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Sex determination of humans by polymerase chain reaction amplification of the DEAD box protein (DDX3X/DDX3Y) gene

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ABSTRACT

Molecular sexing of humans is normally done by PCR amplification of Y chromosomal fragments, or coamplification of homologous fragments from both sex chromosomes. The human sex test is part of commercially available PCR kits which do not always generate accurate and reliable results. Therefore, a simple and precise method for sex determination in humans are a pre-requisite for a number of applications in forensics and for large scale screening. In this study, we have developed a simple, rapid, accurate and reliable test for sex diagnosis in humans using the PCR technique for amplification of the DEAD box protein gene DDX3 (DDX3Y and DDX3Y) that was established in an earlier study as valuable tool for sex determination in cattle meat. This PCR method has demonstrated 100% gender specificity, and more importantly, false positivity and/or false negativity did not occur. We proved the reliability and accuracy of the protocol developed here when the blind test showed 100% concordance. The DDX3-based markers developed here can be used as be simple, precise and relatively cost-effective tools for sex determination in humans for a number of applications in forensics and a wide range of other applications. The human sex-specific markers generated here could be applied to any biological trace in forensic investigations or any other application requiring human sex determination. To our knowledge, this is the first study of its kind to elucidate or test the usability of the targeted gene (DDX3) for determining human gender.

Keywords: DDX3, DEAD box protein, humans, PCR, sex determination

INTRODUCTION

In the event of chemical and nuclear explosions, sudden accidents, natural disasters, and ethnic studies, the first priority in the identification process of a person by forensic investigators is determination of an individual's sexual identity (Laverde 2013, Ramakrishnan *et al.* 2015). Human sex (or gender) determination is important in criminal investigations of missing persons, and in archeology and anthropology

for the exploration of gender differences in past populations and for the study of cultures and human activities (Laverde 2013, Faerman *et al*. 1995, Masuyama *et al*. 2017). Human sex (or gender) determination is also a part and an essential priority of forensic odontology when it is impossible to tradition-nally identify the deceased (Chowdhury *et al*. 2018).

Human sex determination can help when seeking sexual assault evidence as it can serve as confirmation that the "sperm fraction" extracted from swabs and stains do contain male DNA. In this regard, it can also serve as an indicator of the amount of male DNA present in the non-sperm fraction (Reynolds and Varlaro 1996). Ornoy *et al*. (2019) believe that human gender assessment is crucial when the proper definition of the sex is of diagnostic and/or therapeutic importance such as the cases of ambiguous genitalia and intersex.

In criminal investigations, determining sex based on morphology (of the tooth, skull, and other soft tissues in the oral and sub-lumbar region) is very difficult or even impossible in many cases. Sex determination of forensic and archeological samples which lack morphological diagnostic characteristics, such as fragmentary bones and body fluid samples, is also not applicable using morphology (Masuyama *et al*. 2017).

In addition to the use of morphological characteristics in determining human sex, some microscopic methods such as X-chromatin test and Y-chromatin test and detecting Y-specific antigens (Kim *et al*. 2000, Phua *et al*. 2003) have been used for this purpose. Non-radioactive in situ hybridization, fluorescence in situ hybridization (FISH), and primed in situ (PRINS) labeling technique were also used for the same purpose (Delhanty *et al*. 1993, Settin *et al*. 2008). Ornoy *et al*. (2019) described the main needs for the accurate gender determination and the methods that can be employed. Due to the disadvantages of microscopic methods and due to the facts that 1) the morphological patterns of human remains or dead bodies differ with time and external factors, and 2) traditional morphometric analyses fail to identify the gender of incomplete skeletal remains and that of immature individuals (Faerman *et al*. 1995), and 3) DNA fingerprinting has evolved not only in terms of improving its technology, but also in its application beyond the "classical": helping with the estimation of sex, age and ancestry (Adserias-Garriga *et al*. 2018), the best alternative for sex determination of humans in criminal investigations and other events is the analysis of DNA, which exists in two patterns (namely nuclear and mitochondrial DNA) in blood, hairbrush, clothing, Pap smear or biopsy sample (Masuyama *et al*. 2017). Pfeiffer and Brenig (2005) believe that sex identification using genomic DNA extracted from meat, blood, hair or embryo biopsies is some-times an important analytical tool in forensic science or in routine genotyping.

Postmortem human remains must be identified in cases of natural or man-made disasters. This requires comparing hundreds, and sometimes thousands, of ante-mortem data with post-mortem data (Soodan *et al*. 2014). However, human remains may be highly fragmented in such cases, and hence only part of the body may be recovered which makes the identification a tough task (Shafer *et al*. 2006). In these cases, DNA analysis is the best tool for sex determination.

DNA analysis in forensic science requires a sample or source from either an individual (living or dead) or a crime/incident site (Muruganandhan and Sivakumar 2011). Determining the sex of a certain sample from a biological trace (e.g., dental pulp or the dentin of a tooth) of a human using DNA provides forensic investigators with useful information and can be an important part of casework analyses. It can also provide criminal investigators with useful intelligence and can aid the identification of missing person and even of disaster victims whose physical identification may have been destroyed. Gender information, particularly when combined with mitochondrial DNA analysis, can also serve to distinguish biological evidence from two people who share the same DNA type(s) but differ by sex (Reynolds and Varlaro 1996, Dutta *et al*. 2017).

For isolation of DNA and performance of laboratory tests for human sex identification, several biological materials may be used, including blood, dried blood stains, extracted dental pulp, hair bulb, bone tissue, saliva, biopsy sample, and other body tissues. All human body tissues are possible sources of DNA. However, variations only occur regarding the quantity and quality of the DNA extracted from each tissue (Ricardo *et al*. 2007).

Humans are born with 46 chromosomes in 23 pairs. The sex of an individual is determined by a pair of sex

chromosomes. Typically, females have two of the same kind of sex chromosome (XX, the homogametic sex), whereas males have two different kinds of sex chromosomes (XY, the heterogametic sex). Males produce sperm with an X chromosome or a Y chromosome in equal numbers; eggs fertilized by a Y-bearing sperm develop into males and those fertilized by an X-bearing sperm develop into females. Thus, the primary biological role of the Y chromosome is sex determination (Tyler-Smith 2013). The genes involved in sex-determination are thus present in a pair of chromosomes that are indistinguishable under the microscope (Colorado Garzón *et al*. 2012). Therefore, it is possible to determine the sex of an individual by DNA analysis in these sex chromosomes using different methods (Dutta *et al*. 2017).

Over the last few decades, a number of different DNA-based techniques have been developed for determining the human sex. Although these techniques, especially PCR-based methods, have received particular attention, and have proved to be reliable, accurate, sensitive, and fast (Tagliavini *et al*. 1993, Appa Rao *et al*. 1995, Phua *et al*. 2003), several studies revealed that there are weaknesses and limitations (discussed below) of all the sequences and genes targeted for sex determination in humans. Therefore, laboratories should have a routine sex determination protocol and an alternative method to ascertain when first results are doubtful.

Based on the alignment of NCBI reference sequences of the DDX3Y gene (DEAD-Box Helicase 3 Y-Linked, a protein coding gene) (GenBank Accession No. NW_003104786.1) and DDX3X gene (DEAD-Box Helicase 3 X-Linked) (GenBank Accession No. NW_003104743.1), Gokulakrishnan *et al*. (2012) designed two pairs of PCR primers for amplification of the DEAD box protein gene DDX3. The first pair included DDX3-1F (forward) and DDX3-1R (reverse) primers, and the second pair has DDX3-2F as the forward primer and DDX3-2R as the reverse one. DDX3-1F and DDX3-1R primers generated a PCR fragment of 208-bp with the DDX3X gene as target sequence and 184-bp with DDX3Y gene, respectively. Whereas, DDX3-2F and DDX3-2R primers generated a PCR fragment of 171-bp with the DDX3X gene as target sequence and 147-bp with DDX3Y gene, respectively.

In this study, the efficiency of these two pairs of primers in their identification of human sex was investigated. The DDX3-based markers developed here can be used as be simple, precise and relatively cost-effective tools for sex determination in humans for a number of applications in forensics and a wide range of other applications.

MATERIAL AND METHODS

In order to test the efficiency of the DDX3 gene-specific primers, that helped Gokulakrishnan *et al*. (2012) identify sex in cattle meat, in determination of human sex, the amplification of the targeted regions by these primers has first to be optimised on cattle meat of known sex as established by the authors. Therefore, meat samples of cow (4 samples), calf (7 samples) were collected from the local market.

For validation of the efficiency of Gokulakrishnan *et al*. (2012)`s two primer pairs in identification of human sex, 60 fresh human blood samples (30 from women and 30 from men) were collected. A blind test of the protocol developed here was also carried out on ten human blood samples. The utility of human gender-specific markers generated here for detection of human embryo sex using DNAs isolated from 14-15-weeks old amniotic fluids was also detected in this study. For that, four DNA samples of amniotic fluids whose sexes (one female, two males and one hermaphrodite) were determined earlier (Chromo Quant® QF PCR kit v.3, Sweden) were used.

DNA isolation from meat samples of cow and calf

Genomic DNA was extracted from the meat samples of cow and calf according to the protocol of Asahida *et al*. (1996). DNA pelletes obtained were diluted twice to 100 - 150 µl with distilled sterilized water.

DNA isolation from human blood samples

DNA was isolated from human female and male blood samples by silica resin method (Psifidi *et al*. 2015, Price *et al*. 2009, Carpi *et al*. 2011).

Optimization of PCR on DDX3 in targeted animals

PCR was performed in 25 µl of reaction mixture containing PCR buffer [Tris-HCl 100mM (pH 8.8 at 25 °C) (NH4) 2SO4 50mM, Bovin Bromine Serum 0.005% Tween 20 0.00002%], 3 mM (MgSO4), dNTPs [0.15 mM for each of the nucleotides (dCTP, dGTP, dTTP and dATP, Fermentas)], 1 unit of Taq DNA polymerase (Fermentas manufacture), 50-100 ng of DNA and 0.6µ M of each primer. The two primer pairs developed by

Gokulakrishnan *et al*. (2012) were used. These are 1) DDX3-1F (5` aggaagccaggaaagtaa 3`) and DDX3-1R (5` catccacgttctaagtctc 3`), and 2) DDX3-2F (5` tgaggaagccaggaaagtaagtat 3`), and DDX3-2R (5` gcaccaccrtawaccacacaa 3`).

The amplification program using Eppendorf (Germany) machine was as follows (Gokulakrishnan *et al*. 2012): Initial denaturation of the DNA at 94 °C for 5 min followed by 34 cycles, each of which included: 1) denaturation at 94 °C for 45 sec, 2) annealing of primers at 53 °C for 45 sec, 3) extension at 72 °C for 1 min. Final extension for one cycle at 72 °C for 10 min was also carried out. The reaction products were kept at 4 °C until removal from the PCR machine.

PCR products (25 μL each) were then separated by electrophoresis on 2% agarose gel (Q-biogene, USA) that was run at 100 V for 2 h in 1x TAE buffer and visualized under UV lights using Gel Documentation System (GDS8000, UVP). A 100 bp DNA ladder (Vivantis) was used to indicate the positions and sizes of generated bands. The sizes of amplicons were determined compared to the 100 bp DNA ladder used on the same agarose gel.

Optimization of PCR on DDX3 in male and female humans

Following the PCR protocol mentioned above for amplification of DDX3 in animals using the two primer pairs (DDX3-1 and DDX3-2) of Gokulakrishnan *et al*. (2012), those primers were used for amplification of the same targeted regions in one sample of female human and one sample of male human.

Detection of sex-specificity of the developed DDX3-based markers for humans

The primer pair (DDX3-1F and DDX3-1R) that helped accurately, simply, and easily identify the sex of humans was used in the PCR applied on the DNA samples that are representatives of each sex (i.e., 30 male DNA samples and 30 female DNA samples) in order to detect the sex-specificity of the developed markers to humans.

Detection of the efficiency of DDX3-1-based markers for sex determination in amniotic fluids and the blind test

The primer pair DDX3-1F and DDX3-1R was used in PCR on DNA templates of the four amniotic fluid samples for determination of their sexes that were confirmed earlier using a commercially available kit. The protocol developed here was also subjected to a blind test on DNA templates isolated from 10 human blood samples.

RESULTS

Optimization of PCR on DDX3 in targeted animals

DNAs of good quality were obtained from meat samples of cow and calf. This enabled us to amplify the target DNA regions in all of these samples. As for DDX3-1F/DDX3-1R primers, two DNA fragments (208 and 184 bp bands) were observed in PCR products generated for calf (male) samples, whereas only the fragment of 208 bp was observed in the cow (female) samples (Fig. 1).

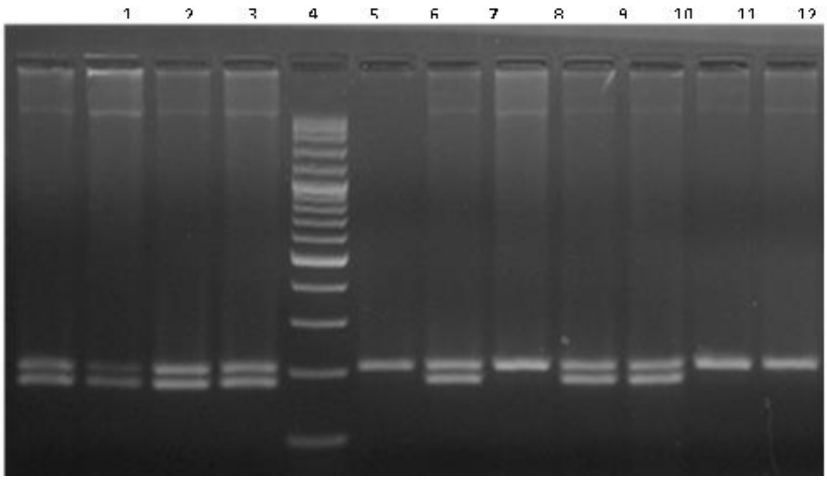

Fig. 1. PCR patterns of amplification products of DDX3-1 gene in 11 cow and calf meat samples. Lanes 1-4, 7, 9 and 10, PCR patterns in 7 calf samples; lane 5, a 100 bp DNA ladder; lanes 6, 8, 11 and 12, PCR patterns in 4 cow samples.

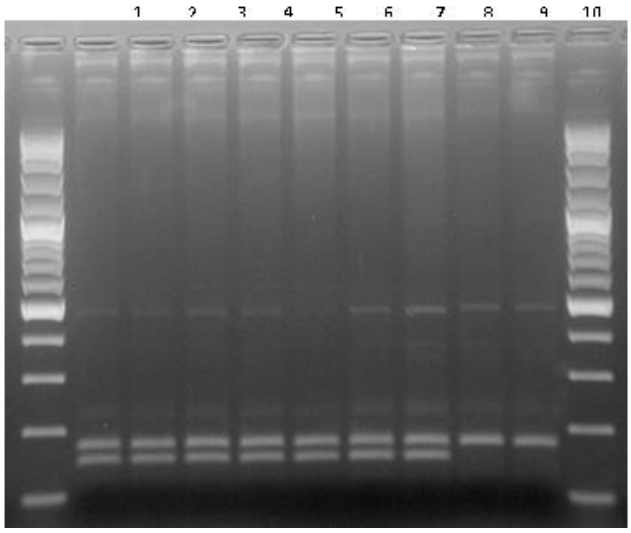

Fig. 2. PCR patterns of amplification products of DDX3-2 gene in 11 animal samples. Lanes 1 and 11, a 100 bp DNA ladder; lanes 2-8, PCR patterns in 7 calf samples; lanes 9 and 10, PCR patterns in 2 cow samples.

When DDX3-2F/DDX3-2R primers were used on meat samples of cow and calf, two fragments (171 and 147 bp) were generated in PCR products of calf (male) samples, whereas only the fragment of 171 bp was observed in the cow (female) samples used (Fig. 2).

Optimization of PCR on DDX3 in male and female humans

The good quality of DNAs extracted from bloods of male and female human samples enabled us to amplify the target DNA regions in all of these samples. Using DDX3-1F and DDX3-1R primers (Fig. 3), a single PCR fragment was generated for the female human sample (208 bp), whereas in the male human sample, two bands were generated; the first one was of the same size (208 bp) of that observed for the female sample and the second was 184 bp. Hence, it was possible to easily and simply differentiate between the two genders of humans using this primer pair.

Detection of sex-specificity of the developed DDX3-based markers for humans

When the primer pair DDX3-2F and DDX3-2R was used for differentiation between human male and female, PCR products with identical patterns were generated in the two genders (Fig. 4).

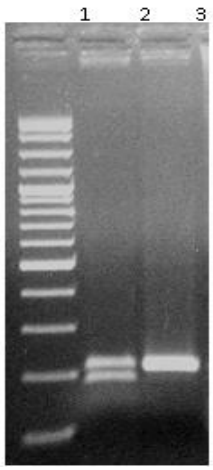

Fig. 3

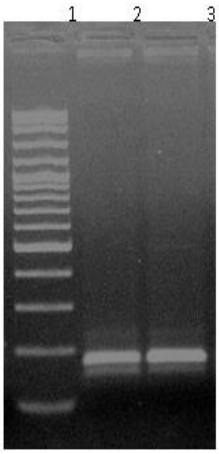

Fig. 4

Fig. 3. PCR patterns of amplification products of DDX3-1 gene in male and female humans. Lane 1, a 100 bp DNA ladder; lane 2, PCR pattern in male human; lane 3, PCR pattern in female human.

Fig. 4. PCR patterns of amplification products of DDX3-1 gene in male and female humans. Lane 1, a 100 bp DNA ladder; lane 2, PCR pattern in male human; lane 3, PCR pattern in female human.

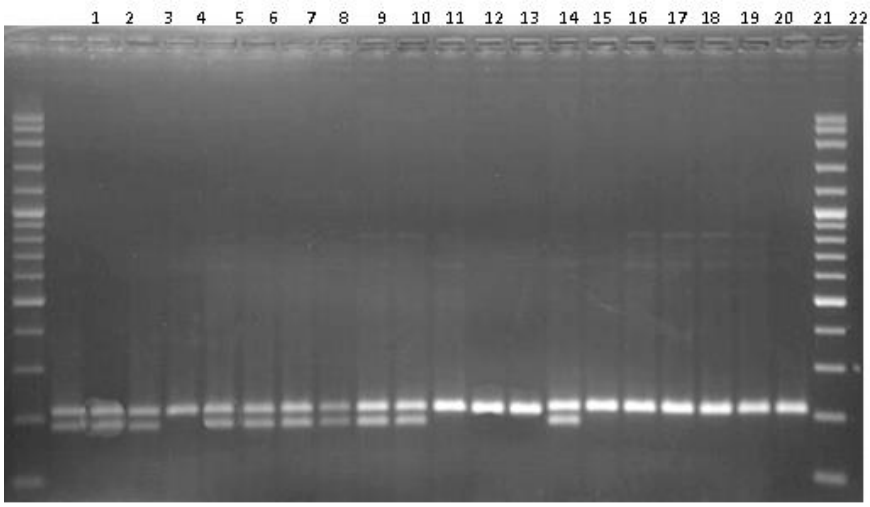

Fig. 5. PCR patterns of amplification products of DDX3-1 gene in male and female humans. Lane 1 & 22, a 100 bp DNA ladder; lanes 2-4, 5-11, and 15, PCR patterns in human male samples; lanes 5, 12-14, and 16-21, PCR patterns in human female samples.

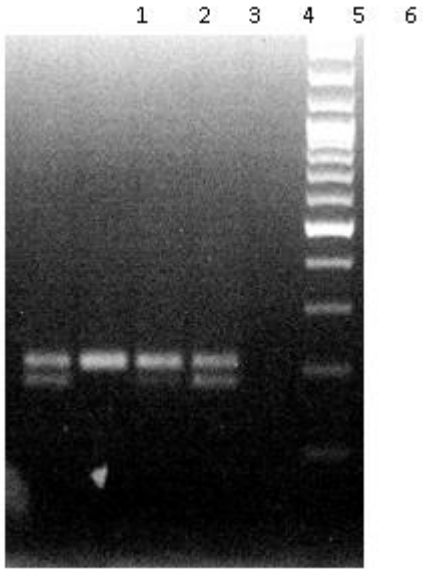

Fig. 6. PCR patterns of amplification products of DDX3-1 gene in humans. Lanes 1 and 4, PCR patterns in amniotic fluids of the women with males; lane 2, amniotic fluid of the woman with female; lane 3, amniotic fluid of the woman with hermaphrodite; lane, 5 a negative control; lane 6, a 100 bp DNA ladder.

PCR that was carried out on DNA templates that represent 60 human samples (30 females and 30 males)using DDX3-1F and DDX3-1R primers generated identical PCR patterns within each gender (Fig. 5)

Detection of the efficiency of DDX3-1-based markers for sex determination in amniotic fluids and the blind test

The primer pair (DDX3-1F and DDX3-1R) that helped accurately and easily identify the sex of humans was used for PCR using four amniotic fluid DNAs as templates. In the amniotic fluid of the woman with hermaphrodite, the two PCR fragments generated for the human male were observed but the smaller fragment (184 bp) was fainter than its counterpart in the normal male as visualized on agarose gel. As for the two amniotic fluids of the women with males, the PCR pattern of male (208 and 184 bp fragments) was observed. The PCR pattern of female (only 208 bp fragment) was detected in the amniotic fluid of the woman with female (Fig. 6). This implies that the sexes of all analyzed amniotic fluid samples were in accordance with their sexes as determined using the commercial kit.

When the protocol optimised here for determination of humans sex based on DEAD box protein gene (DDX3) was subjected to a blind test on DNA templates isolated from 10 human blood samples, 100% concordance was revealed.

DISCUSSION

Analysis of DNA using DNA amplification is possible even at the level of a few cells which may present in forensic samples such as semen stains, vaginal swabs, bloodstains, and head hair bulbs (Pfitzinger *et al*. 1993). DNA regions that differ between male and female individuals are essential features in PCR sex determination (Gokulakrishnan *et al*. 2012). Several PCR-based methods for sex determination have been established for humans by analyzing Y-specific target sequences on the Y chromosome and/or X-specific target sequences on the X chromosome. For example, Pfitzinger *et al*. (1993) have introduced a co-amplification method using X-specific repetitive sequences from DXS424 and Y-specific repetitive sequences from DYZ1 for DNA-based sex determination analysis. In 2001, Honda *et al*. also targeted Y-specific repetitive sequences from DYS14 and DYZ3 for the same purpose.

Witt and Erickson (1989) believe that the only repetitive DNA family that show chromosome specificity is the alphoid satellite family. Those centromeric alphoid repeats have been another target for sex determination in humans as established by Witt and Erickson (1989) when they analysed Y-chromosome-specific and X-chromosome-specific fragments from dried blood specimen. Similarly, in 1995, Neeser and Liechti-Gallati targeted α-satellite DNA from X and Y chromosomes in bloodstains, hair roots cigarette butts, vaginal swabs, and bones. Human sex was also determined in pure and mixed blood samples and stains by Steinlechner *et al*. (2002) through PCR amplification of the X-specific and Y-specific alphoid sequences (XY-PCR).

Other examples of studies that used DNA for sex determination are those in which zinc-finger protein genes (ZF) were analysed. For instance, Aasen and Medrano (1990) have amplified, using universal primers, 447 or 445 bp fragments from male or female genomic DNA corresponding to the ZFY or ZFX genes. Restriction fragment length polymorphism (RFLP) analysis of the fragments yielded specific banding patterns between the two sexes. Reynolds and Varlaro (1996) and Stacks and Witte (1996) also targeted ZF genes in their studies on human sex determination.

Short tandem repeat (STR) typing compares DNA loci from multiple samples. The probes that attach to special areas on the DNA measure the number of repeats of a special unit whose length can be detected by PCR analysis. Discrimination between related and unrelated people is carried out using the difference in autosomal repeated units. Y-STR can be used to determine sex (Pilli *et al* 2018) as illustrated by Delfin *et al*. (2005) who used Y-STR to differentiate between the assailant and victim in males and for proving male sexual harassment in females.

The sex-determining region Y (SRY) gene, which is a Y chromosome-specific sequence, is a master-switch sex-determining gene that is highly conserved in nearly all mammals (Foster and Graves 1994). In most mammals, the male is identified by amplifying the SRY gene (Hawkins 1994). SRY has been used as sex-specific genetic markers in human (Berkovitz *et al*. 1992, Drobnic 2006, Thangaraj *et al*. 2002). Although many genes are involved in sex determination in humans, SRY is the key switch because individuals with SRY develop into males, while when it is absent the individuals develop into females (Tyler-Smith 2013). The discovery of SRY has also been crucial to understanding disorders of sex development (DSD). MCRI (2015) reported that SRY is very important to the whole field of human molecular genetics.

Settin *et al*. (2008) described the PCR-based sex determination identified by the presence (male) or absence (female) of the SRY gene. They revealed that when the results are negative from amplification of only the SRY gene, it cannot be assumed that the individual is female or that there was a mistake in the experimental process. They therefore asserted that this method requires an internal control to verify the absence of the SRY gene or the failure of amplification and thus confirm the validity of the results. Pfeiffer and Brenig (2005) also stressed that in addition to SRY gene, another gene present in both, males and females, should be amplified in the same tube as a positive control in order to assure the absence of SRY gene or PCR faliar and hence verify the results.

One of the most common and routinely used DNA-based sex typing methods that have been proposed to

target X-Y homologous genes with insertion deletions is analysis of the amelogenin gene (AMEL: AMELX and AMELY) (Akane *et al*. 1991, Sullivan *et al*. 1993, Haas-Rochholz and Weiler 1997, Tozzo *et al*. 2013). PCR analysis that target regions of AMEL gene has become the method of choice for sex determination of biological samples (Eleanor 2006). It has been routinely performed in forensic casework, prenatal diagnosis and DNA data basing (Steinlechner *et al*. 2002, Drobnič. 2006).

The AMEL gene is a single-copy gene located on Xp22.1-Xp22.3 and Yp11.2; the simultaneous detection of the X and Y alleles using PCR can lead to gender determination (Tozzo *et al*. 2013). The design of distinct X- and Y-specific PCRs is facilitated by a small deletion in the first intron on the Y allele (Faerman *et al*. 1995).

Added to the widespread use of the AMEL sex test for forensic practice, it is also the most widely used in anthropological studies (Sullivan *et al*. 1993, Dutta *et al*. 2017, Chowdhury *et al*. 2018). The most commonly used amelogenin primer sets were developed by Sullivan *et al*. (1993). These primers target a 6-bp insertion/deletion within an intron of the AMEL gene on the X and Y chromosomes (AMELX and AMELY) and produce 106-bp and 112-bp amplicons for the X and Y chromosomes, respectively (Masuyama *et al*. 2017). The AMEL gene, which exists on both X and Y chromosomes, has been used to determine the sex in cattle (Eleanor 2006) and humans (Leung 2008).

Although it is easy to amplify in the case of the AMEL gene with a moderate difference in amplified product size between males and females, several studies have raised doubts about the reliability of the analysis of the AMEL gene because normal males may be typed as females with this test: AMELY deletions may result in no product of amplification and normal males being typed as female as a result of the test (negative male). Santos *et al*. (1998) reported that a result of a deletion polymorphism, their assay incorrectly typed some males that lacked the Y copy of the AMEL gene as females. The failure rate of the AMEL sex test was 0.018% in another study that was carried out by Steinlechner *et al*. (2002).

With potential difficulties in interpreting the results and gender misinterpretation, the use of only AMEL marker for sex determination in humans may cause troblues in clinical practice and in forensic casework (Tozzo *et al*. 2013). Tozzo *et al*. (2013) environm the different strategies proposed in the literature in case of doubt regarding the presence of deleted AMEL in the DNA profile. They proposed a method for the identification of samples with deleted AMEL that can be applied, as an additional assay, in case of doubt regarding PCR results of sex determination.

In 2000, Roffey *et al*. reported the results from a case where a mutation in the annealing region of the AMEL primers appeared to have resulted in the failure to amplify the amelogenin Y-homolog from a phenotypically normal male. The result was confirmed using two different primer sets that amplify different regions of the AMEL gene. This situation suggests that the genetic determination of sex based on the AMEL sequences from specimens of unknown origin, such as crime scene samples, should not be considered infallible. Michael and Brauner (2004) also environm the failure of amelogenin sex test on a phenotypically normal male. In another study, the success rate of AMEL analysis for human sex genetic identification was only 33.3%. (Zagga *et al*. 2013).

Drobnič (2006) belives that mutational influences or potential discrepancies between biological and legal sexmay may cause the AMEL gene test not to be always accurate on the determination of whether the individual is male or female and therefore, not completely reliable. Therefore, he designed a new pair of primers that target a section of the SRY gene resulting in 96-bp male-specific PCR fragment that can be coamplified with multiplex STR kits which assures unambiguous and quick gender identification.

Dropout of the AMEL Y-specific allele due to an interstitial deletion of the Yp involving the AMELY locus can also cause misidentification of sex genotype. This may have serious consequences in criminal investigations and personal identification processes (Inturri *et al*. 2009). Chowdhury *et al*. (2018) stated that a high prevalence of deletions and other variations in the Y chromosome is quite common, some of which affect Yp11.2, the locus of the AMEL gene. This induces males to be wrongly identified as females because of similarity in AMEL profile.

Because incorrect conclusions regarding the sex associated with the sample using AMEL gene test is common due the frequent large-region deletions in the

Y chromosome, Masuyama *et al*. (2017) developed a new method of sex determination by detecting a single-nucleotide polymorphism in exon 2 of the AMEL gene using amplified product-length polymorphisms (APLP) in combination with the SRY gene as an alternative Y-specific marker. They designed amplicon size to be less than 60 bp to make the method more useful for analyzing degraded DNA samples. Although they bielive that their novel method was the most robust for highly fragmented DNA samples, they could correctly identify the sex of 11 out of 14 individuals when this method was applied to 14 Jomon individuals (3500-year-old bone samples) whose sex had been identified morphologically.

Due to the negatives of SRY and AMEL tests for sex determination in humans, Steinlechner *et al*. (2002) believe that the ideal standard for gender determination is Y-STR (Jia *et al*. 2005) and SRY (sex-determining regions of Y) testing that have to be performed in conjunction with the AMEL testing. This means that for reliable and accurate determination of human sex more than one DNA test is required. Based on this and on the on what stated above regarding the short comings of the sequences and genes targeted for sex determination in humans, laboratories should have a routine sex determination protocol and an alternative method to ascertain when first results are doubtful.

The DEAD-box protein family includes proteins found in most prokaryotes and all eukaryotes. Their essential roles in almost all aspects of RNA metabolism, from transcription, splicing and decay, to translation resulted in their wide spread conservation. Nine conserved motifs have been identified in the helicase core of DEAD-box proteins (Garbelli *et al*. 2011). The DDX3 helicase belongs to the large DEAD-box (Asp-Glu-Ala-Asp) family of ATP-dependent RNA helicases (Kukhanova *et al*. 2020). The human DDX3 helicase was first identified in 1997 (Park *et al*. 1998). It plays crucial roles in tumor proliferation and viral infections and has been identified as a major cofactor for HIV-1 replication (Garbelli *et al*. 2011). DDX3 also appeared to Bol *et al*. (2015) to be one of the most multifaceted helicases with various roles in immunology and cancer. Now, DDX3 is considered as an attractive object for the development of novel pharmaceutical drugs (Kukhanova *et al*. 2020).

In this study, we developed PCR-based markers that show different amplification patterns between male and female humans based on one PCR experiment for amplification of human DDX3 using primers that were designed and used by Gokulakrishnan *et al*. (2012) for determination of sex in catte meat. When female DNA was used as a template for the PCR reaction, only the expected 208 bp X-chromosome-specific fragment was detected, while with male DNA both the expected 184 bp Y-chromosome-specific and the X-chromosome-specific fragments were detected. We confirmed the sex-specificity of developed markers and hence the accuracy and reliability of the method optimised here because there was 100% success rate of genetic sex identification for the human male and female samples analysed.

For generation of developed PCR markers, only one pair of primers was used for the amplification of targeted regions in DNA extracted from male and female human blood samples. For environmentn of amplicons only agarose gel electrophoresis was used and no sophisticated softwares or equipments related to such softwares were needed.

Because there is an urgent need to develop an appropriate strategy to distinguish between negative amplification and non-amplification resulting from failure of PCR, especially when the specialized Y chromosome anchors are used. The advantage with DDX3 gene over SRY gene for human gender identification is its presence on both sex chromosomes. Hence, there is no need of developing a suitable strategy for differentiating no amplification due to PCR failure from the negative amplification (Gokulakrishnan *et al*. 2012). In other words, there is no need for a positive control in the method applied here.

The methods currently used to determine the human sex either need commercially available ready-made kits that are very expensive and/or inaccurate, and therefore they are not always reliable, which requires to accurately determine the human sex to combine more than one method. Analysis of DDX3 gene targeted here does not need kits nor sequencing nor RFLP. Added to that, there is an ease of amplification with a clear difference between males and females in the pattern of amplification profile. More importantly, false positive and/or false negative did not occur. Based on these facts, the DDX3 gene seems to be a unique alternative because of easy mplification with a moderate difference in amplified product size between

male and female as also revealed for sex determination in cattle meat (Gokulakrishnan *et al*. 2012).

Ancient DNA samples are frequently broken down into small fragments and are small in quantity (Pääbo *et al*. 2004) because DNA is easily degraded by environmental factors and microbial attack at archeological sites. Fragmented forensic samples also make it difficult to investigate criminal cases. Therefore, the shortening of PCR products is essential for DNA analyses with highly fragmented samples. Masuyama *et al*. (2017) belive that with conventional methods for sex determination in humans, it is difficult to determine sex from highly fragmented/degenerated DNA samples or from small sample quantities because DNA testing depends heavily on the quality and quantity of DNA samples. The average fragment length of ancient DNA is between 100 and 500 bp (Pääbo 1989, Pääbo *et al*. 2004). The method developed here may be useful even in highly degraded samples of biological traces DNA.

The human sex-specific markers generated here could be applied to any biological trace in forensic investigations or any other application requiring human sex determination. To our knowledge, this is the first study of its kind to elucidate or test the usability of the targeted gene (DDX3) for determining human sexuality.

CONCLUSION

Human gender identification has important applications in forensic casework, prenatal diagnosis, DNA databasing, and blood sample storage (Steinlechner *et al*. 2002, Michael and Brauner 2004). Simple and precise methods for sex determination in humans are a pre-requisite for a number of applications in forensics. In this study, we have developed a simple, rapid, accurate and reliable test for sex diagnosis in humans using the PCR technique for amplification of the DEAD box protein gene DDX3 (DDX3Y and DDX3Y) that was established in an earlier study as valuable tool for sex determination in cattle meat. This PCR method has demonstrated 100% gender specificity, and more importantly, false positivity and/or false negativity did not occur. We proved the reliability and accuracy of the protocol developed here when the blind test showed 100% concordance.

The advantage of this assay is that neither additional control amplicons with a second locus-specific autosomal primer pair nor restriction endonuclease steps or other other DNA analysis tools are necessary for sex determination and control of the PCR reaction. The use of this assay will make the sex determination in humans simpler and much less complicated, since only one pair of primers is required to amplify the different size fragments of the DED box protein gene that are visualised on agarose gel.

Based on the findings of this study, we can suggest that our human sex determination analysis only by PCR using a single pair of primers is a highly sensitive and specific method for gender identification and can be extremely useful for large-scale screening and it has applications in the analysis of biological evidence in forensic science and all fileds where human sex identification is crucial. We also proved the validity of this analysis for determination of sex of human embryos using DNA extracted from amniotic fluids of pregnant women. Since the method developed here was efficient for sex determination of human embryos using DNA extracted from amniotic fluid of pregnant women, it should also be applicable on DNAs extracted from maternal blood plasma taken from pregnant women as early as the 10th week of pregnancy.

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