Kholief et al. Egyptian Journal of Forensic Sciences (2017) 7:29
DOI 10.1186/s41935-017-0030-x

Egyptian Journal of Forensic Sciences

ORIGINAL ARTICLE Open Access

Sex determination from dental pulp DNA among Egyptians

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Abstract

Background: A great challenge arises when sex determination of human remains is needed, especially when only, bone fragments or isolated teeth are available; such as in mass disasters. Thus, the aim of the present work is to extract and analyze the DNA from dental pulp for sex determination of a sample of Egyptian population using the polymerase chain reaction amplification of DYS14 and SRY genes.

Materials and methods: DNA was extracted from the dental pulp of forty freshly extracted teeth (sound and carious) collected from adult Egyptians of both sexes using QIAamp® DNA Investigator Kit. Androgen Receptor gene was PCR amplified as an internal positive control. DYS14 and SRY genes were PCR amplified for sex determination.

Results: DNA extraction was confirmed by detection of the amplified AR gene band (292 bp) in all samples. Sex was successfully determined for all the studied teeth as revealed by amplification of 158 bp band, 778 bp band of the DYS14 and SRY genes respectively.

Conclusion: DYS14 and SRY genes were found to be reliable for dental sex determination regardless of the condition of teeth, whether sound or carious.

Keywords: Sex determination, Dental pulp, DNA, PCR, DYS14 gene, SRY gene

Background

Forensic odontology is a critical subspecialty of forensic medicine as it contributes to proper and accurate identification of individuals in mass disasters and crime scene investigations. Therefore, it is considered as indispensable science for achieving justice (Nuzzolese & Di Vella, 2007; Saxena et al., 2010).

Accurate determination of individual sex exemplifies a valuable clue in human identification, as it immediately excludes approximately one half of the population. The identification based on dental charts fails in some cases due to the lack of ante-mortem records, so DNA typing techniques are needed for exact personal identification (Williams et al., 2004).

Teeth are considered to be the ideal organ to estimate sex from fragmented, decomposed or burnt bodies as they are highly mineralized and most resistant to heat and decomposition. Hence, they represent an excellent source to obtain genetic material (Gaytmenn & Sweet, 2003a; Sweet, 2001).

Dental pulp is a preferable source for DNA extraction, as it is surrounded by hard mineralized tissues, which protects it against postmortem insults (Williams et al., 2004). It consists of connective tissue containing nerves, blood and lymphatic vessels. (Ash & Nelson, 2014) This provides the greatest quantity of DNA, compared to other dental tissues (Pinchi et al., 2011).

With technological advances of molecular biology in forensic medicine, Polymerase Chain Reaction (PCR) offers efficient and sensitive methods for sex determination through the analysis of gender specific sequences on X and Y chromosomes (Akane et al., 1991).

X chromosome contains the Androgen Receptor (AR) gene, that is located on the long arm of X chromosome (Xq 11–12), so this gene can be identified in any male or female DNA sample (Omrani, 2006).

A sex determining region on the short arm of Y chromosome(SRY gene) is located at p11–31, thus, its detection is an indicator of male sex (Skaletsky et al., 2003; Thangaraj et al., 2002a; Jobling et al., 2007). However,

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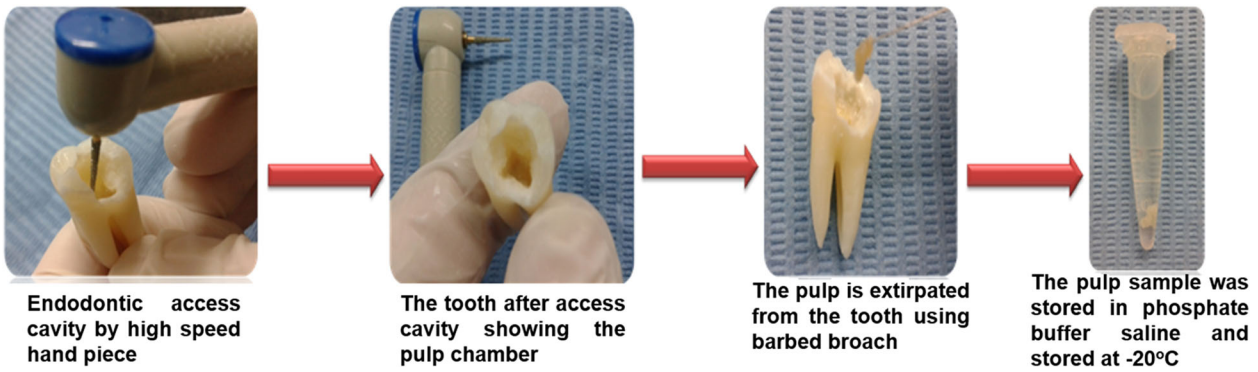

Fig. 1 Extraction of dental pulp tissue by standard endodontic access and pulp storage

much attention has been lately given to the DYS14 gene as a marker located in intron 1 of the multicopy Y-encoded Testis-Specific Protein (TSPY) that plays a role in spermatogenesis. A single Y chromosome contains, on average, more than 50 copies of this gene, each copy carries the DYS14 marker. Thus, it is recommanded to be easily looked for in poor quality DNA or low copy number (Zimmermann et al., 2008).

DYS14 gene was used as a non- invasive method for fetal sex determination from maternal plasma (Fernández-Martínez et al., 2012). Nowadays, it is used for identification of male DNA in mixed male and female samples in case of sexual assaults (Kamodyová et al., 2013).

The purpose of the current study was to extract and analyze DNA from the dental pulp of a sample of Egyptian adult individuals for sex determination using polymerase chain reaction amplification of SRY and DYS14 genes.

Materials and methods

Materials

The present study was conducted on 40 freshly extracted teeth from adult Egyptian patients of both sexes with an age range from 19 to 58 years, attending different dental hospitals and clinics.

All teeth were evaluated using radiographs to verify the presence or absence of pulp space.

Inclusion criteria

Freshly extracted teeth from all quadrants for orthodontic, periodontal or prosthetic reasons were included regardless to the presence or absence of caries.

Exclusion criteria

Previously restored teeth with any filling material, in addition to grossly damaged teeth as well as completely calcified teeth were all excluded from the study.

Methods

Informed consent was obtained from all participants, as well as approval of the Medical Ethics Committee of Faculty of Medicine, Alexandria University.

1. Sample Preparation

All the extracted teeth were decontaminated to eliminate external contaminants present on the exterior surface that could potentially affect the quality of the collected DNA. Handling of the teeth was under aseptic conditions using sterile instruments and disposable gloves. The decontamination process was performed by immersing the teeth in 0.9% isotonic saline solution for 15 min, then washing with distilled water.

2. Pulp Extirpation and Storage: (Fig. 1)

The pulp chamber of each tooth was accessed through the occlusal surface of the teeth by a high speed air driven hand piece. Following that, the pulp was carefully removed using a barbed broach avoiding the shredding effect. Finally, the pulp was placed in a sterile 1.5 ml labeled Eppendorf tube containing phosphate buffer saline and stored at −20 °C till DNA extraction was performed.

Table 1 AR, SRY and DYS14-specific primers sets and base sequences. (Mohammed & Tayel, 2005; Gomaa & Sheta, 2013)

Primer	Sequence 5′-3′
AR Forward	CTCTGGGCTTATTGTAAACTTCC
AR Reverse	GTCCAGGAGCTGGCTTTTCCCTA
SRY forward	GGTGTTGAGGGCGGAGAAATGC
SRY Reverse	GTAGCCAATGTTACCCGATTGTC
DYS14 Forward	CATCCAGAGCGTCCCTGG
DYS14 Reverse	TTCCCCTTTGTTCCCCAAA

Table 2 PCR program for amplification of AR, SRY and DYS14 genes

Steps	PCR Program for AR gene	PCR Program for SRY gene	PCR Program for DYS14 gene
Initial denaturation	95 °C for 10 mins	95 °C for 10 mins	95 °C for 10 mins
Denaturation	95 °C for 20 s	95 °C for 1 min.	95 °C for 30 s
Annealing	72 °C for 1 min.[a]	58 °C for 1 min.	60 °C for 30 s
Extension		72 °C for 1 min.	72 °C for 30 s
Number of cycles	35 cycles	35 cycles	35 cycles
Final extension	72 °C for 7 mins	72 °C for 10 mins	72 °C for 10 mins

[a]Annealing and extension steps are combined together in one step

3. Detection of Androgen Receptor and Sex Determining Genes

All subsequent experiments were performed blindly and sex results were cross matched at the end of the study.

4. DNA Extraction "Solid Phase Extraction":

After complete thawing of the frozen sample, the storage buffer (phosphate buffer saline) was withdrawn and discarded, leaving the dental pulp in the Eppenderof tube.

The extraction procedures were performed according to the QIAamp DNA investigator kit protocol (QIAamp® DNA Investigator Handbook, 2010).

5. DNA amplification:

SRY and DYS14 genes were amplified for sex determination by using a conventional singleplex Polymerase Chain Reaction (PCR) technique. Negative control was rum throughout all the experiments using molecular biological water to detect any possible contamination. AR gene was amplified as an internal positive control.

For each reaction, the following constituents were added to a 200 μl PCR tube containing a PCR bead: (Illustra pure Taq Ready-To-Go PCR Beads, n.d.).

- 0.5 μl (forward) primer, 0.5 μl (reverse) primer (Table 1).
- 5 μl extracted DNA.
- 19 μl sterile molecular biology water to reach a final volume of 25 μl.

Thermal cycling was performed for the three genes using different conditions (Table 2).

Visualization of PCR products: (Denomme et al., 2007)
PCR products were checked via gel electrophoresis on a 2% agarose gel containing ethidium bromide and visualized using UV gel documentation system. The images were captured and saved.

Results

Absence of bands in negative control samples indicated proper decontamination techniques.

All dental pulp samples showed the amplified bands of androgen receptor gene (292 bp) indicating successful DNA extraction as shown in (Fig. 2).

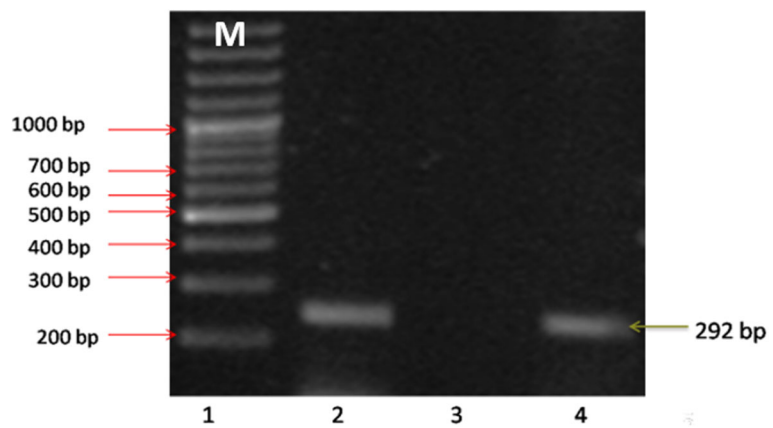

Fig. 2 Singleplex PCR amplification of AR gene on X chromosome (292 bp). Lanes: 1(M) = 100 bp DNA hyperladder, Lanes 2, 4 = bands of AR of either male or female dental pulp samples, lane 3 = negative control (molecular biology water)

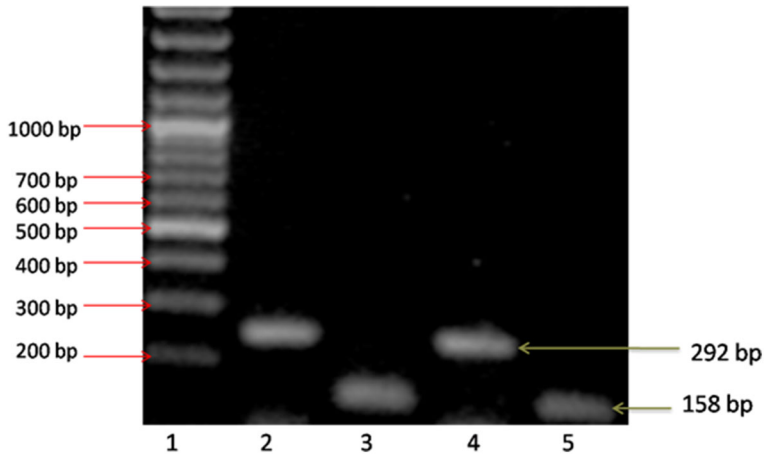

Fig. 3 Singleplex PCR amplification of DYS14 gene (158 bp) and AR gene (292 bp). Lanes: 1(M) = 100 bp DNA ladder, lanes 2, 3 = bands of AR gene at 292 bp and DYS14 gene at 158 bp respectively in the same dental pulp sample, lanes 4, 5 = bands of AR gene and DYS14 gene respectively in another sample. Both samples were of the male sex

Twenty four dental pulp samples showed the amplified bands of DYS14 gene (158 bp) and bands of SRY gene (778 bp) indicating male sex. However, the rest of the samples (16 teeth) showed no bands of either DYS14 or SRY gene in spite of displaying the amplified bands of the positive control AR gene (292 bp) that leads to identification of the female sex (Figs. 3, 4, and 5).

As regards to the tooth status, DNA was successfully extracted as proved by AR gene amplification in all the studied pulp samples, whether from sound or carious teeth. Furthermore, the condition of the teeth did not affect the process of DYS14 or SRY gene where both showed their bands in all the studied male samples, whether extracted from sound or carious teeth (Figs. 6, 7).

At the end of the DNA analysis, results were compared to the original sex data of the dental pulp samples and showed 100% success of the process of sex determination using the studied genes with no false positive or false negative results.

Discussion

With the increasing rate of violence, crimes and terrorism with advancement of weapons used, proper human identification is required for legal and humanitarian reasons which is considered as a challenging field of study and research in forensic science. In this regard, many studies have used the dental pulp for sex determination (Veeraraghavan et al., 2010; Zapico & Ubelaker, 2013; Nogami et al., 2008; Nayar et al., 2014; Naik et al., 2012).

A single tooth may be the sole evidence presented in the scene and being the most durable tissue enclosing a rich vascular network; that is why the tooth pulp was chosen for sex determination in the present study.

Decontamination procedure which was applied in the present study, did not include any bleaching agents or detergents, in contrast to that were applied in the previous studies (Sweet & Hildebrand, 1998; Gaytmenn & Sweet, 2003b; Kemp & Smith, 2005; Marjanovic et al., 2007; Alakoc & Aka, 2009).

(Higgins et al., 2013), proved that bleaching agents decreased the amount of DNA yield in comparison to non- bleach treated teeth. Therefore, in the present work we did not use this method of decontamination to avoid destruction of DNA or dissolution of the pulp.

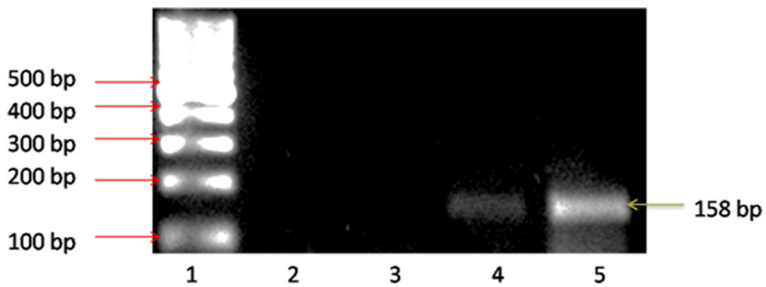

Fig. 4 Singleplex PCR amplification of DYS14 gene (158 bp). Lanes: 1(M) = 100 bp DNA ladder, lanes 2, 3 = no bands at 158 bp denoting female samples, lanes 4, 5 = male samples with bands at 158 bp

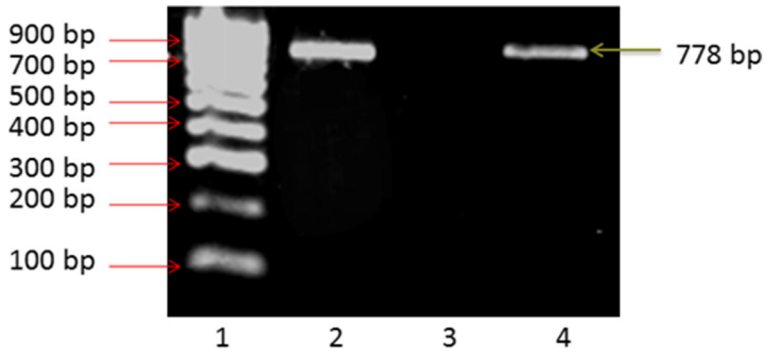

Fig. 5 Singleplex PCR amplification of SRY gene (778 bp). Lanes: 1(M) = 100 bp DNA ladder, lanes 2, 4 = male samples with bands at 778 bp, lane 3 = no band at 778 bp denoting female sample

Actually there were no cases of external DNA contamination encountered in this study, as the androgen receptor gene was successfully amplified in all the samples. This denotes the effectiveness of the used decontamination method.

To access the dental DNA, many authors crushed the whole tooth (Sweet & Hildebrand, 1998; Sivagami et al., 2000; Meyer et al., 2000; Baker et al., 2001; Rubio et al., 2009), while others preferred sectioning the tooth (Gaytmenn & Sweet, 2003b; Murakami et al., 2000; Shiroma et al., 2004; PreseEki et al., 2000). The main drawback of the crushing method is that it completely destroys the teeth which is hindering further investigations. In sectioning the teeth, the access to the pulp chamber, by clear fracture, needs an experienced dentist. It is also difficult to perform teeth sectioning in case of pulp retraction (Tilotta et al., 2010).

In agreement with (Tilotta et al., 2010), the pulp was successfully extirpated in the current study by a standard endodontic access. The main advantage of this method is being conservative, so the tooth can be used for further investigation. It is also performed under air and water spray to prevent heating, whereas crushing or sectioning produces heat which may damage the genetic material (Tilotta et al., 2010). However, the endodontic access method utilized in this study required the need for a skilled dentist where the pulp was extracted with sufficient quality and quantity even from elder teeth.

The DNA extraction protocol used in the current study was based on solid phase extraction using silica- based column in contrary to other studies, where researchers used the classical organic extraction (phenol-chloroform method) (Sweet & Hildebrand, 1998; Murakami et al., 2000; Michaelis et al., 2008; Bulter, 2012).

The phenol chloroform method had many disadvantages including time consumption, difficulty and demanding of large quantity of samples which is not always available in real forensic investigations. Moreover, it requires the sample to be transferred between multiple tubes and this increases the risk of contamination. Also, handling of hazardous chemicals in this method poses a risk on the researchers (Michaelis et al., 2008; Bulter, 2012).

Utilizing the silica- based spin columns resulted in successful extraction of DNA from dental pulp samples

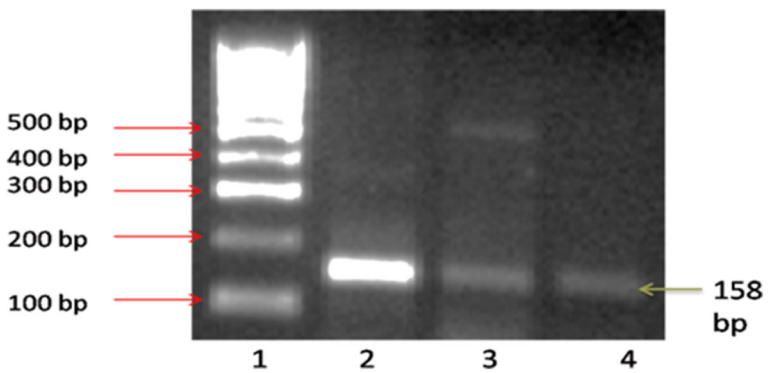

Fig. 6 Singleplex PCR amplification of DYS14 gene (158 bp). Lanes: 1(M) = 100 bp ladder, lanes 2, 4 = male samples from two sound teeth, lane 3 = male sample from a carious tooth

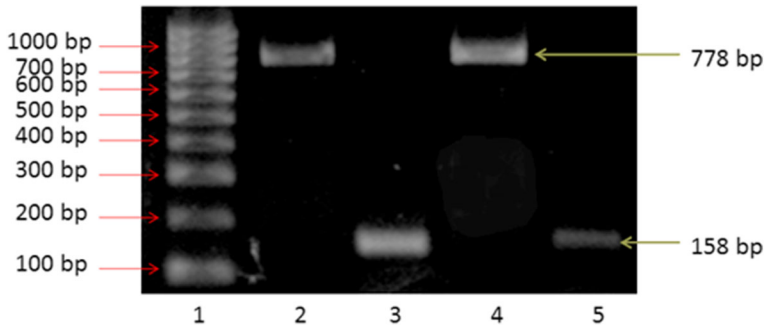

Fig. 7 Singleplex PCR amplification of DYS14 gene (158 bp), SRY gene (778 bp). Lanes: 1(M) = 100 bp DNA ladder, lanes 2, 3 = male samples from one sound tooth, lanes 4, 5 = male samples from one carious tooth

with the ability to determine the desired elution volume that allowed maximum concentration of DNA, which was chosen to be 40 μl in the present study.

Sex determination was successfully achieved in all the studied samples with the use of SRY and DYS14 genes. Moreover, the age of participants had no impact on the potentials for sex determination.

Choosing SRY gene was based on its successful detection in different samples e.g.: blood, saliva, teeth and even from epithelial cells adhered to the bristles of tooth brushes (Vikram Simha Reddy et al., 2011).

Following several studies that showed deletions in the amelogenin gene on Y chromosome and subsequently the wrong identification of males as females, there was a definite need to use other reliable sex determining genetic markers (Kastelic et al., 2009; Kashyap et al., 2006; Chang & Burgoyne, 2003; Thangaraj et al., 2002; Drobnic, 2006; Laverde, 2013).

The multi-copy DYS14 gene was not conventionally used for sex determination in forensic cases, therefore, it was used in this study to verify its efficacy for sex determination from the tooth pulp. (Nakata et al., 2010) and (Blagodatskikh et al., 2010) previously reported its success in identifying sex from prenatal and blood samples. Additionally, (Gomaa & Sheta, 2013) have used DYS14 gene to identify male DNA in a mixture of male and female blood samples in varying proportions.

To compare between the sensitivity of DYS14 gene and SRY gene for sex determination using dental pulp samples, real time PCR would be adventitious in measuring the quantity of the amplified DNA bands. Thus, real time PCR is recommended for future studies to verify the contradictory findings reported by (Blagodatskikh et al., 2010) who documented that SRY gene showed ten times higher sensitivity than DYS14 gene. Whereas, other contradictory studies recommended DYS14 marker for the diagnosis of fetal gender from maternal plasma, and this recommendation was based on their findings which showed higher sensitivity of DYS14 over the SRY gene (Fernández-Martínez et al., 2012; Jacob et al., 2015; Khorshid et al., 2013). Hence, using conventional PCR in the present study provided 100% success in sex determination from all dental pulp samples with no false negative or positive results.

Conclusion

The dental pulp can be considered as a highly reliable source of DNA irrespective to the tooth status whether sound or carious. Particularly, the molar tooth which is considered as a good source of DNA since it is a multi-rooted tooth with the largest pulp volume. In addition, access to the pulp using the standard occlusal endodontic technique provides a significantly large amount of pulp tissue and subsequently sufficient amount of DNA yield with a minimal risk of contamination. Furthermore, DYS14 gene was proved to be as efficient as SRY gene for sex determination from the dental pulp using conventional PCR amplification with no false negative or positive results.

Acknowledgements

After success of the study, we would like to acknowledge the dentist who helped us in teeth extraction from all participants and taught us pulp extraction from teeth. Additionally, a great appreciation must go to all participants who gave approval to give their teeth to be included in the study. Finally, we would like to acknowledge University of Alexandria who funded that project.

Funding

This research was funded by the Faculty of Medicine at Alexandria University, Egypt.

Availability of data and materials

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MK (collection of samples and participation in the practical work, in addition to collection of literature review); SS (writing the discussion section and editing the whole article); RG (the idea of the study, conduction and supervision of the laboratory work and writing methodology and results). All authors read and approved the final manuscript.

Ethics approval and consent to participate

Ethical approval was obtained from the Medical Ethics Committee of Faculty of Medicine, Alexandria University, Egypt. In addition, a detailed written informed consent was obtained from all participants after explaining all details of the nature of the study and emphasizing that all information that will be collected from patients or obtained from the study will be kept confidential and will only be used solely for research purposes.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Publisher's Note

Received: 31 May 2017 Accepted: 4 December 2017
Published online: 16 December 2017

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