NEPALESE NULL AMELY MALES AND THEIR Y-HAPLOTYPES

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Abstract: Amelogenin is the well-liked process for sex typing in recent days. Exceptional failures to amplify AMELY accurate products can cause erroneous gender identification consequence male samples mistakenly identified as females. A total of 9 null AMELY males were noticed in 200 males of 72 Nepalese surnames by means of 4.5% frequency. No amplification of DYS458 Marker in all null AMELY males but great sharing of amplified Y STR alleles among them is a probable sign of the general phylogenetic origin.

Key words: Forensic; Gender determination; Amelogenin; Null AMELY; Y-STR haplotypes etc.

INTRODUCTION:

A precise gender determination is the crucial not only in forensic and medical science but also in other field. Amelogenin system is the most popular method for sex-typing today[10]. Human gender identification, based on the amelogenin gene has important applications in prenatal diagnosis[42], forensic casework[39], archaeological analysis[38,17], preimplantation, DNA databasing, blood sample storage as well as in paternity testing. The amelogenin gene produces a protein important in the development of the tooth enamel matrix[9]. The human amelogenin gene sequenced by Nakahori et al.[28,29] has two homologous alleles, one on the X chromosome (AMELX) and the other on the Y chromosome (AMELY)[28] differing in both size and sequence. The human AMELX gene has a size of 2872 bp and is located at 11.075 Mb on the X chromosome between positions Xp22.1–p22.3 (GenBank accession number M55418) while the human AMELY-gene has a size of 3272 bp and is located at 6.781 Mb on the Y chromosome at Yp11.2 (GenBank accession number M55419;)[6,11,28,29]. Regions on this gene that are sufficiently conserved are amplified for simultaneous detection of the X and Y alleles in gender identification procedures. The amelogenin sex test is based on the identification of single copy X and Y-homologous regions. It was first described for PCR by Nakahori et al.[28,29]. Several PCR primer sets have been developed to use this gene as a sex test [1,2,6,15,16,18,39]. The most commonly used amelogenin PCR-based sex test is the one described by Sullivan et al.[39] in which primers flank a 6 bp deletion within intron 1 of the homologues resulting in 106 and 112 bp PCR products from the X- and Y-chromosome respectively. Since X and Y specific sequences together can be amplified in a single reaction using single primer, X chromosome product itself acts as an internal positive control[10]. Though Amelogenin is an effective method for sex-typing biological samples in most cases; the results are not full proof[10]. Failures to amplify sex chromosome, particularly AMELY specific products can result in incorrect gender identification, in which male samples falsely identified as females. AMELX dropout has also been observed in males in which only the AMELY amplicon is present[35]. AMELY deletion males are of phenotypic interest. Since such males lack the Amelogenin Y gene itself, and may also lack other nearby genes on Yp hence study of such males could throw light on the selective influence that the absence of any such genes might have[20]. We reported 9 null AMELY males out of 200 male samples analyzed from 72 different surnames of Nepal. Therefore calculated null AMELY was 4.5 %. The reality of AMELY dropouts reinforces the essential to analyzed additional sexing markers like *SRY, Y*-STR and STS for consistent gender determination. Null AMELY males DNA samples were further subjected for Y-STR amplification to determine the nature of Y STR haplogroup.

MATERIALS AND METHODOLOGY:

Samples: A total of 200 phenotypic recognized Nepalese male samples (stated in the identification form) received from different court and personel concern for the intention of DNA based paternity issues were the source of this study. All the samples were collected and stored as dried bloodstains on FTA classic card. DNA was purified from 1.2 mm diameter circles of bloodstained FTA papers using FTA purification reagent and TE^{-1} buffer (10mM Tris-HCl, 0.1 mM EDTA, PH 8.0) in accordance with the manufacturer's recommendations.

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Amelogenin Multiplex Typing: The purified and dried FTA punch cards were subjected to PCR for DNA amplification using the AmpF–*!*STR® Identifiler® PCR Amplification Kit (Applied BioSystems, Foster City, CA, USA) containing the 15 Autosomal STR markers with amelogenin marker for gender determination. Procedures followed were adopted from standard manufacture's protocol[4] with minor alterations. Each AmpF–*!*STR® Identifiler® PCR Amplification reaction contained 5 µl AmpF–*!*STR® PCR reaction mix, 2.5 µl AmpF–*!*STR® Identifiler® primer set, 0.5 µl AmpF–*!*STR® AmpliTaq Gold® DNA Polymerse and 4µl of deionized water to a final volume of 12 µl. Amplification was performed in a ABI GeneAmp® 9700 thermal cycler employing the following conditions: 25 cycles at 95°C for 11 minutes (initial incubation); 94°C for 1 minute (denature); 59°C for 1 minute (anneal), 72°C for 1 minute (extend), 60°C for 1 hour (final extension) and 4-25°C for final step. Genotyping of the amplified PCR products was performed on an ABI PRISM® 310 Genetic Analyzer (Applied BioSystems, Foster City, CA, USA). Briefly, 0.5 µl aliquots of PCR product was mixed with 24.5 µl of Hi-Di™ Formamide and 0.5 µl of GeneScan™ – 500 LIZ™ Size standard. One tube was prepared additionally as above, but 1 µl of AmpF–*!*STR® Identifiler® Allelic Ladde was substituted instead of sample. Samples were denaturated at 95^0C for 3 minute and snap-cooled for 3 minutes. Electrophoresis separation was carried out with a 47 cm x 50 µm capillary. Samples were routinely electrokinetically injected for 5 seconds followed by a 28 minutes run at a constant voltage of 15 kV and temperature of 60^0C. The separation medium was the Performance Optimized Polymer 4 (POP-4). The run buffer was a 1:10 dilution of Buffer (10X) with EDTA. Allele designations of Amelogenin together with 15 autosomal STR markers were determined from the electropherograms by comparing with AmpF–*!*STR® Identifiler® Allelic Ladder using GeneMapper ID v3.2 software (Applied BioSystems).

Gender identification with Y-STR: *AMELY* dropout males samples were further subjected for Y-STR amplification, using the AmpF–*!*STR® Y filer™ PCR Amplification Kit (Applied BioSystems, Foster City, USA), following the manufacturer user's manual[5]. Briefly, Y filer enables the simultaneous amplification of 16 locus on the Y-chromosome, namely DYS456, DYS389I, DYS390, DYS389II, DYS458, DYS19, DYS385a/b, DYS393, DYS391, DYS439, DYS635, DYS392, Y GATA H4, DYS437, DYS438 and DYS448. The amplified PCR products were analyzed using an ABI Prism 310 Genetic Analyzer and detailed alleles determined with GeneMapper ID v3.2. .

RESULTS AND DISCUSSION:

Amelogenin gene based gender identification is not fully reliable test and occasionally shows controversial cases. Males were erroneously typed as females due to lack of Y-specific amelogenin amplification. Such result in dire if used in cases of criminal investigation, gender identification in the setting of prenatal diagnosis or in identifying human remains from mass disasters etc. The amelogenin included in AmpF–*!*STR® Identifiler® Allelic Ladder yields peaks of 107bp and 113bp i.e. former peak from the X chromosome and the latter from the Y chromosome. As expected, the majority males exhibit two amelogenin peaks and female contained one peak, corresponding to the X-Y and X chromosomes, respectively with a standard amelogenin test. On the contrary, the DNA of a few males amplified at nearly 107bp (X) but failed to amplify at 113bp (Y). Additionally, the natures of this 107bp peak are similar to that of the female sample (Fig1).

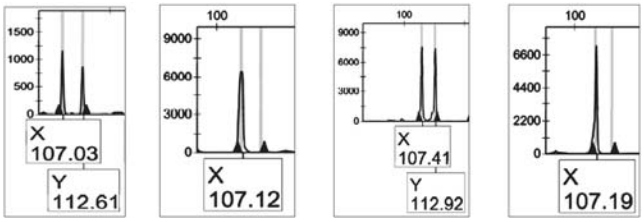

Fig1: Amelogenin amplification (left to right): Allelic ladder, Normal female, Normal male and null AMELY male.

The existence of *AMELY* null allele had been reported globally. The total of 200 males belongs to different 72 Nepalese surnames were studied in this study.The numerical surnamewise details of these are; one each sample from Ansari, Badu, Bhatt, Budha, Chaudhary, Dahal, Dangi, Dev, Dubey, Gelal, Giri, Hussain, Kewat, Lama, Murav, Pariyar, Pun, Purja, Rajbanshi, Raskoti, Sah, Shah and Thakur, two each samples from Ale, Baraili, Basnet, Bhandari, Bhujel, Chonzom, Ghale, KC, Karki, Kohar, Koirala, Kumal, Lonia, Marasini, Mishra, Miya, Nakarmi, Ojha, Pasarwal, Pradhan, Regmi, Rijal, Sapkota, Tamang, Udas, Uprety and Yari, three each samples from Kurmi, Majhi, Paudel and Upadhyay, four each samples from Ahir, Chetri, Malla, Pandey, Rana, Shahi (Khadki) and Sharma, five each samples from Adhikari, Gautam, Ghimire, Pokhrel and Tharu, six each samples of Jha, Rai and Thapa, seven sample of Khadka, nine sample from Shrestha and 24 sample from Gurung. In this study, drop out of the AMELY was detected in 9 out of 200 phenotypically normal Nepalese males. Details of null AMELY males are cited in table 1. Paternal inheritance of null AMELY as well observed in donor of sample 65001B and 67018B. This reason was confirmed by AmpF–*!*STR® Identifiler® PCR Amplification Kit and AmpF–*!*STR® Y filer™ PCR Amplification Kit. The percent of null AMELY males in the Nepalese population (4.5%) in this study is lower than that observed by Santosh et al.[33] in Sri Lankan males (8% i.e. 2 of 24) and Cadenas et al.[12] in the Kathmandu population (6.49% i.e. 5 of 77) but higher than that of males observed in India (3.57%) and Malaysia (0.88%) by Chang et al.[13], India (0.23% i.e. 10 of 4,257) by Kashyap et al.[22], India (1.85% i.e. 5 of 270) by Thangaraj et al.[40], Nepal (1.2% i.e. 9 of 769) by Parkin et al.[30], Israel (1.04% i.e. 1 of 96) by Michael and Brauner[26], Spain (0.13% i.e. 1 of 768) by Bosch et al.[7], Austria (0.018% i.e. 5 of 28,182) by Steinlechner et al.[37] and Italy (0.008% i.e. 1 of 13,000) by Lattanzi et al.[24]. The existence of AMELY dropouts in South East Asia emphasizes the needs to be cautious when interpreting AMELY sexing tests. It reinforces the necessity to include other sexing markers for gender determination in our regional populations[41]. It has been suggested that multiple Y chromosome markers should be analyzed for reliable gender identification[22] in cases where

Table1: Comparisons of Y-STR haplotypes of null AMELY males.

Sample\ Surname	Origin/ District	Y STR Haplotypes																		Max/Min shared alleles*	Reference
		DYS456	*DYS389I*	*DYS390*	*DYS389II*	*DYS458*	*DYS19*	*DYS385a/b*	*DYS393*	*DYS391*	*DYS439*	*DYS635*	*DYS392*	*Y GATA H4*	*DYS437*	*DYS438*	*DYS448*	DYS388	DYSA7.2		
63009C/ Ghimire	Kathmandu	13	12	23	28	-	15	12,16	12	10	13	21	11	11	15	9	19			15/9	Present study
64022C/ Badu	Baitadi	13	12	23	28	-	15	12,15	12	10	13	21	11	11	15	9	19			15/9	
65001B/ Pokhrel	Saptari	13	12	23	28	-	15	12,16	12	10	12	21	11	11	15	9	19			15/10	
65015A/ Gautam	Nawalparasi	13	12	23	28	-	15	12,16	12	10	12	22	11	11	15	9	19			15/9	
65031A/ Ghimire	Okhaldhunga	13	12	24	29	-	15	12,16	12	10	13	21	11	11	15	9	19			14/8	
66012A/ Dahal	Mahottari	13	12	24	29		15	14,16	12	10	13	21	11	11	14	9	19			14/8	
67001A/ Pokhrel	Kaski	13	12	23	28	-	15	12,16	12	10	13	21	11	11	14	9	19			15/8	
67018B/ Kurmi	Nawalparasi	13	12	23	28	-	15	13,16	12	10	11	20	11	11	15	10	10			11/8	
67027B/ Yadav	Rupandahi	13	12	25	28	-	15	14,18	11	11	12	21	11	11	15	9	18			10/8	
Kat16	Kathmandu		12	23	29		15		12	10	12		11					-	7	8/7	Cadenas et al. (12)
Kat28	Kathmandu		12	23	29		15		12	10	13		11					15	7	10/7	
Kat50	Kathmandu		12	23	29		13		12	9	12		11					15	7	7/7	
Kat62	Kathmandu		12	24	29		15		12	10	12		11					15	7	8/7	
Kat66	Kathmandu		12	23	29		15		12	10	13		11					15	7	10/7	
R2	Indian		10	22	27		14		14	10										6/1	Thangraj et al. (40)
M60	Indian		10	22	27		14		14	10										6/1	
B16	Indian		11	24	27		16		12	11										2/1	
K96	Indian		10	25	27		17		12	10										3/2	
A151	Indian		10	21	27		14		14	11										4/2	
I 24	Japanese	11	13	23	30	-	17	12,17	13	11	14	22	11	11	14	11	16			2/14	Kumag ai et al. (23)
F 32	Japanese	15	12	23	28	-	14	12,16	12	10	11	19	12	13	15	10	20			2/14	

*: Comparisons made between each individuals within each study.
-: No amplification.

sex identification is critical. Thangraj et al (2002) advise the inclusion of supplementary Y chromosome markers such as SRY, STR, STS and/or other Y chromosome markers in the existing multiplex STR kits for gender identification.

A primer binding site mutation on AMELY[32,34,35] or large-scale deletions in the Y-chromosomal homologue[24,25,33,37,40] are basis for frequently failure of AMELY amplification. It is rarely due to mutations in the primer-annealing region[32,34] rather a common mutation pattern involves the complete deletion of AMELY. Primarily, two Sri Lankan males were recognized who lacked AMELY through a deletion on the short arm of the Y chromosome[34]. Afterward, AMELY null males with deletion in Yp11.2 region have been found in various populations[12,19,22,24,25,41]. The widespread use of the amelogenin PCR assay provides strong ascertainment of males with apparent interstitial AMELY deletions[20] and the availability of the near-complete sequence of a Y chromosome[36] allows the investigation of the mutational mechanism(s) underlying deletions. Application of male-specific markers like SNPs, STRs, STSs becomes useful in order to delineate the breakpoints of the deletions as well as assess the overall integrity of the Y-Chromosome[12]. Similarly, in order to determine whether amelogenin null profiles caused by primer mismatch due to a point mutation affecting the oligonucleotide binding site(s) targeted Primers, amplification of null amelogenin samples with a different amelogenin primer pair (like AMEL-2F/3R) is benefecial[12]. However, specific reason of AMELY dropout in males could not be determined in the present study.

Amplification of Y-STR indicates the basis of failure of amelogenin typing i.e. either due to mutation in the primer-binding region[8] or due to deletions in the amelogenin region (11.2p) on the Y-chromosome[37]. Y-STR analysis provides further haplotyping resolution, and clustering of haplotypes within a haplogroup can support the idea of common ancestry[20]. The successful amplification of 15 distinct Y-chromosomal STR Markers was observed in all null AMELY samples in the present study. But every one of these samples failed to amplify DYS458 Locus. AMELY null males with no amplification of DYS458 Marker have been found in various populations (Chang et al. 2007; Jobling et al. 2007; Kumagai et al. 2010). DYS458[31] is composed of a polymorphic tetra (GAAA) nucleotide repeat motif and is located at position 7.93 Mb on Yp[23]. Null alleles at this locus have been linked

with amelogenin allele negative men, caused by large scale (>1 Mb) deletions[14]. It often corresponds to specific Y-chromosome binary haplogroup affiliation[12] suggestive of common Y-chromosome lineal ancestries within STR haplotype based data sets[27].

A comparative Y-STR profile of null AMELY males with other study is listed in the Table 1. With the exception of the work by Kumagai et al.[23] in which both null AMELY males possess very different Y- STR haplotypes, comparisons of the Y-STR profiles of null AMELY males for the remaining publications listed in Table 1 displays a greater proportion of similarity within each study. The Y-STR profiles of null AMELY males were sharing a maximum of fifteen alleles and a minimum of eight in this study. The facts that the 5 samples reveal incredibly parallel haplotypes by sharing maximum of 15 alleles. The proportion of sharing alleles within the mountain based surname samples is significantly greater than those samples belong to Terai based surnames. Sharing of maximum 15 alleles among mountain based surname persons in contrast to 11 alleles of Terai based person (Sample 67018B and 67027B) is the sign of well cultural and geographical diversity and demarcation between them. Great sharing of alleles (upto 7 out of 8 similar Loci) between samples of this study with Cadenas et al. (2006) strength the possibility of similar phylogenetic origin. Samples belong to the same Y haplogroup and exhibit identical or very similar Y-STR haplotypes suggests the deletions possibly occurred in the same paternal lineage[12].

CONCLUSION:

The present result of 9 null AMELY males out of a total of 200 samples with the frequency of 4.5 % in Nepalese populations accentuates the need to routinely employ SRY, Y-STR like markers for gender determination in order to obtain an accurate set of inclusion criteria especially in criminal investigations and individual identification. Dropout of similar locus and highly sharing of Y-STR alleles (upto15 alleles in 5 cases) among sample gives general phylogenetic context among them. Applications of specific additional primers like AMEL-2F/3R as well Y – specific STSs, SNPs markers enlighten the factual scenerio.

AUTHORS' CONTRIBUTIONS:

Dinesh k. jha contributed in designing and carrying out of experiments, data analyses and interpretation as well as manuscript preparation. Jiwan P. Rijal provided significant information in manuscript preparation. Nirajan T. Chhetri contributed considerable in execution of experiments. All authors read and approved the final manuscript.

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