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Use of Diverse Extraction Protocols to Decide the Integrity of Deoxyribonucleic Acid (DNA) Samples Extracted from Bovine Bone Samples of Different Ages obtained from an Abattoir in Ikorodu, Lagos State, Nigeria

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ABSTRACT: Techniques for the identification human and non-human biological samples are developing at very high rates with the advent of different DNA extraction methods and polymerase chain reaction (PCR) based assays. This study aimed at using different extraction protocols to determine the integrity of DNA samples extracted from bovine bone samples of different ages collected from abattoir in Ikorodu, Lagos State, Nigeria. The DNA was extracted using CTAB, PCI protocols and a DNA kit (Quick DNA MiniPrep Plus Kit). Bovine mtDNA fragment containing the gene encoding ATPase 8 was amplified via Polymerase Chain Reaction (PCR). The PCR products were analysed on 1.8% agarose gel. It was observed that the DNA samples extracted using the PCI method at 48 h incubation time had the highest purity (1.68) and concentration (336 ng/µl) compared to other extraction methods employed in the study. However, DNA kit extracted samples had mean ± SE purity (1.52 ± 0-05) and concentration (192.25 ± 31.41 ng/µl) values that were higher than CTAB protocol values but lower than PCI protocol values. All isolated DNA samples were PCR-worthy and thus yielded PCR products which were within the expected amplicon size of 126-bp. All DNA extraction protocols employed in this study are stable and efficient for use in the identification of aged non- human bones. This study also revealed that these protocols can be used to isolate PCR amplifiable DNA from old bones.

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Old and new skeletal materials are among the most challenging biological samples for successful genotyping in forensic genetic investigations (Burger *et al*. 1999). Bone material is one of the hardest biological samples used for extraction of DNA. The major objective of molecular tests is to evaluate the concentration and quality of DNA, and these outcomes are of significant importance in subsequent analyses (Pizoń and Jadczak, 2017). The preservation of the DNA in skeletal remains can be affected by temperature, humidity, pH and geochemical properties of the soil and microbial proliferation (Burger *et al*. 1999). The inability to amplify DNA from skeletal remains samples may also be as a result of the presence of inhibitory low molecular weight compounds. These compounds which are co-extracted with the DNA inhibit DNA polymerase in the polymerase chain reaction (Keyser-Tracqui and Ludes, 2005). DNA profiling is considered the core of the human and non-human forensic identification process, thus, in some cases, bone samples are the only accessible biological samples (Abuidrees *et al*. 2016**).** Wildlife forensic science is science that is applied to legal questions involving wildlife crimes and involves the use of molecular biology techniques which includes DNA profiling and sequencing (Sankhla *et al*. 2016). Wildlife forensics relates the suspect, victim and crime scene in concert in a triangular manner with the physical evidence recovered from the crime **(**Siegel *et al*. 2000**).** Wildlife forensics is an emerging field that provides tools that help identify DNA of animal origin. Most forensic laboratories typically do not carry out

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genetic tests with animal DNA, since related methods have not yet been standardized as it is the case with human genetic material (Alaeddini, 2010). Cattle rustling and the violent conflict linked to it has been reported in some parts of Africa (Kaimba *et al*. 2011; Greiner 2013). Cattle rustling has become a major concern and crime in Nigeria, with the northern part being the worst hit (Olaniyan and Yahaya, 2016). This has led to the stealing of numerous number of cattle, destruction of lives and property in the northern parts of Nigeria. This has also significantly affected the security conditions of these area as evidenced in the daily reports of clashes that occur between herders and farmers in these areas. Cattle rustling affects efficient breeding of cattle using methods such of artificial insemination and new breeding genetic techniques (Delgado, 2014).

This study aimed at using different extraction protocols to determine the integrity of DNA samples extracted from bovine bone samples of different ages collected from abattoir in Ikorodu, Lagos State, Nigeria.

MATERIALS AND METHODS

*Bone Samples Collection and Processing***:** Excavated cow bones aged 6 months, 1 year, 1 year and 6 months and 2 years were obtained from an abattoir in Ikorodu, Lagos.

Table 1: Accession number given to samples according to the sample age, incubation period and protocols for DNA extraction

Serial No.	Accession No.	Incubation Period	Sample Age
1	$1C_1$	24 h	0.5 year
2	$2C_1$	24 h	1.5 year
3	$3C_1$	24 h	1 year
4	$4C_1$	24 h	2 years
5	$1C_2$	48 h	0.5 year
6	$2C_2$	48 h	1.5 year
7	$3C_2$	48 h	1 year
8	$4C_2$	48 h	2 years
9	$1P_1$	24 h	0.5 year
10	$2P_1$	24 h	1.5 year
11	$3P_1$	24 h	1 year
12	$4P_1$	24 h	2 years
13	$1P_2$	48 h	0.5 year
14	$2P_2$	48 h	1.5 year
15	$3P_2$	48 h	1 year
16	$4P_2$	48 h	2 years
17	1K	2 h 30 min	0.5 year
18	2K	2 h 30 min	1.5 years
19	3K	2 h 30 min	1 year
20	4K	2 h 30 min	2 years

Key: *1K - 4K = after 2 All DNA samples extracted h 30 min incubation period using kits;*
$1C_1$ - $4C_1$= All DNA samples extracted after 24h incubation period using CetylTrimethylAmmonium Bromide (CTAB) extraction method; $1P_1$-$4P_1$= All DNA samples extracted after 24 h incubation period using Phenol Chloroform Isopropanol (PCI) Extraction Method;
$1C_2$ - $4C_2$= All DNA samples extracted after 48h incubation period using CetylTrimethylAmmonium Bromide (CTAB) extraction method;
1 P_2-$4P_2$= All DNA samples extracted after 48 h incubation period using Phenol Chloroform Isopropanol (PCI) Extraction Method

The estimated ages of each bone sample were provided by local butchers. Before the extraction process, the bones were washed in 8% sodium hypochlorite (bleach), air-dried and ground into a fine powder. The samples were to be subjected to three methods of extraction, which are Phenol: Chloroform: Isoamylalcohol (25:24:1), ascribed **P**, Cetyl trimethyl ammonium bromide (CTAB) + **C**hloroform: Isoamylalcohol (24:1) ascribed **C** and DNA kit (Zymo Research) which was also ascribed **K.** The incubation time for the PCI and CTAB methods were varied, samples were incubated for 24 h and 48 h respectively. The samples extracted using the DNA kits were incubated for 2 h 30 min following the manufacturer's instructions. Approximately 0.5 g of each ground sample was weighed into sample bottles and labelled as shown in Table 1.

DNA EXTRACTION

Phenol Chloroform Isopropanol (PCI) Extraction Method: Approximately 0.5 gram of each ground bone sample was put in 2 ml of 0.5M EDTA (pH 8.0) and incubated at 56 °C for 24 h and 48 h, respectively. The supernatant was then removed and the remaining powder was washed twice with distilled water and once with Tris-EDTA-NaCl (TEN) buffer, which consisted of 10 mM Tris-HCl (pH 8.0), 1mM EDTA (pH 8.0), and 100 mM NaCl. Thereafter, 3 ml of extraction buffer (TEN, 0.5 % SDS, 0.5 mg/ml proteinase K) was added and the sample tubes were incubated at 56 °C for 4 h. Two hundred microliters of the supernatant was pipetted into a new 1.5 ml micro-centrifuge tube and equal volume of phenol: chloroform (1:1, v/v) was added before centrifugation at 13,000 rpm for 5 minutes. The supernatant (200 µl) was transferred into a new tube and 1,200 µl of chloroform was added before centrifugation at 13,000 rpm for 5 minutes. The supernatant was placed in a new tube and 60 µl of 3 M Sodium Acetate and 600 µl of isopropanol were added before centrifugation at 13,000 rpm for 30 minutes. Then, the supernatant was removed and the remaining DNA was dried at 37 °C before adding to 50 µl of distilled water.

CetylTrimethylAmmonium Bromide (CTAB) Extraction Method: Approximately 0.5 g of each bone sample powder was placed in a mortar and 1ml of CTAB extraction buffer (preheated at 65 ºC in a water bath) was added and crushed using a pestle to make a paste, additional 1ml was added and the solution were allowed to incubate for 24 and 48 hours as assigned at room temperature. After incubation, the samples were incubated at 65 ºC for 1 h and vortexed for 5 min, 0.5 ml of the samples was transferred into labeled 1.5 ml Eppendorf tubes, five hundred microliters of 24:1 Chloroform-Isoamyl Alcohol was added and mixed

well to form an emulsion by shaking. The samples were centrifuged for 10 minutes at 15,000 rpm, the aqueous phase was pipetted off into new labeled 1.5 ml Eppendorf tubes, 300 µl of ice-cold isopropanol was added to each tube and allowed to sit in the freezer for 1 hour. The samples were later centrifuged at 15,000 rpm for 3 min, and the liquid was pipetted off carefully, leaving the DNA pellet at the bottom. Afterwards, 700 µl of cold 70 % Ethanol was added to each tube and mixed by inverting once and centrifuged at 15,000 rpm for 1 min, the liquid was pipette off. Additionally, 700 µl of cold 95 % Ethanol was added to each tube and inverted once to mix, then centrifuged at 15,000 rpm for 1 min. The pellets were allowed to air-dry and were re-suspended with 100 µl of distilled water and stored at -20 ºC.

Quick-DNA MiniPrep Plus Kit Extraction Method: Deoxyribonucleic acid samples were extracted from bone powder samples collected by using Quick-DNA Miniprep Plus Kit purchased from Zymo Research through Inqaba, Ibadan, Oyo State, Nigeria. Approximately 25 mg of the bone powder sample was collected into a microcentrifuge tube and 95 µl of water, 95 µl of Solid Tissue Buffer (Blue) and 10 µl of Protinase K were added. The microcentrifuge tube with the bone powder sample and the solution was thoroughly mixed and incubated at 55 °C for 3 hours. The insoluble debris was removed by centrifuging at 12,000 rpm for 1 minutes and transferred into a clean microcentrifuge tube. Double volume (supernatant) Genomic Buffer was added to the supernatant and mixed thoroughly. The mixture was transferred to a Zymo-SpinTM IIC-XL Column in a Collection Tube, centrifuged at 12,000 rpm for 1 minute and the collection tube was discarded with the flow through. Four-hundred microliters DNA pre-wash buffer was added to the column in a new collection tube and centrifuged for 1 minutes at 12,000 rpm. The collection tube was emptied. Seven-hundred microliter g-DNA wash buffer was added and centrifuged for 1 minute at 12,000 rpm. The collection was emptied and 200 µl g-DNA wash buffer was added and centrifuged for 1 minute at 12,000 rpm. The collection tube was discarded with the flow through. The sample was then transferred to a clean microcentrifuge tube and approximately 50 µl DNA elution buffer was added to elute the DNA. It was then incubated for 5 minutes and then centrifuged for 1 minute at 12,000 rpm and stored at -20°C.

*Spectrophotometry***:** Spectrophotometry of the extracted DNA samples was carried out by exposing the samples to UV wavelengths 260 µm and 280 µm using a spectrophotometer (Eppendorf BioPhotometer plus). The optical density of the samples was observed and recorded.

Polymerase Chain Reaction (PCR): The following primers used were developed and validated for the specific detection of bovine DNA: forward, 5' – ACAATGATCTTATCAATATTCTTG-3'; reverse, 5'-CCTTCAAGGGGTGTTTTGTTTTAA-3'. This primer pair amplifies a 126-bp bovine mtDNA fragment containing the gene encoding ATPase 8. The PCR was performed using EdvoCycler (EVTC 22OV 5A) by adding 22 µl of distilled water in sterile tubes, 25 µl of 2 X PCR master mix (Biolabs, New England) and 1 µl (10 µM) of forward primers, 1 µl (10 µM) of reverse primers and 1 µl of template DNA. The reactions were initiated with denaturation at 94 ºC for 5 minutes. For each cycle, the cycling parameters included denaturation at 95 ºC for 45 seconds, annealing at 63 ºC for 45 seconds, an extension for 60 seconds at 72 ºC. The PCR was performed for a total of 30 cycles with a final elongation step at 72 ºC for 10 minutes, and a final hold temperature of 4 ºC. The PCR products were stored at -20 ºC until further analysis on agarose gel by electrophoresis.

Electrophoresis: Approximately 1.8 g of the agarose powder was dissolved in 100 ml of 0.5 x TBE solution by boiling in a microwave oven. It was cooled to approximately 60 °C and ethidium bromide was then added. The mixture was mixed gently and a gel thickness of about 4-5 mm was obtained by pouring into a taped electrophoresis tank (Bio-rad, power pack) with the comb and allowed to solidify for 20 minutes. The rubber dam and the comb were removed carefully to avoid damaging the gel. The tray was placed in a rig and 0.5 x TBE gel buffer was poured into the gel rig to cover the gel by at least 0.5 cm. One microliter of 10M loading dye was mixed with 10 µl of the sample. The mixtures were loaded into the wells. Electrophoresis was then initiated at 60-100 V until the loading dye has migrated three quarter of the gel. Bands of the DNA Plasmid on the agarose gel containing the extracted samples were observed through transillumination with the use of ultra-violet ray transilluminator (Fisher Biotech).

Statistical Analysis: The data obtained were subjected to Microsoft excel and *Statistical Package for The Social Sciences* (SPSS) for descriptive analysis using Excel Microsoft Office 2013 and IBM SPSS Statistics v23 x86 applications respectively.

RESULTS AND DISCUSSION

Spectrophotometry check was carried out to determine the purity and molecular concentration of the extracted DNA samples. At 24 h incubation period, PCI protocol

DNA samples were observed to have a mean ± SE purity of 1.36 ± 0.07 with a mean ± SE concentration of 30 ± 4.42 ng/µl while CTAB protocol DNA samples had a mean ± SE purity of 1.21 ± 0.05 with a mean ± SE concentration of 20.5 ± 4.21 ng/µl. At 48 h incubation period, PCI protocol DNA samples were observed to have a mean ± SE purity of 1.66 ±0.01 with a mean ± SE concentration of 315.50 ±11.62 ng/µl while CTAB protocol DNA samples had a mean ± SE purity of 1.44 ± 0.07 with a mean ± SE concentration of 21.75 ± 8.76 ng/µl. However, the DNA kit DNA samples' mean ± SE purity (1.52 ± 0-05) and concentration (192.25 ± 31.41 ng/µl) values were higher than CTAB protocol values but lower than PCI protocol values (Table 2). Pearson's correlation analysis was employed to determine the correlation among the spectrophotometric parameters of the three protocols employed in this study. This analysis revealed that there was a positive correlation between Purity-PCI (48 h) and Conc.-CTAB (48 h) (ng/µl) at 0.05 level of significance (Table 3). However, that there was no correlation between other spectrophotometric parameters of the three protocols employed in this study ($p>0.05$).

Table 2: Descriptive statistics of spectrophotometric readings

Protocol	Parameters	Minimum	Maximum	Mean ±S.E	Variance
CTAB (24 h)	Conc.(ng/µl)	12.00	30.00	20.50 ± 4.21	71.00
	Purity	1.09	1.32	1.21 ± 0.05	0.01
PCI (24 h)	Conc.(ng/µl)	17.00	36.00	30.00 ± 4.42	78.00
	Purity	1.21	1.51	1.36 ± 0.07	0.02
CTAB (48 h)	Conc.(ng/µl)	12.00	48.00	21.75 ± 8.76	306.92
	Purity	1.23	1.53	1.44 ± 0.07	0.02
PCI (48 h)	Conc.(ng/µl)	286.00	336.00	315.50 ±11.62	539.67
	Purity	1.63	1.68	1.6625 ±0.01	0.00
DNA Kit	Conc. (ng/µl)	123.00	262.00	192.25 ± 31.41	3946.25
	Purity	1.43	1.65	1.52 ± 0.05	0.01

***Key**: CTAB= CetylTrimethylAmmonium Bromide (CTAB) extraction method; PCI=Phenol Chloroform Isopropanol (PCI) Extraction Method*

Table 3: Correlation output of spectrophotometric parameters of the three protocols

Parameter	Purity PCI (24 h)	Conc. PCI (24h) (ng/µl)	Purity CTAB (24 h)	Conc. CTAB (24h) (ng/µl)	Purity PCI (48 h)	Conc. PCI (48h) (ng/µl)	Purity CTAB (48 h)	Conc. CTAB (48h) (ng/µl)	Purity Kit	Conc. Kit (ng/µl)
Purity PCI (24 h)	1	-0.4	0.8	0.28	0.56	0.29	-0.63	-0.44	0.42	0.1
		0.6	0.2	0.72	0.44	0.72	0.37	0.56	0.58	0.9
Conc. PCI (24 h) (ng/µl)	-.04	1	-0.8	0.6	-0.05	0.39	-0.45	0.19	0.66	0.83
	0.6		0.2	0.4	0.95	0.61	0.55	0.81	0.34	0.17
Purity CTAB (24 h)	0.8	-0.8	1	-0.01	0.12	-0.28	-0.08	-0.1	-0.09	-0.51
	0.2	0.2		0.99	0.88	0.72	0.92	0.9	0.91	0.49
Conc. CTAB (24 h) (ng/µl)	0.28	0.6	-0.01	1	-0.15	0.06	-0.72	0.4	0.9	0.58
	0.72	0.4	0.99		0.85	0.94	0.28	0.6	0.1	0.42
Purity PCI (48 h)	0.56	-0.05	0.12	-0.15	1	0.9	-0.58	-0.97*	0.28	0.49
	.044	0.95	0.88	0.85		0.1	0.42	0.03	0.73	0.52
Conc. PCI (48 h) (ng/µl)	0.29	0.39	-0.28	0.06	0.9	1	-0.68	-0.82	0.48	0.79
	0.72	0.61	0.72	0.94	0.1		0.32	0.18	0.52	0.21
Purity CTAB 48 h	-0.63	-0.45	-0.08	-0.72	-0.58	-0.68	1	0.35	-0.94	-0.82
	0.37	0.55	0.92	0.28	0.42	0.32		0.65	0.06	0.18
Conc. CTAB 48 h (ng/µl)	-0.44	0.19	-0.1	0.4	-0.97*	-0.82	0.35	1	-0.03	-0.31
	0.56	0.81	0.9	0.6	0.03	0.18	0.65		0.97	0.69
Purity Kit	0.42	0.66	-0.09	0.9	0.28	0.48	-0.94	-0.03	1	0.84
	0.58	0.34	0.91	0.1	0.73	0.52	0.06	0.97		0.17
Conc. Kit (ng/µl)	0.1	0.83	-0.51	0.58	0.49	0.79	-0.82	-0.31	0.84	1
	0.9	0.17	0.49	0.42	0.52	0.21	0.18	0.69	0.17	

***Key**: CTAB= CetylTrimethylAmmonium Bromide (CTAB) extraction method; PCI=Phenol Chloroform Isopropanol (PCI) Extraction Method*

Plate 1 shows the gel image of all the PCR products of DNA samples extracted with the three different protocols used in this study at 24 h incubation period. The bands on lanes indicate the samples for each protocol while band on lane L indicates the ladder. Plate 2 shows the gel image of all the PCR products of DNA samples extracted with CTAB and PCI protocols respectively at 48 h incubation period. Lanes $1P_2$ to $4P_2$ indicate all PCI samples, lanes $1C_2$ to $4C_2$ indicate all CTAB samples, while lane L indicates the DNA ladder. Several DNA extractions protocols have been developed for the isolation of DNA from skeletal remains for their nuclear or mitochondrial typing (Loreille *et al*. 2007; Irwin *et al*. 2012). Most of these protocols which require considerable amounts of bone materials are laborious (Draus-Barini *et al*. 2013). The purity and concentration of nucleic acids is determined by spectrophotometric measurement of the quantity of

UV light absorbed by DNA nucleobases and derived from A260/A280 ratio.

Plate 1: Gel Electrophoresis image of the PCR products of DNA samples extracted with different protocols at 24 h incubation period.

***Key**: L= ladder; 1P$_1$= 0.5 year old PCI sample; 2P$_1$=1.5 year old PCI sample; 3P$_1$=1 year old PCI sample; 4P$_1$= 2 year old PCI sample; 1C$_1$ = 0.5 year old CTAB sample; 2C$_1$= 1.5 year old CTAB sample; 3C$_1$= 1 year old CTAB sample; 4C$_1$= 2 year old CTAB sample; 1K= 0.5 year old Kit sample; 2K=1.5 year old Kit sample; 3K= 1 year old Kit sample; 4K= 2 year old Kit sample*

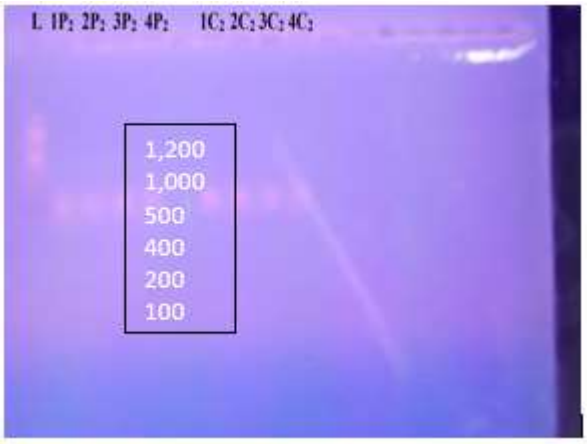

Plate 2: Gel Electrophoresis image of the PCR products of DNA samples extracted with CTAB and PCI protocols at 48 h incubation time.

***Key**: L= ladder; 1P$_2$= 0.5 year old PCI sample; 2P$_2$=1.5 year old, PCI sample; 3P$_2$=1 year old PCI sample; 4P$_2$= 2 year old PCI sample; 1C$_2$ = 0.5 year old CTAB sample; 2C$_2$= 1.5 year old CTAB sample; 3C$_2$= 1 year old CTAB sample; 4C$_2$= 2 year old CTAB sample.*

In this study, it was observed that the DNA samples extracted using the PCI method at 48 h incubation time had the highest purity (1.68) and concentration (336 ng/µl) compared to other extraction methods employed in the study. However, the purity value was below the acceptable purity range of 1.8-2.0. The DNA suitable for molecular analyses has A260/A280 ratio between 1.8 and 2.0 (Davoren *et al.* 2007). Phenol/chloroform/isoamyl alcohol is the most often used DNA extraction protocol in forensic science laboratories (Caputo *et al*. 2013). The organic PCI extraction protocol, though utilizes hazardous chemicals extracts DNA with the high level of purity (Hasan *et al.* 2014). The DNA samples extracted from both 24 hours and 48 hours incubation time using organic PCI protocol were of better quality and concentration compared to the DNA samples extracted using CTAB protocol. Additionally, there was no correlation between most of the spectrophotometric parameters of the three protocols employed in this study ($p>0.05$) except for Purity-PCI (48 h) and Conc.-CTAB (48 h) (ng/µl) that correlated at 0.05 level of significance. This contrasts with the report of Ye *et al.* (2004) who stated that the CTAB DNA extraction yielded better results than the PCI method of DNA extraction. This study also supports the report of Hasan *et al.* (2014) who documented that DNA of high quality can be extracted with complete demineralization without the use of cryogenic or mechanical grinding. From this study, it was also revealed that all extracted DNA samples yielded PCR products which were within the expected amplicon size of 126-bp. This implies that each was suitable for isolating PCR-worthy DNA. This agrees with the findings of Verma *et al*. (2016) who were able to carry out PCR amplification of their desired gene accurately for the identification of Chital (*Axis axis*) species.

Conclusion: The DNA extraction protocols employed in this study are stable, reliable, robust, and efficient for use in the identification of aged non- human bones. Isolation of PCR amplifiable DNA from old bones is a very powerful tool in forensic science as anthropological methods of identification of skeletal remains are not always sufficient.

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