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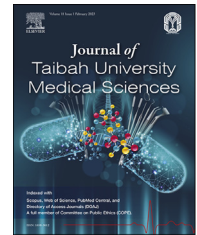

Original Article

The significance of amelogenin loci from toothpicks as forensic evidence for sex determination

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الملخص

أهداف البحث: أصبح تحليل الحمض النووي الريبي منقوص الأكسجين هو المعيار الذهبي في التعرف على الأفراد من خلال فحص الطب الشرعي. هناك العديد من المصادر الأولية والثانوية لعينات الحمض النووي في مجال الطب الشرعي. تشمل المصادر الثانوية العناصر التي يستخدمها الشخص مثل الملابس وفرشاة الأسنان وأعواد الأسنان. في الدول الآسيوية مثل كوريا واليابان وإندونيسيا، ينتشر استخدام أعواد الأسنان. يمكن استخدام طرف عود الأسنان المستخدم في تنظيف جزيئات الطعام في المناطق بين الأسنان لالتقاط عينات من الحمض النووي من اللعاب. استخدام عينات اللعاب لتحليل الحمض النووي في تحديد الجنس يعتمد على التكرار الترادفي القصير مع موضع الأميلوجينين. الغرض من هذه الدراسة هو فحص تركيز ونقاء الحمض النووي على أعواد الأسنان كدليل في التحقيقات الجنائية، بالإضافة إلى إمكانية استخدامه لتحديد الجنس.

طرق البحث: تم توجيه ثمانية أشخاص لتنظيف الجانب البيني لأسنانهم الخلفية باستخدام عود أسنان. علاوة على ذلك، تم الاحتفاظ بكل عينة من أعواد الأسنان لمدة صفر يوما (كعنصر تحكم)، و 14 يوما، و 20 يوما. تم تحديد النقاء وتركيز الحمض النووي لكل عينة من خلال فحص الحمض النووي. بعد تحديد تركيز ونقاء الحمض النووي من كل عينة، تم إجراء الرحلان الكهربائي في مواضع الأميلوجينين لتحديد الجنس.

النتائج: أظهرت هذه الدراسة أن متوسط تركيز الحمض النووي في أعواد الأسنان تراوح بين 425.25 - 796.25 ميكروغرام / مل. متوسط نقاء الحمض النووي في هذه الدراسة يتراوح بين 1.09 - 1.13 ميكروغرام / مل. ينتج جين الأميلوجينين 112 زوج قاعدي و 116 زوج قاعدي من المضخم النووي من الكروموسومات إكس و واي.

الاستنتاجات: يمكن تحديد الجنس باستخدام الحمض النووي باستخدام موضع الأميلوجينين، وهو بروتين موجود في الكروموسومات الجنسية (إكس و واي). لا يزال من الممكن استخدام قيمة تركيز الحمض النووي لعود الأسنان في هذه الدراسة لدعم الطب الشرعي بعد 20 يوما في درجة حرارة الغرفة.

الكلمات المفتاحية: أميلوجينين؛ تحليل الحمض النووي؛ العدل؛ الهوية القانونية؛ تحديد الجنس

Abstract

Objective: DNA analysis has become the gold standard of all identification methods in forensic science. There are several primary and secondary sources of DNA samples in the field of forensics. Primary sources can be obtained directly from individuals, whereas secondary sources of DNA samples include items used by someone such as clothes, toothbrushes, and toothpicks. In Asian countries such as Korea, Japan, and Indonesia, toothpicks are often used, as the tip of toothpicks used to clear food particles in the interdental regions can be utilized to capture DNA samples from saliva. The use of saliva samples for DNA analysis in sex identification is based on short tandem repeats with the amelogenin (AMG) locus. The purpose of this study was to examine the concentration and purity of DNA on toothpicks as

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evidence in criminal case investigations, as well as its potential as sex determination material.

Methods: Eight subjects were instructed to clean the interproximal side of their posterior teeth using a toothpick. Each toothpick sample was kept for 0 days (as a control), 14 days, and 20 days. The purity and DNA concentration of each sample were determined through DNA examination. After determining the concentration and purity of DNA from each sample, electrophoresis of the AMG loci was performed for sex determination.

Results: This study showed that the average concentration of DNA on toothpicks ranged from 425.25 to 796.25 µg/ml, and the average purity of DNA ranged from 1.09 to 1.13 µg/ml. The AMG gene produces 112 and 116 base pair amplicons from the X and Y chromosomes.

Conclusion: Sex determination using DNA can be done using AMG loci, a protein found on the sex chromosomes (X and Y). The value of DNA concentration on toothpicks could be used to support forensic identification after 20 days at room temperature.

Keywords: Amelogenin; DNA analysis; Justice; Legal identity; Sex determination

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Introduction

DNA is the smallest unit of organisms.[1,2] In the last three decades, DNA analysis has become an important tool in forensic science. DNA profiling is based on short tandem repeats (STRs) and aids in human identification from biological samples. It is a reliable and robust identification parameter, and is also legally accepted as evidence in criminal cases.[3,4,5] There are two types of DNA sources in the field of forensic sciences: primary and secondary. Primary DNA sources can be obtained directly from individuals, whereas secondary sources can be collected from a person's relatives. Body fluids such as saliva, blood, semen, and urine represent important components in DNA human identifi-cation.[6]

Forensic DNA examination for sex determination is frequently required for disaster victim identification, missing person investigations, and sexual assault cases. Y-specific target sequence analysis on the Y chromosome is an effective method for sex determination and estimating the ratio of male to female DNA in mixed forensic samples.[6,7] DNA is generally used to solve crimes in one of two ways. When a suspect is identified, a DNA sample from that person can be compared to evidence from the crime scene. The results of this comparison may aid in determining whether the suspect committed the crime. In cases where a suspect has not yet been identified, biological evidence from the crime scene can be analyzed and compared to offender profiles in DNA databases to assist in identifying the perpetrator. Crime scene evidence can also be linked to other crime scenes through the use of DNA databases.[8,9]

In forensic situations involving mass disasters, transportation accidents, missing persons, or sexual assault, sex determination can be helpful. The DNA of the victim can be used to track down the remains of a corpse utilizing samples from numerous sources such as teeth, oral epithelial tissue, and saliva. Amelogenin (AMG) is a sex typing marker that is commonly utilized. AMG is an epithelial protein whose gene is found on both the X and Y chromosomes.[10] Due to variation in the X and Y chromosomes, a group of proteins may be utilized to identify sex.[11]

Every object at the crime scene has important meaning as forensic evidence. This includes everything used by the perpetrator or victim for the last time such as clothes, combs, toothbrushes, toothpicks, miswak, and tableware.[12] Objects exposed to saliva are good DNA sources for forensic identification. Saliva collection is considered a robust, non-invasive procedure for obtaining samples both in the laboratory and field.[10,11]

The presence of food remnants, dental plaque, and calculus in the oral cavity shows the degree of oral hygiene. The miswak and toothpicks are the most widely used tooth cleaning instruments.[12,13,14]Toothpicks are commonly used in Asian coun-tries such as China, Korea, Japan, and Indonesia.[16,17] The Mini-stry of Health of Indonesia also recommends toothpicks as one of the interdental cleaning instruments.[2,15] DNA samples from saliva can be easily collected from the tip of toothpicks that are used to remove food debris in the interdental areas.[18,19]

DNA sex determination has opened new avenues of exploration for modern forensic studies. The application of DNA examination using saliva samples in sex determination is based on STRs with AMG loci.[20] This study aims determined the quantity and quality of DNA on toothpicks as evidence in criminal investigations. Then an appropriate quantity and quality of the DNA were examined for sex determination through electrophoresis of AMG loci.

Materials and Methods

This study was conducted in the Human Genetic Laboratory of Universitas Airlangga (Surabaya, Indonesia). Eight subjects (four males and four females) who met the following inclusion criteria participated in this study: aged 18–23 years old, with complete permanent posterior teeth and no severe crowding of the anterior and posterior teeth. The number of samples in this study was limited because this study was conducted during the coronavirus disease 2019 (COVID-19) pandemic, so we were constrained in taking samples from the oral cavity. We carried out sampling according to strict health protocols.

Each subject was asked to eat fibrous foods and drink mineral water. Then they were instructed to clean the interdental surfaces of the posterior teeth with the toothpicks

provided. The process of cleaning the interdental surfaces was repeated three times with different toothpicks. Each toothpick sample was placed in an envelope, kept at room temperature, and divided into three groups: Group A (control day 0), Group B (day 14), and Group C (day 20). As the control sample, DNA quality and quantity were directly examined in Group A, while Groups B and C were stored for 14 and 20 days, respectively. All samples were stored in controlled conditions at a room temperature of 24 °C and humidity of 66%.

The DNA extraction process was started by soaking the toothpicks separately overnight in eight sterile centrifuge tubes. To dissolve and settle DNA samples from the toothpicks, each tube was filled with 5 cm^3 distilled water. After 24 h, the samples were centrifuged for 10 min at 12,000 rpm. Then the supernatants were discarded. DNAzol was added to the DNA sample solution, followed by vortexing, incubating for 15 min, and centrifugation at 8000 rpm for 10 min. The supernatant was mixed with isopropanol and centrifuged for 10 min at 12,000 rpm. The pellet was washed with 70% ethanol before being centrifuged again at 12,000 rpm.[21] Then the pellet was resuspended in 50 ml sterile aquadest before undergoing a 30 s spindown for the spectrophotometer. Final DNA extraction and isolation were performed using the Invitrogen™ DNAzol™ reagent.

The DNA purity of this study was determined using the optical density (OD) and OD260/280 ratio, and the DNA concentration was determined using a dilution factor of 700 ml (10 ml DNA and 690 ml distilled water). The DNA concentration was calculated using the following formula: OD260 dilution factor for 50 μg/ml double-stranded DNA. The results of the DNA concentration and purity tests were statistically analyzed using one-way repeated measure analysis of variance in SPSS version 20.0. In addition to determining DNA purity and concentration, visualization of AMG loci in electrophoretic gels was also performed to observe differences in base pair (bp) bands for sex determination on the DNA from toothpick samples.

Electrophoresis was performed after the acrylamide gel was prepared. The gel was made by mixing 3 cm^3 acrylamide reagent (Sigma–Aldrich, St. Louis, MO, USA) with 8 cm^3 Tris-borate-EDTA (TBE) Buffer 0.5× (Promega Co., Madison, WI USA) in an Erlenmeyer flask, followed by homogenization. A 200 μl solution of ammonium persulfate (Sigma–Aldrich) was added, followed by homogenization in the Erlenmeyer flask. The prepared gel solution was placed in a 0.5× TBE buffer solution electrophoresis chamber. A volume of 5 μl PCR DNA sample was pipetted into the gel column. After adding the solution, electrophoresis was performed at 100 V for 60 min to allow the negatively charged DNA to migrate to the positively charged opposite end, resulting in band contrast.

Results

The average DNA concentrations in Groups A, B, and C were 425.25, 796.25, and 531.12 μg/ml, respectively. A comparative test of DNA concentration revealed that there was a significant difference among Groups A, B, and C ($p < 0.05$). Because the p value was <0.05, we used the Tukey Honest Significant Difference post hoc test. The results of Groups A and B showed significant differences, but there were no significant differences between Groups A and C and Groups B and C (see Figure 1)

This study showed that, in addition to DNA concentration, the average purity of DNA in Groups A, B, and C was 1.11, 1.13, and 1.09 μg/ml, respectively (see Figure 2). The comparative test results for DNA purity revealed no significant differences among Groups A, B, and C ($p > 0.05$), so it is not used post hoc test.

The electrophoresis results of this study were visualized at the AMG loci to determine the sex of the toothpick samples. Electrophoresis was conducted with the sample with the lowest concentration to determine if the lowest concentration in this study could still be seen in DNA bands on the electrophoresis visualization results. Figure 3 shows that five

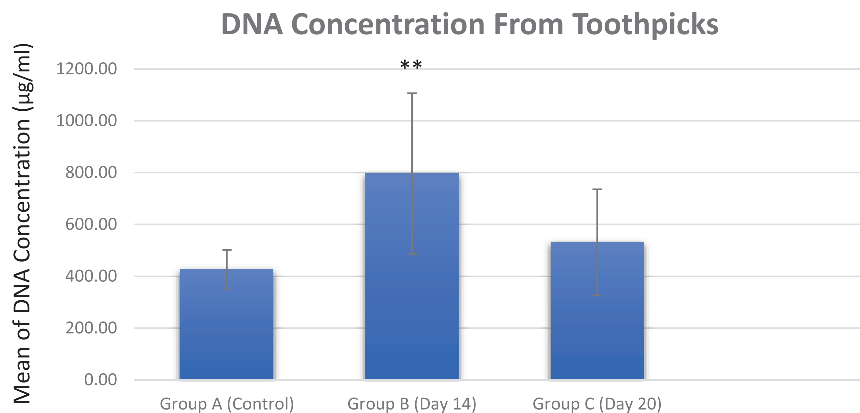

Figure 1: Descriptive analysis and one-way ANOVA with post hoc Tukey test result of DNA concentration from toothpicks. **: showed a statistically significant difference, with $p < 0.01$ vs control. DNA concentration of Group A: 425.25 ± 76.48 μg/ml; group B: 796.25 ± 309.89 μg/ml; and group C: 531.13 ± 204.88 μg/ml. The graph represents the mean ± SEM of the data.

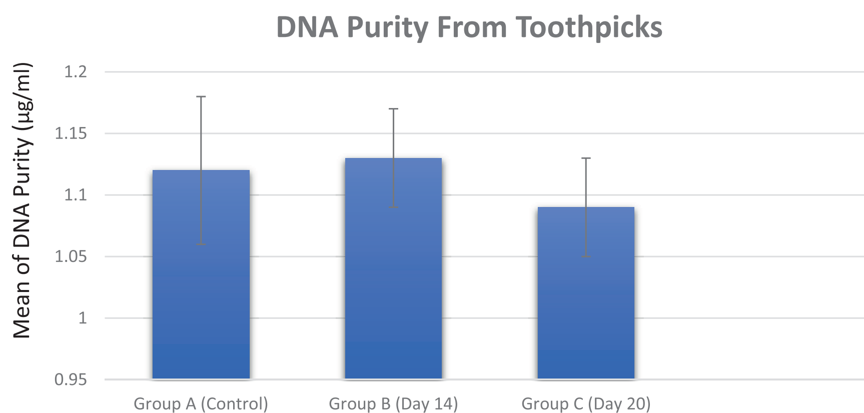

Figure 2: Descriptive analysis and one-way analysis of variance test result of DNA purity from toothpicks. The comparative test results for DNA purity revealed no significant difference, with p = 0.703. DNA purity of Group A: 1.12 ± 0.06 µg/ml; Group B: 1.13 ± 0.04 µg/ml; Group C: 1.09 ± 0.04 µg/ml.

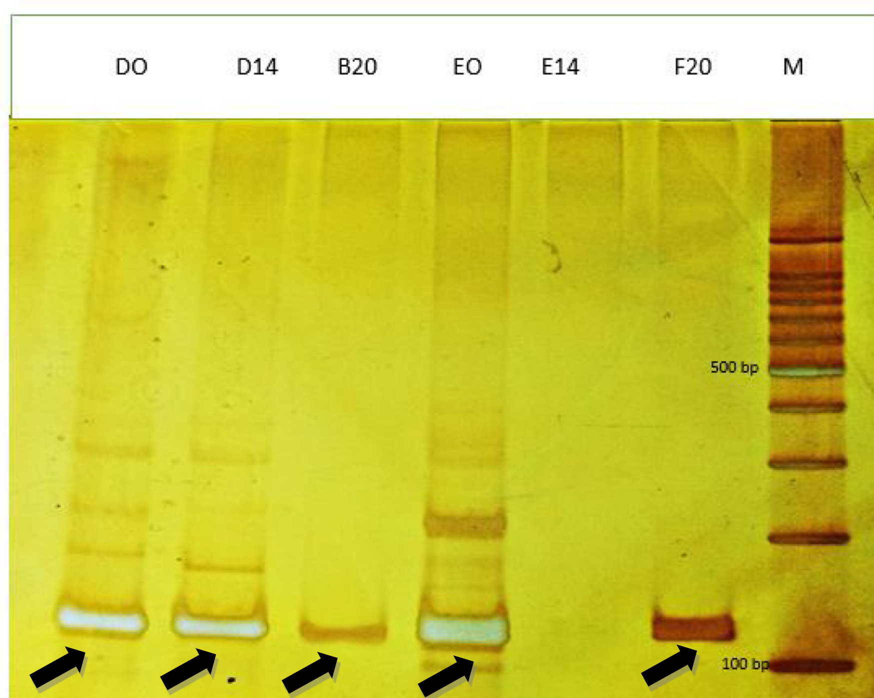

Figure 3: Electrophoresis visualization of the amelogenin loci. M = base pair (bp) marker; D0 = sample with the lowest concentration on day 0 (female). D14 = sample with the lowest concentration on day 14 (female); B20 = sample with the lowest concentration on day 20 (female); E0 = sample with the lowest concentration on day 0 (male); E14 = sample with the lowest concentration on day 14 (male); F20 = sample with the lowest concentration on day 20 (male); the arrows indicate that the amelogenin gene generates 112 bp for X and 116 bp for Y.

samples clearly showed DNA bands, while one sample did not show DNA bands at all.

Discussion

Spectrophotometry is used to determine the concentration and purity of DNA using ultraviolet absorbance ratios at 260 and 280 nm.[22] A DNA purity ratio of 1.8–2 is considered good. The DNA purity ranged from 1.09 to 1.13 µg/ml in this study, and statistical analysis revealed no significant difference in DNA purity among Groups A, B, and C ($p > 0.05$). Previous research has determined that the minimum value of DNA purity is 1–2 µg/ml, with excellent values ranging from 1.8 to 2 µg/ml.[3] This indicated that the level of DNA purity in this study met the minimum requirements and that the PCR amplification process can be continued.

It was also shown in this study that the average DNA concentration of all sample groups ranged from 425.25 to 796.25 µg/ml. This result is more valuable than that of Bharath

et al.,[23] which showed that the DNA concentration of epithelial cells in acrylic removable partial dentures was 32.37 μg/ml. Furthermore, statistical analyses revealed significant differences in DNA concentration among Groups A, B, and C ($p < 0.05$). Even though there were significant differences among sample groups, the value of the toothpick DNA concentration in this study can still be used to support forensic identification after 20 days at room temperature.

The identification process correctly identifies the victims of a disaster or incident where there are multiple fatalities or where the identity of deceased victims is in dispute before returning them to their families. Identification parameters such as sex, age, and paternity are critical in establishing an individual's identity. In forensic identification, determining the victim's sex and age can help in categorizing the victim group. Various methods have been considered reliable for sex determination, such as DNA AMG loci analysis.[24]

The electrophoresis results at the AMG loci revealed that each sample had distinct bands. The E14 bands, however, were not visible in the sample. The reason is not that the concentration was too low. In fact of the four samples that underwent electrophoresis, sample D14 had a lower concentration but the DNA band was still clearly visible. This condition can be caused by several factors, one of which is that the annealing temperature is close to but lower than the ideal annealing temperature.[25] The next possibility is that DNA has been degraded. Despite the small number of DNA samples, PCR is very sensitive and only requires a small amount of DNA to produce good results. As a result, PCR works well. However, agarose gels are based on visual detection and are therefore much less sensitive. It requires much more DNA to be visualized in a gel than for a successful PCR to work.[26] The reason for this is that the DNA band does not appear when the electrophoresis results on the agarose gel are visualized.

The AMG gene generates 112 and 116 bp amplicons from the X and Y chromosomes, respectively. Particular observations on electrophoresis results are required to distinguish the location of the X and Y bands because they only differ by four bp, resulting in only thick bands for males (XY chromosomes) and thinner bands for females (XX chromosomes).[1] This is a shortcoming in studies of sex determination using agarose gel visualization of AMG loci. However, if the AMG test fails to determine sex, the DXYS156 loci method can be used.[10] The limitation of this study was the small number of samples due to the COVID-19 pandemic.

The clinical implications and future perspectives related to this study can help support the police in determining the sex of the alleged perpetrator if the evidence is found in the form of toothpicks at the crime scene, even if discovered after 20 days.

Conclusion

Sex determination using DNA can be done using the loci of AMG, a protein found on the sex chromosomes (X and Y). The value of toothpick DNA concentration shown in this study could be used to support forensic identification after 20 days at room temperature.

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Conflict of interest

The authors have no conflict of interest to declare.

Ethical approval

This research was approved by the Ethical Clearance Committee, Faculty of Dental Medicine, Universitas Airlangga (Permit No. 155/HRECC.FODM/III/2020).

Authors contributions

AK designed the study, analyzed and evaluated the data, wrote the first draft of the paper, and revised the final text. BNR collected the data, developed the research question, conducted a literature search and review, and critically examined the manuscript. AC developed the manuscript's methodology section, collected the data, and reviewed the final draft. The manuscript's content and similarity index are the responsibility of all authors, who have critically examined and approved the final draft. All authors have critically reviewed and approved the final draft and are responsible for the content and similarity index of the manuscript.

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