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ORIGINAL ARTICLE

"GenderPlex" a PCR multiplex for reliable gender determination of degraded human DNA samples and complex gender constellations

Anna Esteve Codina · Harald Niederstätter · Walther Parson

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Abstract The amelogenin test integrated in most commercial polymerase chain reaction (PCR) multiplex kits is routinely used in the forensic field for gender determination of DNA samples. It has been demonstrated that this test is not entirely reliable. Males with deletions in the homologous amelogenin part on the Y chromosome (AMELY) were erroneously typed as females due to lack of Y-specific amelogenin amplification. Also, primer binding site mutations that result in a failure to amplify the AMELY or the X-chromosomal part (AMELX) have been observed. For clarification of such phenomena, a new PCR multiplex (GenderPlex) is presented, co-amplifying two different regions of the amelogenin gene (55/58 and 106/112 bp for the AMELX and AMELY alleles, respectively), a 93-bp sequence stretch of the *SRY* gene and four mini-X-STR loci DXS7424, DXS8378, DXS6803 and GATA172D05 (maximum product size less than 140 bp). This strategy helps with the evaluation of samples for the presence of amelogenin-based primer site mutations and confirms a male genotype by the absence of heterozygote X-STR alleles and the presence of an SRY-related peak. The short amplicon sizes of all involved loci proved to be beneficial in a study on artificially degraded DNA. Furthermore, we demonstrate by means of sensitivity, human specificity and mixture studies that the multiplex is suitable for investigations in the forensic scene. Finally, the performance of the GenderPlex was evaluated on a west Eurasian population sample from Austria comprising 166 male and 104 female individuals.

Keywords GenderPlex · PCR multiplex · Gender determination · Degraded human DNA samples · Complex gender constellations

Introduction

The amelogenin gene has two homologous alleles, one on the X chromosome (AMELX) and the other on the Y chromosome (AMELY), differing in both size and sequence. The AMELX gene is located between positions Xp22.31–p22.1 (GenBank accession number M55418) and AMELY is located at Yp11.2 (GenBank accession number M55419; [1]). A commonly used polymerase chain reaction (PCR) primer pair developed by Sullivan et al. [2] amplifies a 106-bp long sequence stretch in intron 1 of AMELX and a 112-bp target within AMELY. Since the female genotype is usually XX, only a single AMELX peak is observed when testing females, whereas males usually possess both X and Y chromosomes and, therefore, exhibit two peaks with a standard amelogenin test. This test is integrated in almost all commercially available STR multiplex kits that are routinely used in medical genetic laboratories, forensic casework, prenatal diagnosis and transplantation medicine. But the amelogenin sex test is not entirely reliable. It has been reported that it produces misleading results when a primer binding site mutation is present on AMELX or AMELY [3–6] and—more frequently—fails to amplify AMELY due to large-scale deletions in the Y-chromosomal homologue [7–19]. Even though the frequency of these cases is reported to be low in Caucasian populations, they

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A. Esteve Codina · H. Niederstätter · W. Parson (✉)
Institute of Legal Medicine, Innsbruck Medical University,
Müllerstrasse 44,
6020 Innsbruck, Austria
e-mail: walther.parson@i-med.ac.at

can reach appreciable levels in other populations [20, 21]. In some cases, the investigative aspect of the gender of a sample is so important that one may desire to use an alternative approach, such has been proposed by, e.g. [22] or [23]. In this study, an assay is pursued that uses redundant X and Y chromosome-specific testing.

Materials and methods

X-STR selection

Possible X-STR candidates were found at "ChrX-STR.org" ([24]; http://www.chrx-str.org/), a forensic web-database for X chromosome STRs. The selection of our loci was based on criteria involving small amplicons (sizes <140 bp), high heterozygosity levels, balanced frequency distribution and high polymorphism content. The compatibility of the primer sequences for co-amplification was checked with AutoDimer [25]. Chromosomal localisations of the X-STRs were determined with UCSC In-Silico PCR (http://genome.ucsc.edu/) and their linkage group affiliations are according to [26].

GenderPlex PCR amplification

The four X-STR loci DXS7424, DXS8378, DXS6803 and GATA172D05, a 93-bp segment of the *SRY* gene and two targets in the amelogenin region were co-amplified in a total reaction volume of 20 μL including 1x PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 5 μg non-acetylated BSA and 2 U AmpliTaq Gold DNA polymerase (Applied Biosystems AB, Foster City, CA, USA). Primer sequences and their assay concentrations are given in Table 1. To promote the non-template addition of dATP by the *Taq* polymerase, PIGtails [27] were added to the primer sequences for some X-STR markers, i.e. a GTTTCTT sequence was attached at the 5′ end of the reverse primers of DXS7424, DXS8378 and DXS6803 and a 5′ GTTT sequence was included in the forward primer of SRY. PCR was performed in a thermal cycler (BioRad, Hercules, CA, USA) using 30 cycles of 95°C for 15 s, 54°C for 30 s and 72°C for 45 s and a final incubation at 72°C for 60 min following initial denaturation at 95°C for 10 min. Of the amplification products, 2-μL aliquots were combined with 20 μL deionised formamide including 0.5 μL internal lane standard (GeneScan 500 LIZ), heat-denatured at 95°C for 3 min and subjected to laser-induced fluorescence capillary electrophoresis on an ABI PRISM 3100 Genetic Analyzer using POP6 and default conditions. Data were analysed using GeneScan Analysis version 3.7 and GenoTyper version 3.6 (both AB). In the sensitivity, mixture and degraded DNA studies, singleton amplifications were performed.

Allelic ladder construction and sequencing

For sequence analysis and generation of the X-STR allelic ladder, non-fluorescent singleplex amplifications were carried out on 174 samples from male Austrian platelet donors [28]. The DXS8378 alleles 9–15 were re-amplified from the allelic ladder included in the Mentype Argus X-UL kit [29]. Cycle sequencing reactions were performed on enzymatically treated PCR products using 30 cycles of 96°C for 10 s, 50°C for 5 s and 60°C for 4 min following an initial step at 96°C for 1 min. Sequencing primers are given in Table S1 of the Electronic supplementary material. We modified the nomenclature for the locus DXS7424 ([30]; $(TAA)_n$) to the notation $(AAT)_n$ following the recommendations of the International Society for Forensic Genetics [31].

Table 1 Primer sequences and assay concentrations used in the GenderPlex

Primer	Concentration in assay (nM)	Primer sequence	Label	Reference
AmeloSull_F*	100	CCCTGGG*CTCTG*TAAAGAATAGTG	FAM	[2]
AmeloSull_R	100	ATCAGAGCTTAAACTGGGAAGCTG		
SRY_GTTT_F	150	GTTTAGGCACAGAAATTACAGGCCATGC		This study
SRY_R	150	TGCAATTCTTCGGCAGCATCTTCG	FAM	
AmeloShort_F	200	ACCCCTTTGAAGTGGTACCAGAGCAT	FAM	This study
AmeloShort_R	200	GAACAAAATGTCTACATACYGGTGG		
DXS7424_F	400	AAAACAGGAAGACCCCATC	VIC	[39]
DXS7424PIG_R	400	GTTTCTTGGCTAAGAAGAATCCCGCACA		
DXS8378_F	300	TTAGGCAACCCGGTGGTCC	VIC	[24]
DXS8378PIG_R	300	GTTTCTTACAAGAACGAAACTCCAACTC		
DXS6803_F	300	GAAATGTGCTTTGACAGGAA	NED	[24]
DXS6803PIG_R	300	GTTTCTTCAAAAAGGGACATATGCTACTT		
GATA172D05_F	500	TAGTGGTGATGGTTGCACAG	PET	[40]
GATA172D05_R	500	ATAATTGAAAGCCCGGATTC		

Italicized bases indicate positions for which sequence variation was found in this study (nt 8: C>A on one Y chromosome; nt 10: C>T on one X chromosome)

Results and discussion

We developed a PCR multiplex (GenderPlex) for the unambiguous gender determination of human DNA samples. Within the assay, two different regions of the amelogenin gene are amplified together with a 93-bp target sequence in the *SRY* gene and the four mini-X-STR loci DXS8378 (chrX: 9330261–9330378, p22.31, linkage group 1), DXS6803 (chrX: 86317826–86317938, q21.31, linkage group 2), DXS7424 (chrX: 100505512–100505604; q22.1, linkage group 2) and GATA172D05 (chrX: 113061249–113061368, q23, linkage group 2). The first segment in the amelogenin gene (located in intron 1) is amplified by the commonly used Sullivan primers [2], yielding 106 and 112 bp PCR products for AMELX and AMELY, respectively. Moreover, to make the system more robust, a new pair of primers was designed to amplify another region of the gene (located in intron 2/exon 2) yielding 55 and 58 bp amplicons for AMELX and AMELY, respectively. Independently from this study, the same GAT insertion/deletion was also targeted by [32] in a pyrosequencing assay for amelogenin-based sex determination. With this approach, the sex of a sample can be evaluated using multiple targets. In this way, the negative effect of primer binding site mutations on successful amplification of alleles can be significantly minimised, as pseudo null alleles are unlikely to occur in both reactions of the amelogenin assay. Thus, at least one of the two amplification systems would correctly report the gender of the sample. In addition, the presence of the SRY amplification product would confirm the male gender of a sample whereas its absence would usually verify the female nature of a sample. These three targeted amplicons alone would characterise a female sample only by lack of amplification products (i.e. failure to amplify both AMELY and SRY). Therefore, we added four X-STR loci selected by short amplicon lengths (DXS6803: allele 10, 109 bp–allele 14.3, 128 bp; DXS7424: allele 10, 76 bp–allele 19, 103 bp; DXS8378: allele 6, 102 bp–allele 14, 134 bp; GATA172D05: allele 6, 108 bp–allele 13, 136 bp) and high levels of heterozygosity. One would expect at least some of these loci to be heterozygous in females. In fact, in a total of 104 investigated females, no sample displayed homozygous allelic states for all four X-STR markers.

The GenderPlex was applied to samples from eight phenotypic male individuals that gave ambiguous amelogenin results with the AmpF*ℓ*STR SGM Plus PCR amplification kit (AB). Of these samples, seven failed to amplify the AMELY Sullivan PCR product (X0 males) and one sample that did not yield the AMELX amplicon (0Y male). Of the seven X0 males, six were published previously [8] and all of them gave a positive signal for the SRY peak. However, the lack of any positive AMELY result was confirmed for both amplicons using the GenderPlex (Fig. S1 of the Electronic supplementary material; Table 2). Together with the absence of a second peak for all four X-STR markers, it is likely that these males (Y-STR minimal haplotype reported in [8]) harbour a deletion polymorphism on the Y chromosome that caused the erroneous amelogenin-based gender prediction. The sixth X0 male from [8], i.e. X0 2 (Table 2) failed to yield both AMELY PCR products in the GenderPlex and exhibited heterozygous allelic states for all four X-STRs. However, a clear SRY peak was observed for this sample. Taking the previously observed absence of Y-STR amplification for this individual showing a male phenotype [8] into consideration, these results suggest a lack of the Y chromosome accompanied by a translocation of SRY to another chromosome. Finally, the seventh X0 male (X0 7; Table 2) gave a typical male profile with the GenderPlex, displaying AMELX and Y products for both primer pairs. A C>A transversion at nucleotide position 8 in the Y-chromosomal binding site of the forward Sullivan amelogenin primer was found by sequencing analysis. For the 0Y sample, a C>T transition at position 10 in the X-chromosomal Sullivan forward primer binding site explained the lack of the AMELX peak when using the SGM Plus kit. In these cases, the GenderPlex was found to be more robust against sequence variations in the Sullivan primer binding sequences, and a full male profile was obtained for the SGM Plus 0Y sample.

Table 2 GenderPlex genotypes of samples from west Eurasian individuals showing male phenotype and ambiguous amelogenin results with the AmpF*ℓ*STR SGM Plus PCR amplification kit (Applied Biosystems, Foster City, CA, USA)

Sample	Amelogenin short	SRY	Amelogenin Sullivan	DXS7424	DXS8378	DXS6803	GATA172D05
X0 1	X, 0	SRY	X, 0	16	11	13.3	8
X0 2	X, 0	SRY	X, 0	14, 16	11, 12	11, 12.3	8, 10
X0 3	X, 0	SRY	X, 0	15	11	10	8
X0 4	X, 0	SRY	X, 0	14	12	12	6
X0 5	X, 0	SRY	X, 0	16	11	12	10
X0 6	X, 0	SRY	X, 0	15	11	12.3	11
X0 7	X, Y	SRY	X, Y	16	10	14.3	8
0Y	X, Y	SRY	X, Y	15	12	11	12

Sensitivity study

A sensitivity study was carried out using the GenderPlex with initial genomic DNA amounts of 10 ng, 5 ng, 2.5 ng, 1.75 ng, 875 pg, 438 pg, 219 pg, 109 pg and 55 pg. Two studies were performed in parallel, one for a male sample and one for a female sample. Both in-house DNA extracts from single donor venous blood were quantified with real-time PCR as described in [33]. Unambiguous and reproducible results were obtained with 109 pg of genomic DNA using 30 PCR cycles for both sexes. Some interpretable profiles were also obtained with 55 pg of initial template DNA. However, for this DNA amount, allelic drop-out was observed for DXS6803 and SRY.

Degraded DNA study

An experiment on artificially degraded DNA (2 ng/GenderPlex assay) showed that DNase I treatment (1.2 μg genomic DNA, 2.4 U Turbo DNase; Ambion, Austin, TX, USA) for 4 min at 20°C resulted in a complete loss of the DXS6803 allele for a male sample, which was not the case for a female sample. After 8 min of DNase digestion, only the lowest molecular weight systems such as AMELX, AMELY (55 and 58 bp) and SRY amplicons (93 bp) were observed. The female sample still gave slightly better results after 8 min DNase treatment. Even larger amplicons were present, albeit with reduced peak heights. We have been using the enzymatic DNA fragmentation model for the evaluation of other PCR-based tests [34, 35] and found that the GenderPlex gave best results due to the very short amplicons generated.

Human specificity study

DNA samples from 41 different vertebrate species were amplified with the GenderPlex in order to determine human specificity (Table S2 of the Electronic supplementary material). As expected, samples from chimpanzee, gorilla, and orangutan gave amplification products for almost all loci. AMELX versions of 55 and 106 bp were present for the three primates, but none of them yielded AMELY products, although a SRY peak was present for the chimpanzee. Generally, no amplification products were observed for the remaining animal species tested with the exception of some peaks that were observed outside the human allele categories and might represent X-STR specific bands. In some cases, the conventional amelogenin assay [2] amplified a single peak for the amelogenin locus which corresponds to a 102- to 103-bp amplification product, very similar in size to the 106-bp human AMELX peak. This phenomenon has been described earlier [36]. It has also been demonstrated that the amelogenin test cannot be used for the determination of the sex in animals [37] and our results prove that GenderPlex does not provide information about the gender of the tested animals.

Mixture study

DNA admixture studies—comprising a male/male mixture, a female/male mixture with shared alleles, a female/male admixture with a minimised number of shared alleles and a male/female mixture, all at ratios of 100:0, 99:1, 95:5, 90:10, 80:20, 50:50 and 0:100—were performed. All pure and admixed samples were used at a concentration of 2 ng per 20 μL assay and 30 cycles of PCR amplification were applied. DNA mixtures between males showed drop-out of non-shared X-STR alleles for the minor contributor at the 5% proportion. In female/male DNA mixtures, the minimum male contribution allowing for the detection of the SRY and the AMELY peaks was 5%. In some cases, also the tri-allelic patterns of non-shared X-STR alleles allowed for mixture detection. This was again the case when the minor male contribution amounted to at least 5% of the total DNA input. In the male/female DNA admixture, the detection limit for the female minor fraction was nearer 10% than 5%.

Population study

We tested DNA from 270 individuals (166 male and 104 female west Eurasian platelet donors from the Austrian Blood Bank) to evaluate the performance of the GenderPlex. The results of the X-STR study are summarised in Tables S3 (allele frequencies), S4 (male profiles) and S5 (female profiles) of the Electronic supplementary material. In one male individual, the locus GATA172D05 failed to amplify, both with the amplification primers and the sequencing primers, which suggests a possible deletion

Table 3 GenderPlex results for commonly used reference DNA preparations

Reference DNA	Amelogenin Short	SRY	Amelogenin Sullivan	DXS7424	DXS8378	DXS6803	GATA172D05
007	X, Y	SRY	X, Y	16	10	12.3	6
K652	X, X	–	X, X	17, 17	10, 10	10, 10	12, 12
9947	X, X	–	X, X	14, 16	10, 11	11.3, 12	10, 10
9948	X, Y	SRY	X, Y	16	11	13	6

rather than a primer binding site mutation. Furthermore, the as yet unobserved allele 6 was found for locus DXS8378 during the construction of the allelic ladder (different DNA pool) but not in the population study. The gender determination was unambiguous in all tested samples. Levels of heterozygosity in females for the X-STR loci DXS7424, DXS8378, DXS6803 and GATA172D05 were calculated (PowerStats) and the observed heterozygosity rates were 67%, 73%, 81% and 76%, respectively. In a total of 104 investigated females, we observed 102 unique X-STR genotypes, whereas in the male samples, 112 out of 166 samples gave unique X-STR profiles. None of the investigated females was homozygous in all four X-STRs, which confirms the usefulness of these markers as a positive control for the presence of a female DNA. As the incorporation of reference DNA is crucial to the validation of any DNA typing protocol [38], the GenderPlex profiles for the commonly used control DNA samples 007 (AB), K652 (Promega, Madison, WI), 9947A (Promega) and 9948 (Promega) are given in Table 3. Interestingly, the female cell line K562 has lost heterozygosity for all investigated X-STR loci.

Conclusions

The amelogenin test that is included in most commercial PCR multiplex kits is generally a reliable tool to determine the gender of a sample. In rare cases that can sometimes reach higher frequencies when affected Y lineages are under investigation, this test produces false-positive female DNA profiles or fails to amplify the AMELX. In such cases, misleading information to investigative leads may be offered or problems with reporting the evidence may arise. Based on the requirement to further investigate observed instances of such results, we developed GenderPlex, a multiplex PCR that combines two independent systems for the amelogenin locus with the amplification of a SRY-specific PCR product and four X-STR loci. The presence of the SRY peak in support of single peaks at the X-STRs confirms a male phenotype although deletion events may have occurred in the Y-specific amelogenin region. A female DNA profile would be confirmed not only by the absence of the SRY peak but also by expected heterozygosity in at least one of the four X-STRs. We acknowledge the fact that female genotypes may exist that display only single peaks in all four X-STR loci; however, we conclude this to be a rare event given the high observed heterozygosity values of the loci and the fact that such a profile has not been observed in 104 randomly selected women. The described multiplex seems fit for forensic purpose as the amplicon sizes of all involved loci are small enough to deal with degraded DNA, the sensitivity of the assay is comparable to other multiplex systems and mixtures of both sexes can be detected.

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