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Forensic Science International: Reports 4 (2021) 100248

Contents lists available at ScienceDirect

Forensic Science International: Reports

journal homepage: www.sciencedirect.com/journal/forensic-science-international-reports

PCR based method for sex estimation from bone samples of unidentified South African fetal remains

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ARTICLE INFO

Keywords:
G6PD
SRY
PCR Sex estimation
Forensic Identification
Genomic DNA isolation
Perinatal remains

ABSTRACT

Fragmentary bone in disaster victim identification is reliant on a robust and objective method. Current methods employing traditional phenotypic analysis for sex estimation of forensic fetal remains are limited and often not possible due to the lack of distinctive cranial and pelvic traits. Thus, a molecular sex estimation method optimized for bone samples would enhance current techniques. Sternal rib samples were dissected from unclaimed perinatal decedents, presenting at varied stages of decomposition and unconfirmed circumstances of death, who were admitted to the Johannesburg Forensic Pathology Service (JFPS) and included in the Johannesburg Forensic Paediatric Collection (JFPC). A molecular protocol was designed and optimized using genomic DNA from SW480 cells and five individuals of unknown provenance and known sex (estimated during medicolegal autopsy) by targeting the X-linked G6PD and Y-linked SRY genes. The sex estimated at autopsy was confirmed using the optimized protocol. Nine (9) unidentified individuals were included and assigned sex based on the method described. The development of a sex typing protocol targeting the G6PD and SRY loci provides a new PCR method for sex estimation of human remains from 25 mg of bone fragment.

1. Introduction

Advances in genetic fingerprinting and molecular assay development for forensic identification are aiding the disciplines of anatomy and physical anthropology. In unique scenarios, post-mortem modification can alter osteology, thereby making traditional biological profiling techniques difficult [3,4,11]. Molecular approaches have the potential to positively identify skeletal remains with respects to sex typing, ancestry and species [20]. As an important contributor to the biological profile, sex estimation can provide insights into population distribution and aid in the identification of forensic remains. The current phenotypic parameters used to estimate the sex of individuals are based on skeletal remains and include the assessment of established morphological and metric sex differences on the pelvic, long bone and cranial skeletal elements in adults. While the fetal ilium has proven to be an appropriate diagnostic element for sex typing due to dynamic nature of its development and growth *in utero* and following birth [13], traditional morphometric anthropological sex estimation from fetal remains is often not possible due to the lack of distinctive morphological features and markers necessary for these analyses. As such, DNA-based sex estimation has become instrumental in identifying fragmentary or fetal remains [19]. The most common genes used in methodologies for sex estimation are sex determining region y (SRY), Zinc finger protein (ZF) and amelogenin (AMEL) genes [3,21]. In South Africa, only the ZF and AMEL genes have been used for sex identification. The amelogenin structure and properties make it a good candidate to use in numerous studies for sex estimation, and is the standard used in forensic profiling STR tests. Amelogenin genes are mostly used with fetal remains where anthropological methods are limited due to the lack of sexually dimorphic osteological landmarks and by the South African police service (SAPS) through the silica extraction method [2].

Historically, two molecular methods of sex estimation were developed from The Raymond Dart collection of Human Skeletons (University of Witwatersrand) using miscellaneous archaeological skeletal material. These methods provided means for sexing of ancient DNA (aDNA) by sex specific single nucleotide polymorphisms (SNPs) and an addition of an indel (6 bp) [9]. DNA was extracted from the cancellous bone from the intercondylar fossa. In line with previously reported protocols, targeting

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https://doi.org/10.1016/j.fsir.2021.100248
Received 21 July 2021; Received in revised form 18 November 2021; Accepted 24 November 2021
Available online 27 November 2021
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the amelogenin gene, the quality of DNA and the taphonomic conditions that the sample was exposed to impact the outcome of assays ([14,18]).

Sex estimation using the amelogenin gene has been demonstrated in infanticide victims from the late Roman era [6] and by Lassen and colleagues [12] who extracted ancient DNA from long bones in Aegerten stillborn and neonatal remains. Ancient DNA was extracted from long bones through an automated phenol/chloroform method and polymerase chain reaction (PCR) amplified with a primer system targeting a part of the amelogenin gene located on the sex chromosomes. Molecular results were compared to the morphometric data. The sex determining region Y (SRY) has also been extensively used with anthropological remains to ascertain sex and confirm morphometric observations. Researchers compared the anatomical method results found on the diameter of the femoral head and femoral mid-shaft circumference of 20 modern Egyptian adults. A multiplex method for DNA sex estimation using the SRY gene was performed, and results compared to anatomical data. The SRY data confirmed deductions made through osteological means [7].

Recently, developments in next generation sequencing (NGS), single nucleotide polymorphisms (SNPs) and proteomics have been applied to the forensic and archaeological context. In many respects, these technologies provide solutions to problems such as PCR inhibitors, degraded DNA samples, mixed DNA samples, ethnic biases, and mutations in primer binding sites [8,16,17] However, these technologies are time consuming and expensive. In addition, further validation of proposed protocols is necessary due to the nature of forensic samples. Low copy number and compromised samples presents obstacles to the application of next generation sequencing [1,10,22]. Forensic genetics still depends heavily on PCR based methods [21]. Therefore, to assist the forensic identification of unknown fetal, perinatal and postnatal skeletal remains, a simple, inexpensive PCR based sex typing protocol from DNA isolated from bone was developed from decedents admitted for medico-legal autopsy.

2. Materials and methods

2.1. Sampling and sample storage

The sample comprised of fourteen (14) perinatal decedents sourced from the Johannesburg Forensic Pathology Service (JFPS) medico-legal mortuary and the Johannesburg Forensic Paediatric Collection (JFPC). Ethical approval was granted through the Rhodes University Ethical Standards committee (RU-HSD-15–05–0004) and the Human Research Ethics committee of the University of the Witwatersrand (HREC-medical: M160280). Human perinatal bilateral rib samples equivalent of 5 × 10 mm were excised from individuals of unknown provenance.

Individuals sourced from the Forensic Pathology services were frozen at the medico-legal mortuary prior to thawing. Rib samples were then isolated from these individuals and placed in a microcentrifuge tube with 1 ml of RNAlater and stored at 4 °C for 24 h prior to − 80 °C storage and nucleic acid extraction and subsequent PCR analyses. Individuals sourced from the Forensic Paediatric Collection were initially fixed in formalin and then stored in the School of Anatomical Sciences for subsequent analysis. Rib samples from these individuals were isolated and placed in a microcentrifuge tube without RNAlater and stored directly at − 80 °C prior extraction.

Preliminary samples were obtained from five individuals of suspected infanticide and known sex (established visually during routine autopsy) were included in the optimization phase of protocol development. Test samples were obtained from nine individuals of unknown sex due to advanced decomposition at autopsy. These individuals were sexed using the protocol developed.

2.2. Genomic (g) DNA isolation

Isolation of SW480 cell line and perinatal genomic DNA (gDNA) (optimization samples and test samples) was conducted using the Quick-gDNA miniprep kit (Zymo Research, USA) as per the manufacturer's instructions. The gDNA purity and quality was confirmed by absorbance at 260 nm using the Nanodrop 2000 spectrophotometer and stored at − 20 °C until required. For formalin fixed perinatal samples, the Quick-DNA FFPE kit (Zymo Research, USA) was used as per the manufacturer's instructions.

2.3. Generation of plasmids for sex typing controls

Glyceraldehyde 3-phosphate dehydrogenase (G6PD) and sex-determining region Y (SRY) primers are given in Table 1. The G6PD primers were designed to amplify a 198 bp fragment, while the SRY primers amplified a 556 bp fragment (Table 1).

To generate plasmids for positive controls in sex typing, genomic DNA (gDNA; 100 ng) was isolated from the SW480 (male) cell line and used as the template for PCR-based amplification of the sex-specific amplicons.

PCR reactions consisted of 10 μM forward and reverse primers (Table 1) and GoTaq mastermix in 25 μl volumes and were subject to an initial denaturation of 2 min at 95 °C, and 30 cycles of 95 °C denaturation for 30 s, a gradient annealing for 30 s at 52 °C, 55.1 °C, 56.9 °C and 60 °C followed by a 72 °C extension for 1 min and a final extension of 72 °C for 5 min. PCR products were visualized by ethidium bromide (10 μg/ml) staining on 0.8% w/v agarose gel in TAE (40 mM Tris base, 20 mM acetic acid and 1 mM ethylenediaminetetraacetic acid [pH 8.0]).

The PCR product was gel purified using Zymoclean DNA gel recovery kit (Zymo Research, USA) as per the manufacturer's instructions and ligated into the pTZ57R vector by TA cloning following the manufacturer's instructions for the InsTAclone PCR cloning kit (ThermoScientific, USA) before transformation into *E. coli* JM109 or DH5α competent cells. Plasmids were extracted from colonies using the Zyppy miniprep plasmid extraction kit (Zymo Research, USA) according to manufacturer's instructions and screened by PCR and restriction digestion analysis using *Hin*dIII and *Eco*R1 (10 U) and analysed by ethidium bromide staining (10 μg/ml) on an 0.8% (w/v) agarose gel in TAE for the presence of the SRY and G6PD inserts. Plasmid sequences were confirmed by Sanger sequencing (Inqaba Biotec, South Africa).

2.4. Sex typing protocol optimization

The sex typing PCR protocol was optimized using gDNA from SW480 cells and the preliminary study samples. Reactions were set up with 100 ng gDNA template, 100 μM primers, GoTaq mastermix (Sigma Aldrich, South Africa) containing 1x reaction Buffer pH 8.5, 400 μM dATP, 400 μM dGTP, 400 μM dCTP, 400 μM dTTP and 4 mM $MgCl_2$) in 50 μl reactions with parameters of initial denaturation for 2 min at 95 °C, followed by 32 cycles of 95 °C for 30 s, 56.9 °C for 30 s and 72 °C extension for 1 min, and then a final extension of 72 °C for 5 min. Results were visualized by an ethidium bromide (10 μg/ml) stained 0.8% w/v agarose gel in TAE (data not shown). This optimized protocol was performed on test samples (nine) of unknown sex at autopsy.

3. Results

3.1. Plasmid controls

For the X chromosome, a PCR product at approximately 200 bp, corresponding to the predicted size of the X-linked G6PD amplicon, was detected in lanes which contained samples from the test reactions (Fig. 1A). The lanes containing control reactions lacking template did not show any bands, suggesting specific amplification of the PCR product. The PCR reactions using the primers designed from the X-linked G6PD gene produced a product (191 bp) at all four annealing temperatures (60, 56.9, 55.1, 52 °C) tested (Fig. 1A). The PCR products were ligated into the pTZ57R plasmid by TA cloning and the resultant

Table 1
Primers designed and used in the protocol.

Gene	Direction	Primer Sequence (5' to 3')	Product Length (bp)	Genbank accession number
G6PD	Forward Reverse	GCAACAGATACAAGAACGTGAAGCT GCCTCGGCTGCCATAAATATAGG	191	NM_001042351.2
SRY	Forward Reverse	CTGGCACCTTTCAATTTTGTCG GTGTGGCTTTCGTACAGTCATC	556	NM_003140.2

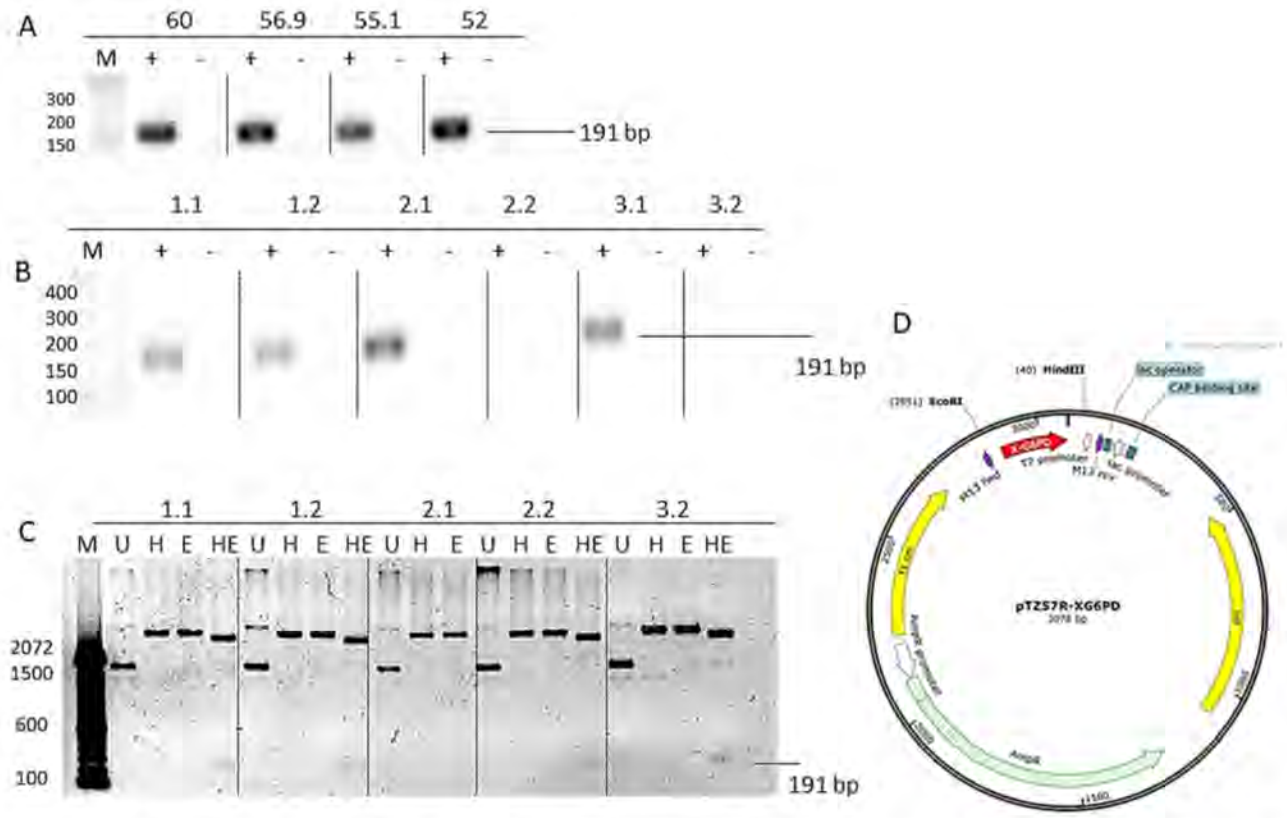


Fig. 1. Confirmation of insertion of G6PD X-linked amplicon into pTZ57R vector for plasmid controls. A) Ethidium bromide stained 0.8% agarose gel of gradient PCR of G6PD primers using annealing temperatures of 60, 56.9, 55.1 and 52 °C and SW480 cell line cDNA as template. + : reaction prepared with template, -: reaction prepared without template. B) Ethidium bromide stained 0.8% agarose gel of PCR using putative pTZ57R-XG6PD containing plasmids as template. pTZ57R-XG6PD clones: 1.1, 1.2, 2.1, 2.2, 3.1 and 3.2 C) Double restriction digestion of putative pTZ57R-XG6PD plasmids with *Eco*RI and *Hind*III enzymes. U: Uncut reactions; H: *Hind*III cut reactions E: *Eco*RI cut reactions; HE: Double digestion reactions; M: DNA ladder. D) Plasmid map of pTZ57R-XG6PD plasmid, generated in Snapgene.

clones screened for the presence of the insert by PCR (Fig. 1B) and restriction digestion (Fig. 1C), followed by agarose gel electrophoresis.

Plasmid DNA in reactions (1.1, 1.2, 2.1 and 3.1) produced an amplicon with the G6PD primers indicating the presence of the G6PD insert in these clones (Fig. 1B). Single and double restriction digestion (*Hind*III and *Eco*RI enzymes) reactions also confirmed the presence of G6PD insert at 191 bp in selected plasmids (Fig. 1C). Plasmid 3.1 which resulted in a positive signal in the PCR assay (Fig. 1B) was excluded from restriction digests due to low DNA concentration. Plasmid 2.1 was not included in a double digest (Fig. 1C) due to low DNA concentration. Plasmids 1.1 and 1.2 were selected and further confirmed by sequencing. A plasmid map of the plasmid was generated to illustrate the presence of the amplicon in the PTZ57R vector (Fig. 1D).

A PCR product, corresponding to the predicted size of the SRY amplicon (556 bp) was obtained at an annealing temperature of 56.9 °C (Fig. 2A). The PCR product from the SRY gene was cloned into the pTZ57R plasmid by TA cloning. The plasmid clones obtained were screened using PCR (Fig. 2B) and restriction digestion (Fig. 2C), followed by agarose gel electrophoresis. Plasmids 1.1, 1.2 and 2.1 showed bands at 556 bp after PCR using SRY specific primers suggesting the presence of the SRY amplicon in these clones.

3.2. Molecular sex typing of human fetal remains

The X-linked G6PD and Y-linked SRY primers were tested using the optimized PCR protocol on five samples of perinatal gDNA for which the sex had been recorded at autopsy. The molecular confirmation of sex is represented in Fig. 3, where panel A represents detection of the X chromosome (due to the 191 bp G6PD amplicon) while B represents detection of the Y chromosome (due to the 556 bp amplicon). A single band at 191 bp indicates a female (XX) sex typing, while a band at both 191 bp and 556 bp indicates a male (XY) individual. Due to the limited amount of sternal tissue and gDNA extracted; the optimization of the sex typing protocol resulted in small amounts of gDNA remaining for the confirmation of sex (Fig. 3), therefore only 20 ng of gDNA was used in the PCR reaction for individual 449.

The two faint bands present in panel A for individual 449 is the result of a low template concentration but indicate that this method can accommodate small quantities of gDNA template. When comparing molecular sex with that recorded on the autopsy report, positive correlation was found for four of the individuals. Individual 706 was noted as female at autopsy. However, following this assay and visualization by agarose gel electrophoresis, a faint band was present for the Y-linked primers at 556 bp. Therefore, this individual was re-categorized as male.

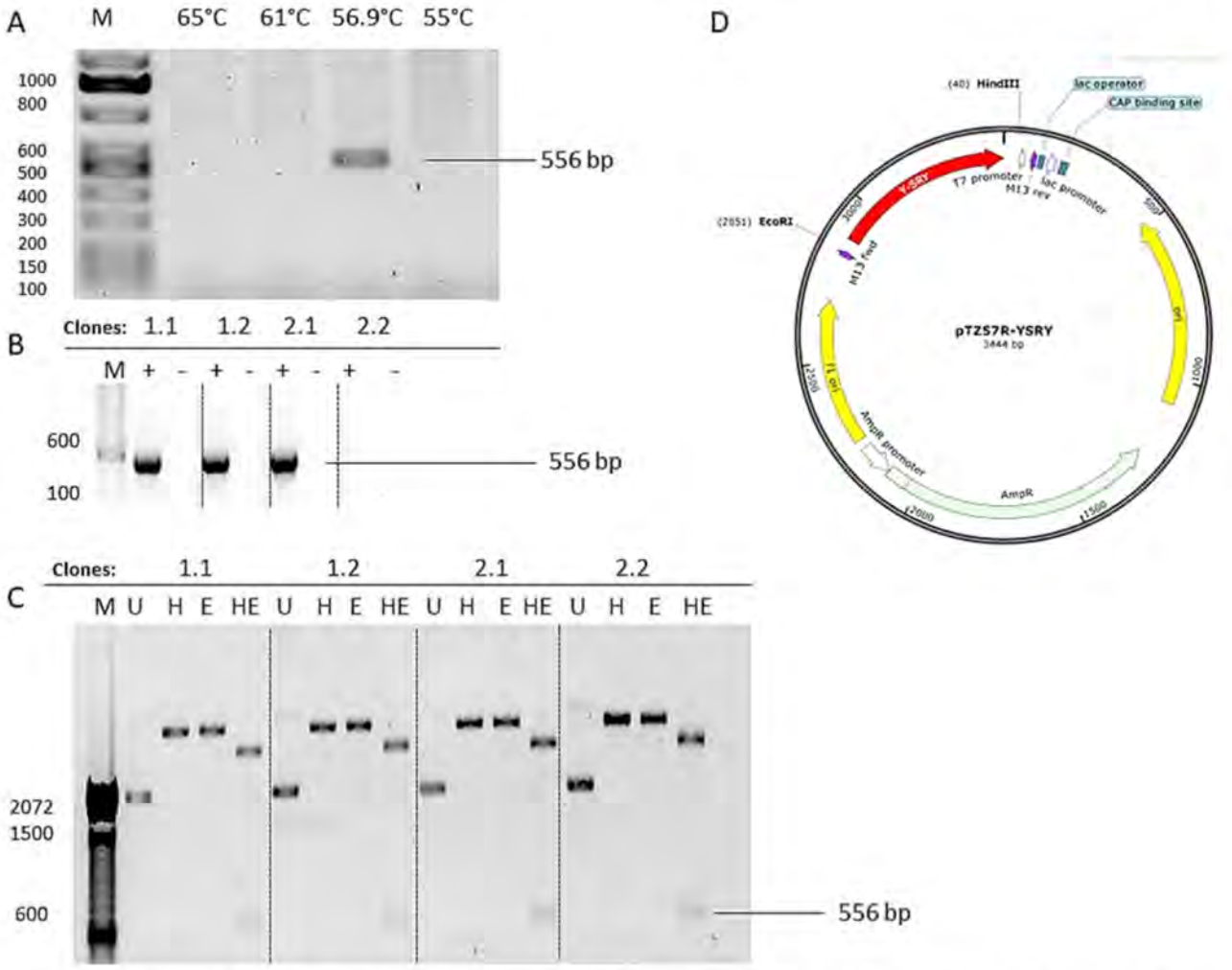


Fig. 2. Confirmation of insertion of the SRY Y-linked amplicon into the pTZ57R vector for plasmid controls. A.) Ethidium bromide stained 0.8% agarose gel of gradient PCR with primers designed to amplify a region of the SRY gene using a range of annealing temperatures (65, 61, 56.9 and 55 °C) from SW480 cell line gDNA. + : reaction prepared with template, -: reaction prepared without template. B.) Ethidium bromide stained 0.8% agarose gel of PCR using putative pTZ57R-YSRY plasmids using SRY primers. pTZ57R-YSRY clones: 1.1, 1.2, 2.1 and 2.2. C.) Double Restriction digestions of putative pTZ57R-YSRY plasmids with *Eco*RI and *Hind*III enzymes. U: Uncut reactions; H: *Hind*III cut reactions E: EcoR1 cut reactions; HE: Double digestion reactions; M: DNA ladder. D.) Plasmid map of pTZ57R-YSRY plasmid generated in Snapgene.

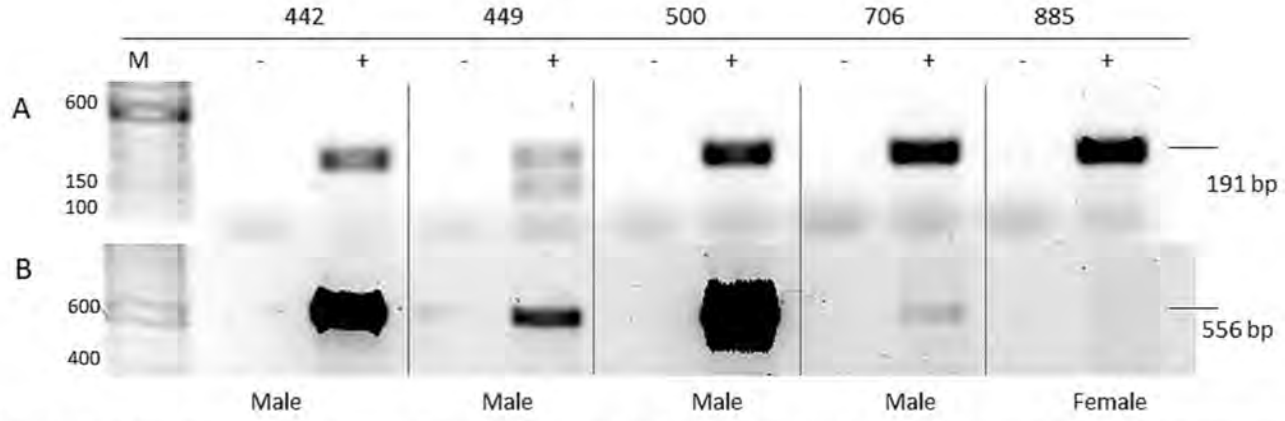


Fig. 3. Confirmation of sex in selected individual samples using X-linked G6PD AND Y-linked SRY optimized primers. A.) Ethidium Bromide stained 0.8% agarose gel of PCR protocol optimized for X-linked G6PD primers targeting perinatal gDNA B.) Ethidium Bromide stained 0.8% agarose gel of PCR protocol optimized for Y-linked SRY primers targeting perinatal gDNA. + : reactions prepared with template. -: reactions prepared without template. Female = band at 191 bp only. Male = band at 556 bp only.

The gDNA used in sexing of unknown perinatal remains derived from three individuals from the JFPS and six formalin fixed individuals from the JFPC. Following PCR, reactions were visualized by agarose gel electrophoresis. The positive control plasmids (G6PD and SRY) are represented by strong bands at 191 bp (X-linked G6PD) and 556 bp (Y-linked SRY) and confirm that the PCR reaction worked correctly. Negative controls reactions were used with the respective primers and no gDNA template (Fig. 4).

Each sample of unknown gDNA was added to separate reactions with X-linked primers and Y-linked primers to estimate sex (Fig. 4). The positive control plasmids produced strong amplicons at the expected sizes for the X and Y linked primers. These positive signals also confirm that the PCR reaction worked correctly. Bands were present at only 191 bp and not 556 bp for the nine unknown individuals. There was a minor difference in band intensity between mortuary (21/2017, 279/2017, 553/2017) and formalin preserved individuals (312/2012, 435/2012, 845/2011, 368/2011, 1564/2012 and 232/2012). These individuals were categorized as female.

4. Discussion

In compliance with the legal requisites cited in the regulations of Forensic Pathology Services, unidentified remains, unclaimed fetal or perinatal remains were included in the study and processed after 30 days of custody at the service within the jurisdictional area [15]. As such, the individuals included in these assays were admitted to the JFPS and JFPC at an advanced stage of decomposition. These remains were frozen, or formalin fixed (JFPC) prior to dissection of rib samples and DNA isolation. Therefore, the integrity of DNA is highly questionable. It should be noted that these decedents were admitted for medico-legal autopsy without any medical history, collateral information about identity or circumstance of death particulars. The limited amount of information associated with the decedents largely stems from the circumstances in which their remains are found in the post-mortem context, this may include scenarios described as abandonment at birth, concealment of birth or suspected infanticide. While these circumstances are a prevalent public health and social issue within South Africa [5], this unique situation presents a challenge in developing solutions without reference to established methods based on similar modern samples of infanticide origin. As such the development of the proposed study method imitates real world scenarios such as varied taphonomic and environmental conditions, cause of death and post-mortem interval prior to admission to a medico-legal facility and demonstrates that it can be successfully applied to small amounts of low-quality DNA.

Despite the limitation in sample size, the findings present successful sex estimation from degraded genomic DNA from bone using the under-reported G6PD gene. There is a paucity of knowledge and methods available which are exclusively formulated for modern forensic fetal bone samples. When one compares this silica-based method with the literature reported, the study sample is forensic in nature and not archaeological. This is particularly true when one considers molecular protocols applied to fetal or neonatal bone tissue. The work by Faerman and colleagues utilized both a chelex and silica-based purification method, with 121 bone samples assigned sex using the traditional phenol/chloroform method and chelex extraction [6,12]. The current study's findings are comparable and in agreement with previously reported work. Thus, it may be suggested that researchers use a combination of approaches when targeting method development and

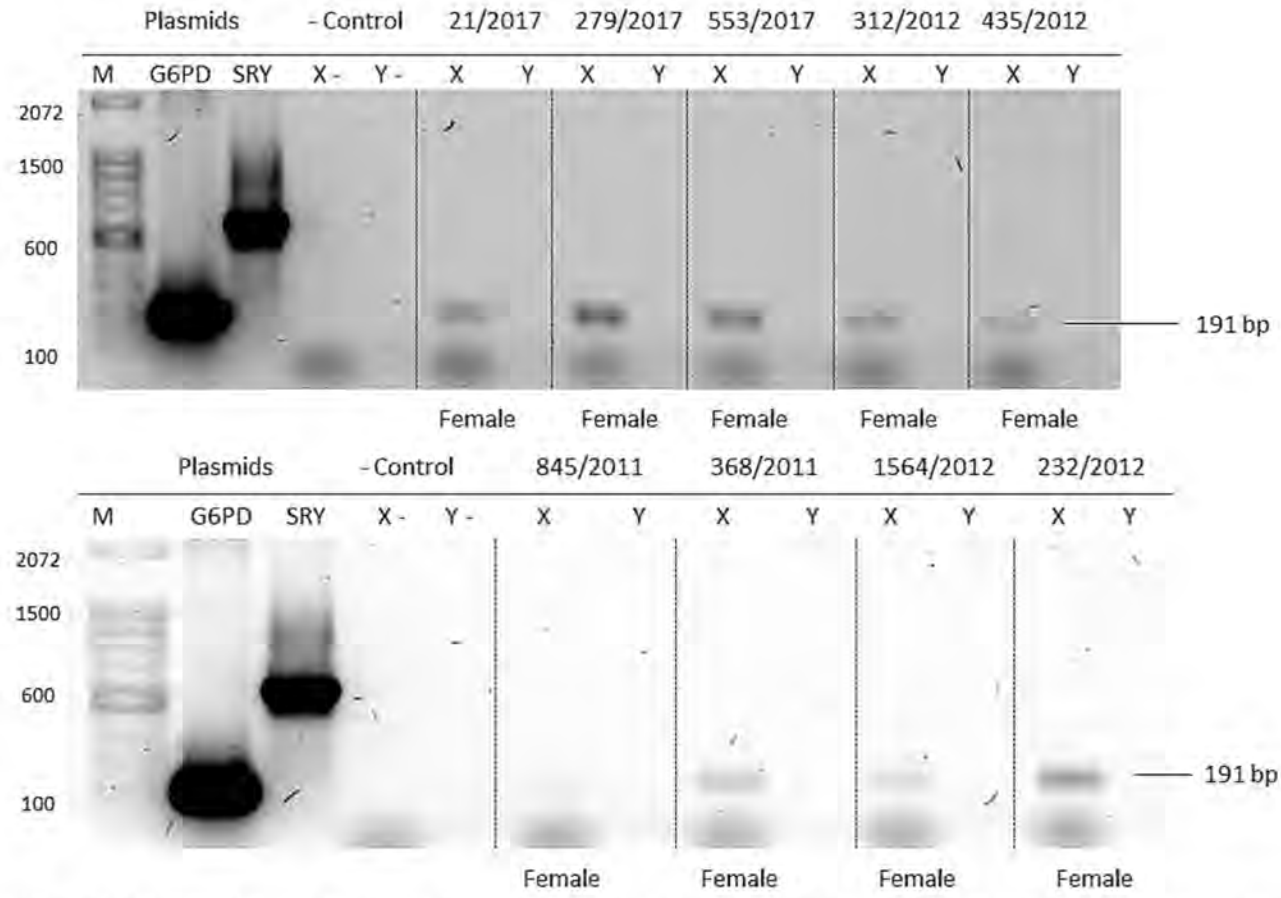


Fig. 4. Sex typing of unknown individuals by PCR. Ethidium Bromide stained 0.8% agarose gel of PCR products indicating sex of unknown perinates included in the study. G6PD and SRY = plasmid controls at 191 bp (X-linked) and 556 bp (Y-linked). Control = No template added in PCR reaction. X = X-linked primers only. Y = Y-linked primers only.

validation, especially on purification of DNA from degraded or less than desirable sources. Many of the current methods employed within forensic workflows are based on ancient DNA, adult samples [14,18] or degraded samples and the Dabney method developed to amplify short fragments [21] which could be problematic to downstream analyses. Therefore, a PCR based methodology is still relevant and utilitarian in the field of forensic anthropology. Alternatives to the amelogenin marker has been proposed by Dash and colleagues [3]. The use of the G6PD marker needs further experimentation and validation with studies on forensic fetal biomarkers. The degree of ossified tissue needs to be considered as during gestation, skeletogenesis and the stage there of will impact the ratio of cartilage to immature bone. It is recommended that the homogenization phase is adapted to ensure successful lysis of bone tissue.

5. Conclusion

This study presented a simple method to PCR amplify degraded DNA from unclaimed and unidentified fetal and perinatal remains for the purpose of sex estimation. This method relies on the inclusion of PCR stabilizing agents due to low template concentration of degraded forensic samples. As circumstance of death is varied in this criminal setting, further validation of the above method is recommended to test reliability, universal implications, and its range of applications within forensic workflows. As such it is advised that bone tissue samples be excised and preserved as soon as possible after death as a long post-mortem interval will impact purification, results and sex typing of remains. This practical and low-cost method is readily adaptable to any homogenization equipment and commercially available isolation protocols and reagents.

Funding information

This work was supported by funding through the Sandiswa Imbewu (Upando Ubungqina, Rhodes University, South Africa).

CRediT authorship contribution statement

Roxanne Thornton: Conceptualization, Method, Formal Analysis, Investigation, Data curation, Writing – original draft preparation, Visualization. **Erin Hutchinson:** Validation, Formal Analysis, Writing – review & editing, supervision. **Adrienne Edkins:** Conceptualization, Method, Validation, Formal analysis, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors thank Professor Jeanine Vellema (University of the Witwatersrand) for access to the unidentified and unclaimed perinatal individuals included in the study.

References

[1] D. Ballard, J. Winkler-Galicki, J. Wesoly, Massive parallel sequencing in forensics: advantages, issues, technicalities, and prospects, Int. J. Leg. Med. 134 (4) (2020) 1291–1303.
[2] M. Bidmos, V. Gibbon, G. Strkalj, Recent advances in sex identification of human skeletal remains in South Africa, South Afr. J. Sci. 106 (11/12) (2010) 1–7.
[3] H.R. Dash, N. Rawat, S. Das, Alternatives to amelogenin markers for sex determination in humans and their forensic relevance, Mol. Biol. Rep. (2020).
[4] C. Cunningham, L. Scheuer, S. Black, Developmental Juvenile Osteology, second ed., Elsevier Academic Press, 2016.
[5] L. du Toit-Prinsloo, C. Pickles, Z. Smith, J. Jordaan, G. Saayman, The medico-legal investigation of abandoned fetuses and newborns—a review of cases admitted to the Pretoria Medico-Legal Laboratory, South Africa, Int. J. Leg. Med. 130 (2) (2016) 569–574.
[6] M. Faerman, D. Filon, G. Kahila, C.L. Greenblatt, P. Smith, A. Oppenheim, Sex identification of archaeological human remains based on amplification of the X and Y amelogenin alleles, Gene 167 (1–2) (1995) 327–332.
[7] I.F. Gaballah, A.M. Shehab, K.A. Bayoumi, Sex estimation in femurs of modern Egyptians: a comparative study between metric measurements and SRY gene detection, Egypt. J. Forensic Sci. 4 (4) (2014) 109–115.
[8] D. Gaudio, D.M. Fernandes, R. Schmidt, O. Cheronet, D. Mazzarelli, M. Mattia, T. O'Keeffe, R. Feeney, C. Cattaneo, R. Pinhasi, Genome-Wide DNA from degraded petrous bones and the assessment of sex and probable geographic origins of forensic cases, Sci. Rep. 9 (1) (2019) 8226.
[9] V. Gibbon, M. Paximadis, G. Strkalj, P. Ruff, C. Penny, Novel methods of molecular sex identification from skeletal tissue using the amelogenin gene, Forensic Sci. Int. Genet. 3 (2009) 74–99.
[10] D. Jordan, D.E. Mills, Past, present, and future of DNA typing for analyzing human and non-human forensic samples, Front. Ecol. Evol. 9 (2021) 1–7.
[11] M.J. Iscan, M. Steyn, The Human Skeleton in Forensic Medicine, third ed., Charles C.Thomas, USA, 2013.
[12] C. Lassen, S. Hummel, B. Herrmann, Molecular sex identification of stillborn and neonate individuals (" Traufkinder ") from the burial site Aegerten, Anthropol. Anz. 1 (2000) 1–8.
[13] L.H. Luna, C.M. Aranda, Á.M. Monge Calleja, A.L. Santos, Test of the auricular surface sex estimation method in fetuses and non-adults under 5 years old from the Lisbon and Granada reference collections, Int. J. Leg. Med. 135 (3) (2021) 993–1003.
[14] A. Mannucci, K.M. Sullivan, P.L. Ivanov, P. Gill, Forensic application of a rapid and quantitative DNA sex test by amplification of the X-Y homologous gene amelogenin, Int. J. Leg. Med. 106 (4) (1994) 190–193.
[15] Regulations rendering of Forensic Pathology Service, 2007, In: National Health Act, 2003, No. 61, Cape Town: Staatskoerant (31), No. 30075.
[16] P. Skoglund, J. Storå, A. Götherström, M. Jakobsson, Accurate sex identification of ancient human remains using DNA shotgun sequencing, J. Archaeol. Sci. 40 (12) (2013) 4477–4482.
[17] N.A. Stewart, R.F. Gerlach, R.L. Gowland, K.J. Gron, J. Montgomery, Sex determination of human remains from peptides in tooth enamel, Proc. Natl. Acad. Sci. USA 114 (52) (2017) 13649–13654.
[18] A.C. Stone, G.R. Milner, S. Pääbo, M. Stoneking, Sex determination of ancient human skeletons using DNA, Am. J. Phys. Anthropol. 99 (2) (1996) 231–238.
[19] R.M. Thomas, Sex determination using DNA and its impact on biological anthropology. Sex Estimation of the Human Skeleton, Elsevier, 2020, pp. 343–350.
[20] D.H. Ubelaker, Recent advances in forensic anthropology, Forensic Sci. Res. 3 (4) (2018) 275–277.
[21] C. Xavier, M. Eduardoff, B. Bertoglio, C. Amory, C. Berger, A. Casas-Vargas, J. Pallua, W. Parson, Evaluation of DNA extraction methods developed for forensic and ancient DNA applications using bone samples of different age, Genes 12 (2) (2021) 146.
[22] Y. Yang, B. Xie, J. Yan, Application of next-generation sequencing technology in forensic science, Genom. Proteom. Bioinforma. 12 (5) (2014) 190–197.