at different ADD (Chapter 4), Although, this was not the perfect comparison as ideally, whole bodies should be exposed to high ambient temperature, but it was not possible under UK weather conditions.

Results showed that the shorter 70 bp amplicon was present with high frequency as long as muscles were available for collection with the exception of few samples at 37 °C and 47 °C (Tables 6.2 and 6.3). The larger amplicons drop out rapidly, both in terms of days

and ADD when compared to field samples. This potentially was caused by high humidity inside the closed polypropylene tubes but is also likely to be due to a more rapid degradation of DNA at higher temperatures.

During multiplex amplification it was observed in rabbit samples incubated at 37 °C that there was no 4-plex profile at day seven and day nine and appeared again on day 13 (Table 6.2), this might be due to the that these samples were not extracted properly i.e. human error was to blame. It was also observed in some samples that the peak heights of larger amplicons were below the threshold level (50 RFU) and therefore, not sized, CE was repeated for few of these samples by increasing the PCR products (2 µl) and double injection time (20 s) as recommended by previous researchers (Lawlor et al., 1991; Handt et al., 1994) but it did not have an effect. It was observed that that by reducing the detection limit in analysis matrix to 30 RFU, the size and peak heights could be obtained for few samples, but this might not be acceptable for forensic cases.

Finally, the results demonstrated in this chapter indicated that the amplification of 70 bp was more persistent compared to larger amplicons with exception of few samples at 37 °C and 47 °C. As small length of SNP loci amplified more efficiently than the larger loci present in the STR system (Gill et al., 1998), therefore, it would be advisable to use SNPs in highly degraded soft muscle tissue samples commonly encountered during mass disaster situations (Gill et al., 2004; Budowle and Eisenberg et al., 2005; Butler et al., 2007).

6.6 Conclusion

The designed 4-plex multiplex demonstrated a high level of sensitivity in obtaining amplification from heavily degraded DNA samples of incubation experiments. The amplification of larger amplicons (194 bp, 305 bp and 384 bp) in pig and rabbit reduced more rapidly compared to 70 bp. The persistence of 70 bp in most of the samples incubated at different temperatures suggested that soft muscle tissues exposed to different temperature could be used to perform SNP analysis.

When controlled incubation data was converted to ADD and results of DNA degradation were compared with samples obtained at different ADD from decomposing pig and rabbit under field conditions, it was found that the drop-out in amplification of the 305 bp and 384 bp amplicons occurred more rapidly when samples were incubated under laboratory conditions compared to the field samples. Naturally the field conditions are much cooler, and so it is not possible, without further experimentation, to say whether the same would be seen in field samples and how much of the enhanced degradation was due to the high humidity within the closed plastic tubes.

CHAPTER 7

GENERAL DISCUSSION and FUTURE WORK

7.1 General Discussion and Future Work

The aim of this study was to assess DNA persistence in muscle tissues in relation to ADD. This study was carried out bearing in mind the mass disaster situations where forensic scientist often come across whole carcases, fragmented bodies and bodies with high insect activity. Pig and rabbit were selected as model organisms as they were previously used in many decomposition studies (Robins and Furey et al., 2001; Larkin et al., 2010; Simmons and Moffatt, 2010) to estimate PMI. DNA persistence was assessed in relation to ADD as it provides a measure of both time and temperature and also been used to estimate PMI (Megyesi and Haskell 2005; Slone et al., 2007; Adlam and Simmons 2007).

A 4-plex multiplex PCR based assay was developed that would amplify efficiently both fresh and degraded DNA samples of pig, rabbit and human. The absence of any detected discordance between independent analyses demonstrates that the amplification procedure of this multiplex is reliable and reproducible for pig, rabbit and human DNA samples. This multiplex is also cost effective and can amplify a similar size range of PCR amplicons covered by commercially available STR kits and can be used in forensic analysis to assess DNA persistence in decomposing human bodies and experimental studies.

However, due to high sensitivity, throughput and lower cost, PicoGreen® dsDNA quantification was used to quantify DNA. The inconsistency observed in the results with respect to DNA quantity with increasing ADD is likely due to the non-specificity of PicoGreen® dye as it binds to any dsDNA regardless of species. The increase in DNA quantity at later stages of decomposition was likely due to the bacterial and fungal growth on the decomposing tissues. This has also been described by Larkin et al., (2010). In some cases, amounts of DNA were also found to decrease with ADD, making any valid conclusions about DNA quantity and ADD difficult. In fact, there have been a number of studies that have found lack of consistency with PicoGreen®. Haque et al., (2003) reported that inconsistency in PicoGreen® quantification results might be due to the variability in laboratory sample handling steps or due to residual (unmodeled) factors. The author also reported the amount of variance attributed to the entire laboratory handling procedures was 4.3% and intra-assay (between replicates) coefficient of variance (CV) was 13.6%. Rengarajan et al., (2002) also evaluated the sensitivity and linearity of PicoGreen® quantification and demonstrated that the intra-

assay CV of PicoGreen® was 8.3%. It was reported that the smaller volumes of DNA (2.0 µl -5.0 µl) used for PicoGreen® quantification also introduced more sample variability due to the sampling effect or increased failure to transfer smaller volumes (Ahn et al., 1996; Singer et al., 1997; Rengarajan et al., 2002; Haque et al., 2003).

Due to the inconsistency in DNA quantification results using PicoGreen®, statistical analysis was performed using only 4-plex multiplex amplification data. DNA persistence in muscles was assessed in relation to ADD by including all factors such as species, days, temperatures, whole carcasses, body fragments and suspended tissues as random variables and results suggested that there was no difference in DNA persistence in whole carcases versus suspended tissues and whole carcass versus body fragments. However, there was a significant difference in DNA persistence in body fragments versus suspended tissues. Furthermore, the amplification of 70 bp was clearly more persistent compared to larger amplicons with increase in ADD.

The results obtained from field experiments suggested that muscle tissues if available should be collected for DNA profiling, since even if degraded, a SNP profile could be obtained. The results also suggested that the isolation of tissues from insect activity as quickly as possible (even if immediate storage is not possible) may be beneficial for DNA persistence. The significant result between body fragments and suspended tissues support this because suspended tissues had mesh keeping out insects. Seasonal variation in DNA persistence was observed. This was due to maggot mass growth in decomposing bodies especially at high temperature (10 °C-23 °C) in summer (Slone et al., 2007; Adlam and Simmons 2007). Insect activity free (suspended tissues) used during these experiments suggested that the isolation of tissues from insect activity as quickly as possible (even if immediate storage is not possible) would be a better choice to delay decomposition and hence increase DNA persistence, but no significant difference between whole carcasses and suspended tissues was difficult to explain in this context.

These experiments were performed in temperate climate and it was not possible to get high ambient temperature (27 °C, 37 °C and 47 °C). Therefore, controlled incubation experiments were carried out to assess DNA persistence in muscles. It was observed that the larger amplicons (194 bp, 305 bp and 384 bp) droped-out more rapidly compared to 70 bp. This is likely due to the high temperature, bacterial and fungal growth and the action of hydrolytic enzymes (Eglington et al., 1991; Lindahl, 1993;

Gill-King et al., 1996; Hofreiter et al., 2001). Further outdoor experiments should be performed at high ambient temperatures to assess DNA persistence in muscles.

Preservation and handling of samples during mass disaster situations is an important factor for successful DNA analysis (Prinz et al, 2005). The immediate access to mass disaster sites is not always possible; similarly, cold storage is not always available. Therefore, in this study, some preservation methods were evaluated to preserve DNA in fresh and partially decomposed tissues stored at room temperature for one year. The findings suggested full 4-plex multiplex amplification can be obtained successfully from tissues preserved in 96% ethanol, cell lysis solution (with or without the addition of 1% sodium azide (bacteriostatic agent) at room temperature for one year. Similarly, full multiplex amplification was obtained from dehydrated tissues stored at 4 °C for one year. The failure in amplification of larger amplicons (194 bp, 305 bp and 384 bp) in formalin preserved samples suggested that formalin was not suitable for long term storage of tissues at room temperature. This system should therefore be considered as an additional method during DVI to preserve fresh and partially decomposed samples.

In the present study, RAG-1 gene was selected for 4-plex multiplex design. Although this gene is highly conserved across different mammals, future work should include confirmation of the target region via DNA sequencing. Also, in the 4-plex multiplex, the predicted 70 bp was sized much smaller (approximately 65 bp). This is likely due to the florescent dye attached to the primer as different dyes have been demonstrated to affect the mobility of DNA fragments, Alternatively, different performance optimised polymers (POP) have been shown to size fragments differently (Butler, 2005), For future studies the use of different size standards to carry out fragments analysis and the sequencing of the PCR products would be beneficial. Finally, potential future work could assess DNA preservation in post-mortem muscle tissue samples preserved at room temperature without any preservative solution.

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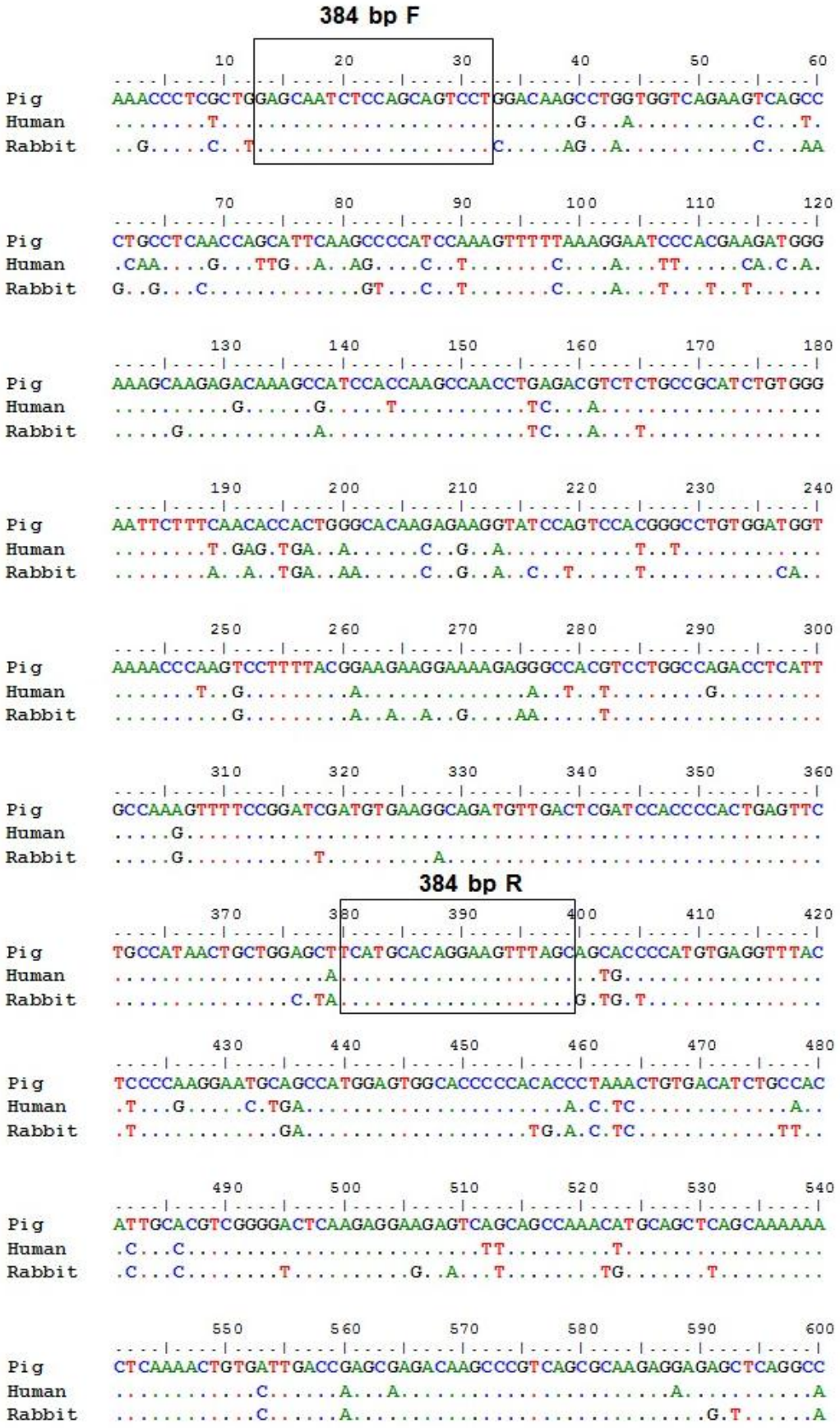
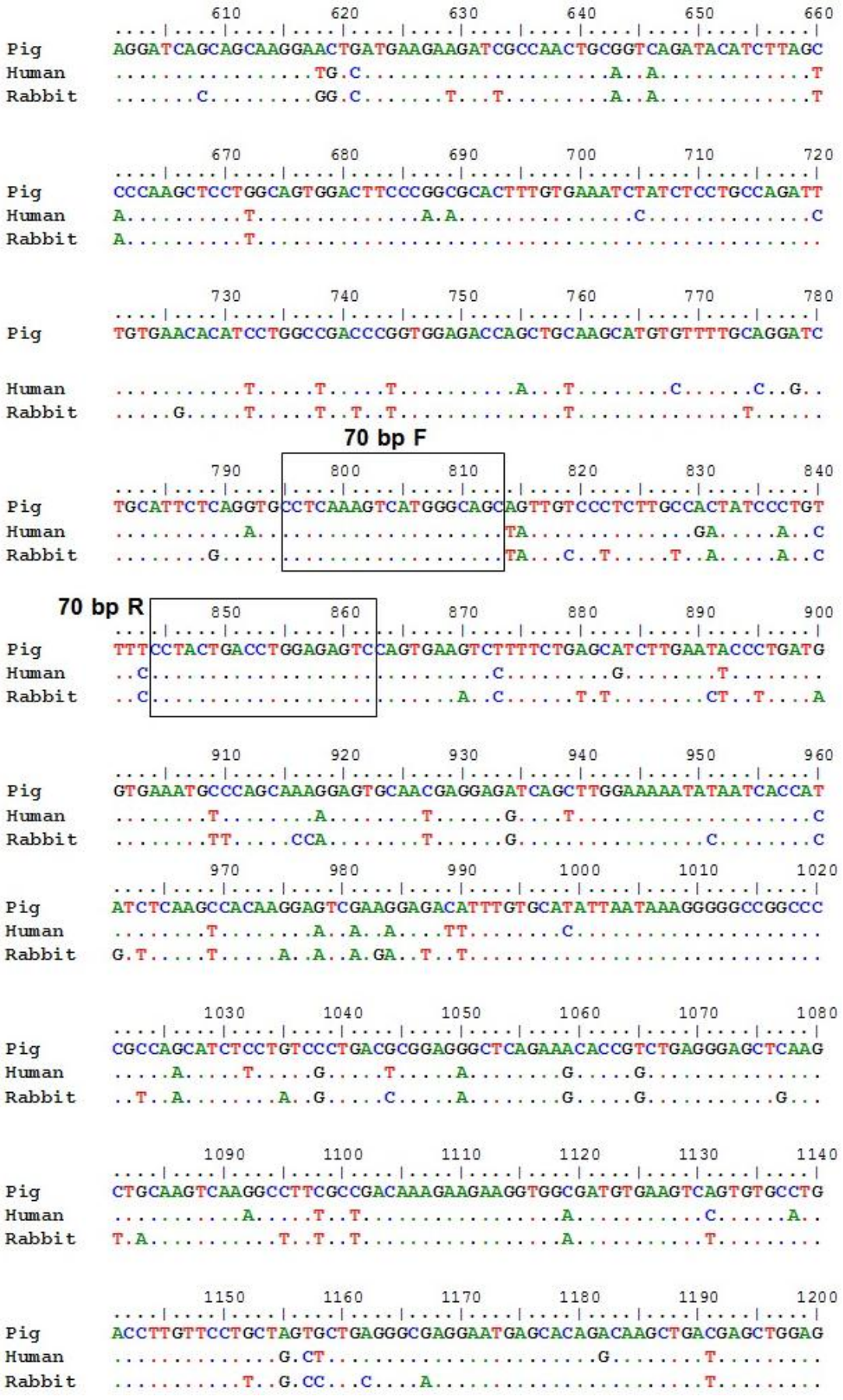
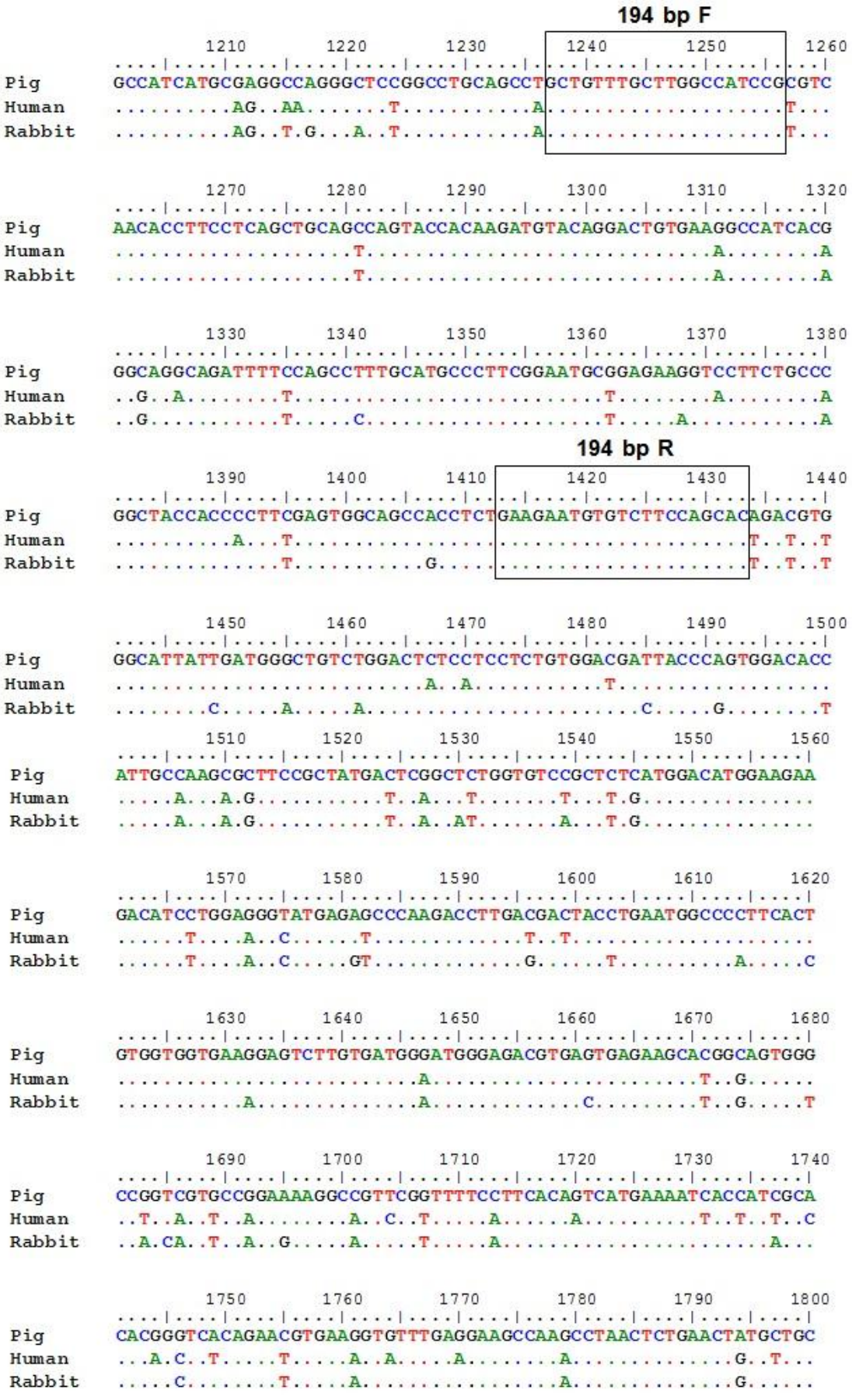
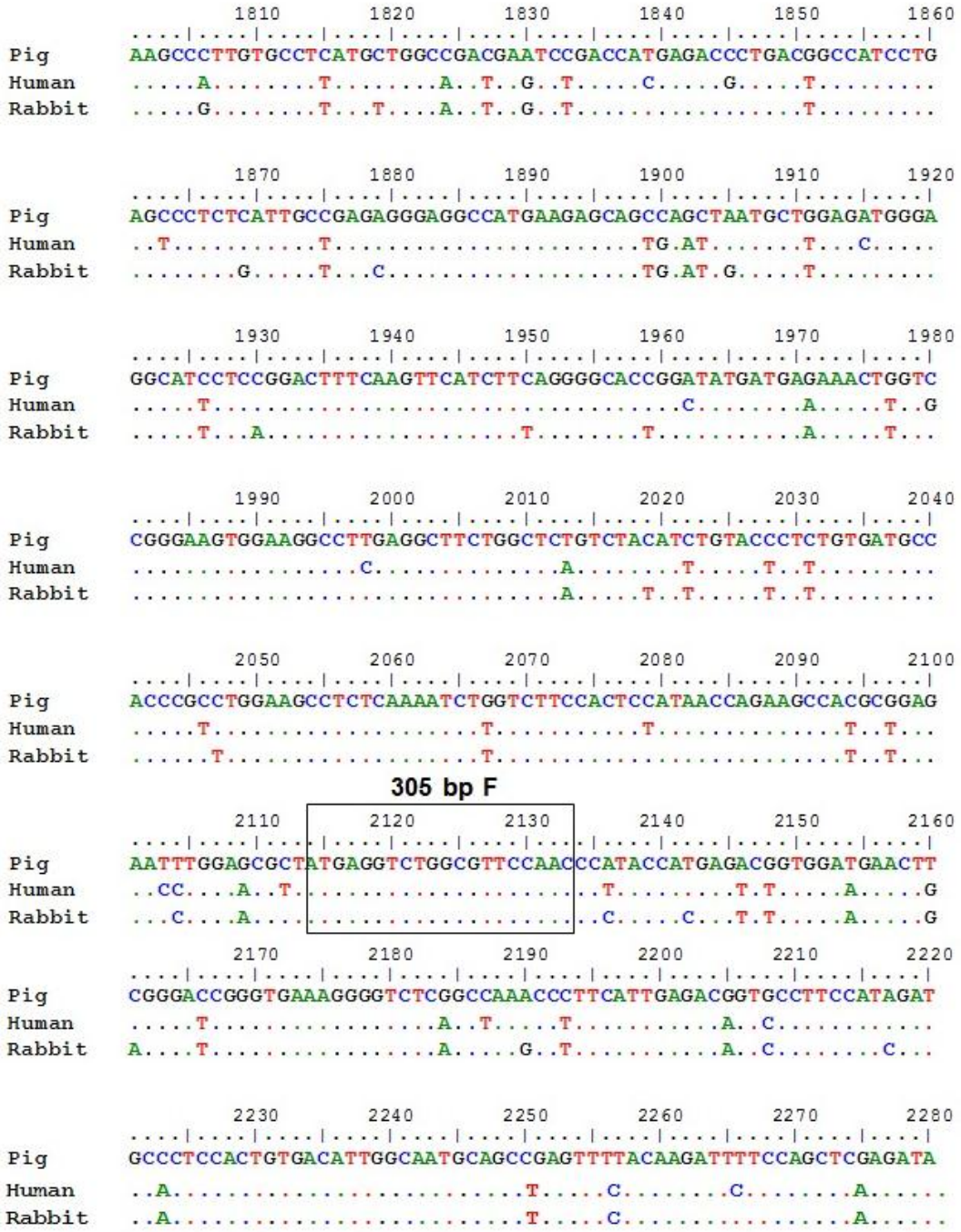
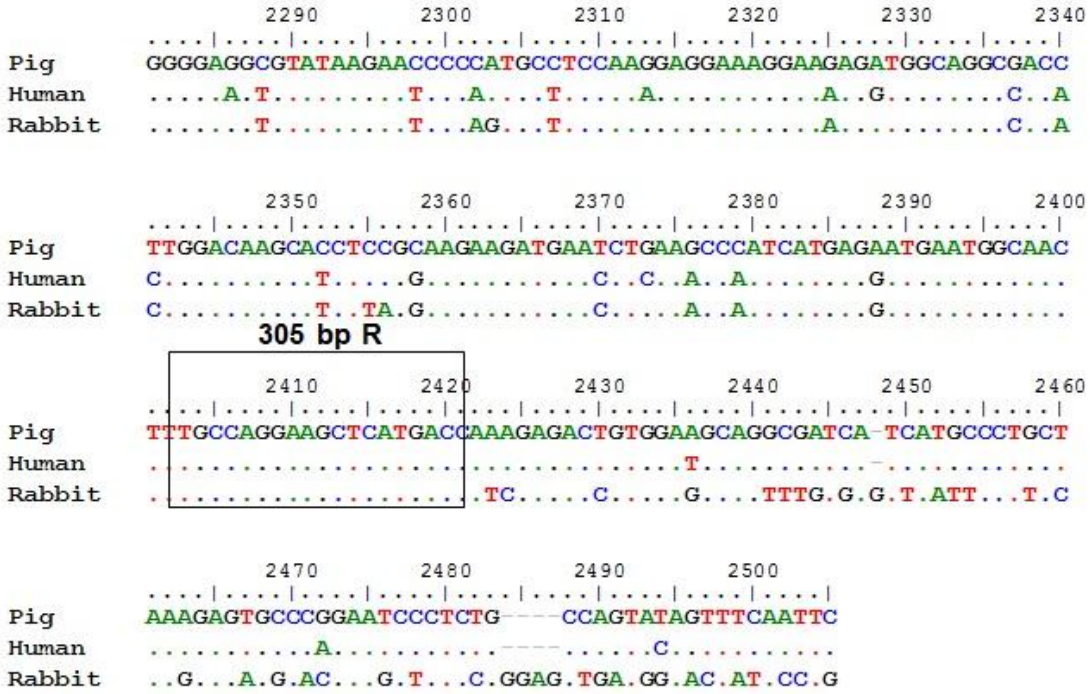
APPENDIX I


384 bp F
384 bp R
Pig
Human
Rabbit


610 620 630 640 650 660
Pig AGGATCAGCAGCAAGGAACTGATGAAGAAGATCGCCAACTGCGGTCAGATACATCTTAGC
Human .....TG.C.....A..A.....T
Rabbit .....C.....GG.C.....T...T.....A..A.....T
670 680 690 700 710 720
Pig CCCAAGCTCCTGGCAGTGGACTTCCCGGCGCACTTTGTGAAATCTATCTCCTGCCAGATT
Human A.....T.....A.A.....C.....C
Rabbit A.....T.....
730 740 750 760 770 780
Pig TGTGAACACATCCTGGCCGACCCGGTGGAGACCAGCTGCAAGCATGTGTTTTGCAGGATC
Human .....T.....T.....T.....A...T.....C.....C..G..
Rabbit .....G.....T.....T..T..T.....T.....T.....
70 bp F
790 800 810 820 830 840
Pig TGCATTCTCAGGTGCCTCAAAGTCATGGGCAGCAGTTGTCCCTCTTGCCACTATCCCTGT
Human .....A.....TA.....GA.....A..C
Rabbit .....G.....TA...C..T.....T..A.....A..C
70 bp R
850 860 870 880 890 900
Pig TTTCCTACTGACCTGGAGAGTCCAGTGAAGTCTTTTCTGAGCATCTTGAATACCCTGATG
Human ..C.....C.....G.....T.....
Rabbit ..C.....A..C.....T.T.....CT..T....A
910 920 930 940 950 960
Pig GTGAAATGCCCAGCAAAGGAGTGCAACGAGGAGATCAGCTTGGAAAAATATAATCACCAT
Human .....T.....A.....T.....G....T.....C
Rabbit .....TT.....CCA.....T.....G.....C.....C
970 980 990 1000 1010 1020
Pig ATCTCAAGCCACAAGGAGTCGAAGGAGACATTTGTGCATATTAATAAAGGGGGCCGGCCC
Human .....T.....A..A..A....TT.....C.....
Rabbit G.T.....T.....A..A..A.GA..T..T.....
1030 1040 1050 1060 1070 1080
Pig CGCCAGCATCTCCTGTCCCTGACGCGGAGGGCTCAGAAACACCGTCTGAGGGAGCTCAAG
Human .....A.....T.....G.....T.....A.....G.....G.....
Rabbit ..T..A.....A..G.....C.....A.....G.....G.....G...
1090 1100 1110 1120 1130 1140
Pig CTGCAAGTCAAGGCCTTCGCCGACAAAGAAGAAGGTGGCGATGTGAAGTCAGTGTGCCTG
Human .....A.....T..T.....A.....C.....A..
Rabbit T.A.....T..T..T.....A.....T.....
1150 1160 1170 1180 1190 1200
Pig ACCTTGTTCCTGCTAGTGCTGAGGGCGAGGAATGAGCACAGACAAGCTGACGAGCTGGAG
Human .....G.CT.....G.....T.....
Rabbit .....T..G.CC...C....A.....T.....


194 bp F
194 bp R
Pig
Human
Rabbit


1810 1820 1830 1840 1850 1860
Pig AAGCCCTTGTGCCTCATGCTGGCCGACGAATCCGACCATGAGACCCTGACGGCCATCCTG
Human .....A.....T.....A..T..G..T.....C.....G.....T.....
Rabbit .....G.....T...T....A..T..G..T.....T.....
1870 1880 1890 1900 1910 1920
Pig AGCCCTCTCATTGCCGAGAGGGAGGCCATGAAGAGCAGCCAGCTAATGCTGGAGATGGGA
Human ..T.....T.....TG.AT.....T...C.....
Rabbit .....G.....T...C.....TG.AT.G.....T.....
1930 1940 1950 1960 1970 1980
Pig GGCATCCTCCGGACTTTCAAGTTCATCTTCAGGGGCACCGGATATGATGAGAAACTGGTC
Human .....T.....C.....A.....T..G
Rabbit .....T...A.....T.....T.....A.....T...
1990 2000 2010 2020 2030 2040
Pig CGGGAAGTGGAAGGCCTTGAGGCTTCTGGCTCTGTCTACATCTGTACCCTCTGTGATGCC
Human .....C.....A.....T.....T..T.....
Rabbit .....A.....T..T.....T..T.....
2050 2060 2070 2080 2090 2100
Pig ACCCGCCTGGAAGCCTCTCAAAATCTGGTCTTCCACTCCATAACCAGAAGCCACGCGGAG
Human .....T.....T.....T.....T..T...
Rabbit .....T.....T.....T..T...
305 bp F
2110 2120 2130 2140 2150 2160
Pig AATTTGGAGCGCTATGAGGTCTGGCGTTCCAACCCATACCATGAGACGGTGGATGAACTT
Human ..CC....A..T.....T.....T.T.....A.....G
Rabbit ...C....A.....C.....C...T.T.....A.....G
2170 2180 2190 2200 2210 2220
Pig CGGGACCGGGTGAAAGGGGTCTCGGCCAAACCCTTCATTGAGACGGTGCCTTCCATAGAT
Human .....T.....A..T.....T.....A..C.....
Rabbit A....T.....A.....G..T.....A..C.....C...
2230 2240 2250 2260 2270 2280
Pig GCCCTCCACTGTGACATTGGCAATGCAGCCGAGTTTTACAAGATTTTCCAGCTCGAGATA
Human ..A.....T.....C.....C.....A.....
Rabbit ..A.....T.....C.....A.....



Appendix I Shown above is the recombination activating gene (RAG-1) showing conserved regions for 70 bp, 194 bp, 305 bp and 384 bp in pig, rabbit and human. The dots shows the conserved regions.

APPENDIX II

A. Courses Attended

- Excel for Researchers
- Central induction event
- Reflective skills, presentation and conference techniques
- Analytical methods
- Analytical methods
- Time and Project Management
- Reference manager and Bibliographic
- Teambuilding
- Health, safety and Ethics
- Technical/Scientific Writing
- Reflective Skills
- Legal, business and other aspects
- The theory/Philosophy of Ethics
- Practical Ethics and Governance
- Teaching tool kits
- SPSS2
- Careers in Academia Workshop, Birmingham
- Data protection
- Research techniques for life sciences, UCLan
- Gene Mapper software tutorial
- A guide to poster design
- Scientific Writing, University of Sheffield, UK
- GRAD School, University of Essex, UK.
- Applied Biosystems Symposium on application of Y- Chromosome analysis in Court. Vienna, Austria.

- Public lecturer on Genetic Fingerprinting, Past, Present and Future delivered by Professor Sir Alec Jeffrey, Lancaster Town Hall, UK.

B. Membership of National and International Research Societies

- American Academy of Forensic Sciences (AAFS), USA.
- European Society of Human Genetics (ESHG), Vienna, Austria.
- International Society for Applied Biological Sciences, (ISABS), Czech Republic.
- International Society for Forensic Genetics **(**ISFG), Germany.
- British Association for Human Identification, (BAHID), UK.
- The Genetics Society, Edinburgh, UK**.**
- Postgraduate research society, University of Central Lancashire, Preston, UK.

C. Conferences Attended

International Conferences

- 24th World Congress of the International Society for Forensic Genetics (ISFG), August 29th-September 3, 2011, University of Vienna, Austria-Poster presentation.

- European Society of Human Genetics (ESHG) conference, May 28- 31, Amsterdam, the Netherlands-Poster presentation.

- 8th International Livestock & Poultry Congress, March 27-28, 2007, at Aiwan-e - Iqbal Complex, Lahore, Pakistan

National Conferences and Meetings

- Annual Science and Technology conferences, 6th-8th June, 2011, University of Central Lancashire, Preston, UK-Poster presentation.
- Annual Science and Technology conference, 30th June 2010, University of Central Lancashire, Preston, UK- Poster presentation..
- Annual Science and Technology conference, 30th June 2009, University of Central Lancashire, Preston, UK.
- Genetics Society, 12th November, 2010, the Royal Society, London, UK.

- Launch conference of Cancer at UCLan, Tuesday, 24th May, 2011, University of Central Lancashire, Preston, UK.

D. Publications

Abstract Published in National and Internal Conference Proceedings

The research abstract published in the following conferences

- European Journal of Human Genetics and ESHG conferences, June 2012, Germany. (Control No. 2012-A-2304-ESHG).
- American Academy of Forensic Sciences (AAFS), 64th annual scientific meeting in Atlanta, Georgia, February, 20 - 25, 2012, USA.
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DNA degradation in post-mortem soft muscle tissues in relation to accumulated degree-days (ADD)

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ABSTRACT

In order to assess DNA degradation in the model organisms chosen (pig and rabbit), two nuclear genes, Connexin 43 and RAG-1, were aligned to identify conserved regions. Primers were designed to amplify 70 bp, 194 bp, 305 bp and 384 bp amplicons. The primers were also designed to amplify human DNA, which allowed the use of commercially purchased DNA standards to be used as controls. Following DNA extraction PCR analysis was performed using the four primers sets in a multiplex (4-plex): the PCR was optimised so that it worked over a wide range of template amounts (0.1–75.83 ng). The multiplex (4-plex) PCR was found to work efficiently in triplicate samples with all three species down to 0.3 ng of DNA template. This multiplex has been used to assess whether DNA degradation can be predicted by accumulated degree-days (ADD), which provides a measure of both time and temperature. Full 4-plex profiles were generated until day 7 (112 ADD) from whole carcasses and body fragments. Future work will include; development of real-time PCR quantification assays, DNA fragment analysis and DNA preservation.

1. Introduction

Extracting DNA from muscle tissue can be important for forensic investigations, in particular, when attempting to use DNA profiling to identify human remains. DNA degradation in biological samples starts rapidly after death with fragmentation of DNA caused by endogenous nuclease activity and hydrolytic attack [1]. The persistence of DNA in soft muscle tissue has been shown to correlate with ADD, which is the cumulative total of the average daily temperatures. One study demonstrated that DNA survives for 101 ADD in summer and 138 ADD in winter. However, these observations were based on limited data [2]. Therefore, further research on ADD will be helpful for a better understanding of the likelihood of successfully retrieving DNA from post-mortem muscle tissue.

2. Materials and methods

2.1. Experimental design

To access DNA degradation in soft muscle tissues, 60 rabbit carcasses were used in the first pilot study: 12 triplicate sets of rabbit whole carcasses (36 carcasses) were placed in direct contact with the ground and covered by a wire mesh to prevent scavenger access; 12 triplicate sets of rear legs (36 rear legs) were cut from 18 rabbits and put alongside the whole carcasses. This experiment was performed at the University of Central Lancashire (UCLan) field site called TRACES—the Taphonomic Research in Anthropology: Centre for Experimental Studies. The environmental temperature and humidity was recorded every hour using a data-logger.

2.2. Sample collection

Muscle tissue samples (1 cm^3) were collected from whole carcasses, and body fragments in triplicate, starting from day zero until no soft tissue remained and stored at −20 °C until processing. Samples collected at day zero were used as positive controls.

2.3. DNA extraction

DNA extraction was carried out using DNeasy® blood and tissue kit according to the manufacturer's instructions.

2.4. Primer design, optimisation and multiplexing

Two nuclear genes, Connexin 43 and RAG-1, were aligned to identify conserved regions in human, pig and rabbit. Primers were designed to amplify 70 bp, 194 bp, 305 bp and 384 bp amplicons. The singleplex and multiplex (4-plex) PCR was optimised to work with different primer concentrations (0.05–0.4 μM).

3. Results

Multiplex (4-plex) PCR was optimised successfully on ABI 310 Genetic Analyser for rabbit DNA samples and also worked with pig

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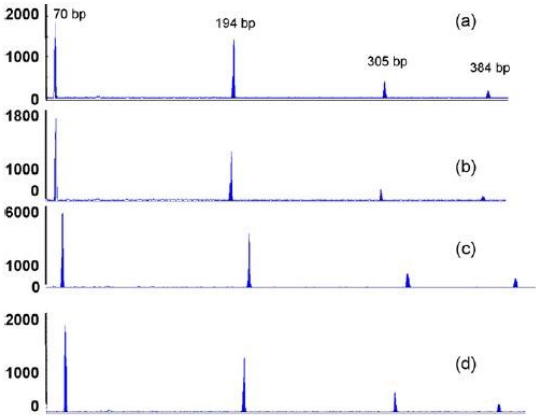

Fig. 1. Electropherograms from (a) body fragments at 13 ADD, (b) 112 ADD, (c) whole bodies at 13 ADD and (d) 112 ADD.

and human DNA. The multiplex profiles were obtained for DNA samples extracted from muscle taken from fragmented bodies at 13 ADD and 112 ADD (Fig. 1a and b). Similarly DNA extracted from muscle taken from whole bodies gave full 4-plex profiles at day 1 (13 ADD) and day 7 (112 ADD) (Fig. 1c and d). After day seven no soft tissues remained in either fragmented or whole bodies.

4. Discussion

This research was conducted to provide empirical evidence to supplement the advice available to the forensic community for the collection of muscle tissues for forensic analysis; this type of collection is normally carried out to determine the identity of individuals following mass disasters, such as plane crashes or natural disasters [3].

The primer sets developed allows us to assess the amount of intact DNA in rabbit, pig and human. The data showed that DNA profiles (4-plex) could be recovered from samples several days post-mortem, for as long as soft muscle tissues remained. This supports the collection of soft muscle tissue whilst it is present. The data presented are all from a temperate environment; it is not clear whether DNA persistence will be present for similar ADD in warmer climates [4].

Conflict of interest

None.

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