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TECHNICAL NOTE

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Y-STRs in Forensic Medicine: DNA Analysis in Semen Samples of Azoospermic Individuals

ABSTRACT: The incidence of rape has increased, especially in metropolitan areas, such as the city of São Paulo. In Brazil, studies about it have shown that the majority of this type of crime is committed by the relatives and persons close to the victim. This has made the crime more difficult to be denounced, as only 10% of the cases are reported to competent police authorities. Usually, cytological exams are carried out in sex crime investigations. The difficulty in showing the presence of spermatozoa is frequent, but it does not exclude the presence of male DNA. The absence of spermatozoa in material collected from rape victims can be due to several factors, including the fact that the agressor suffers from azoospermia. This condition can be the result of a successful vasectomy. As the majority of DNA in the ejaculation sample is from spermatozoa, there is much less DNA to be analyzed. This study presents the application of Y-STRs (DYS19, DYS389I, DYS389II, DYS390, DYS391, DYS392, and DYS393) in DNA analysis of sperm samples from 105 vasectomized men. The study demonstrated a great variation in DNA concentration. DNA extraction and amplification was possible in all sperm samples even in the absence of spermatozoa. The same profile was observed, for each individual, from DNA extracted from blood, pre- and postvasectomy semen samples. The use of markers specific for Y chromosome in sex crime cases, especially in the absence of spermatozoa, is very important, mainly because in most situations there is a small quantity of the agressor's DNA in the medium and a large quantity of the victim's DNA.

KEYWORDS: forensic science, DNA typing, Y-STR, sexual assault, azoospermy, human identification

In the Brazilian Penal Code (Article 13), rape is defined as penis penetration in the vagina without the woman's consent. Other types of sexual violence, including those against men (which are the minority of sexual crimes) are classified as violent attacks to dishonor an individual. It is also considered rape when a sexual assault has occurred after the victim has been given a drug by the aggressor to render her unconscious (drug-facilitated assault), a crime that has been frequently reported (1–3). Rape is considered one of the most violent crimes and is classified as a hideous crime. Sexual assaults, such as rapes followed or not by death, have increased considerably, especially in metropolitan areas, such as the city of São Paulo. In Brazil, statistical data on this subject are very scarce. The majority of cases are not even reported because of the moral, social, and emotional aspects that involve the victim. Studies on such a subject in Brazil show that $<10\%$ of the cases are reported to police authorities (4). One of the limiting factors for clarifying rape crimes is the lack of spermatozoa collected from vaginal material, which makes the identification of genetic material from the perpetrator difficult. This difficulty is even greater in cases of azoospermic individuals where the loss of viable sperm in the semen is related to the individual's constitution or due to elective surgery; vasectomy is a contraceptive method widely carried out in Brazil. The Y-chromosome short tandem repeat (STR) typing has become an important tool in forensic analysis, first because of the ease of amplification of male DNA from a mixed sample when one of the donors is a male, and the other a female, even when the concentration of female DNA is much higher than that of the male and second, because it bypasses the need for carrying out the differential extraction of sperm and nonsperm material (5–11). In cases where multiple males are contributors, the number of donors can be estimated because of the haploid nature of Y-STRs.

The DNA Commission of the International Society of Forensic Genetics (ISFG) has published guidelines and recommendations concerning the use of Y-STRs polymorphisms in human identification (12,13). This paper presents the application of Y-STRs in the analysis of azoospermic semen samples from vasectomized men.

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Materials and Methods

Samples

After informed consent, 105 men who wanted to undergo vasectomy surgery were assessed. They consented to donate

semen samples before and after the vasectomy and 5 mL of peripheral blood.

DNA Extraction

DNA was extracted from 5 mL of peripheral blood by a salting-out procedure (14) and from ejaculated samples by a phenol/chloroform method.

After liquefaction, the semen samples were centrifuged at 300 × *g* for 10 min. The supernatant (homologous seminal plasma) was decanted, leaving about 1.0 mL of the homologous seminal plasma. The cell pellet was resuspended in the residual homologous seminal plasma by using a vortex. The DNA was extracted from 250 μL of concentrated semen samples and was resuspended in 100 μL of TE (10 mM Tris: 0.1 mM ethylenediaminetetraacetic acid [EDTA]).

After the surgery and confirmation of azoospermy by sperm examination (according to techniques standardized by the World Health Organization) (15), the DNA was extracted again from the semen samples of those patients, as described above. In this case, the cell pellet was resuspended in 0.5 mL of the homologous seminal plasma. The DNA was also resuspended in 100 μL of TE (10 mM Tris: 0.1 mM EDTA).

DNA Quantitation

DNA was quantified by spectrometry (Ultrospec III, Pharmacia, Piscataway, NJ).

PCR Amplification

The amplification of the loci DYS19, DYS389I, DYS389II, DYS390, DYS391, DYS392, and DYS393 was performed according to Kayser et al. (16). The amplification reaction was carried out in two multiplex reactions, one triplex (DYS391, DYS392, DYS393) and one tetraplex (DYS19, DYS389I, DYS389II, and DYS390). One primer of each pair was labeled with a fluorescent dye. In a final volume of 25 μL, 50 ng of genomic DNA was mixed with 200 μM of dNTP, 2.0 mM $MgCl_2$, 2.5 U of Taq polymerase (Amersham Biosciences, Piscataway, NJ), 2.5 μL of the 10 × reaction buffer provided by the manufacturer and with the forward and reverse primers. For the triplex reaction, the primer concentration was 7.0 pmol for DYS391, 8.5 pmol for DYS392, 3.0 pmol for DYS393 and for the tetraplex reaction, 7.0 pmol for DYS19, 6.0 pmol for DYS389I/II and 4.0 pmol for DYS390. The samples were subjected to 30 cycles of amplification in a 9700 thermal cycler (Applied Biosystems, Foster City, CA). The conditions of amplification were 94°C, 5 min; 35 cycles of 94°C 1 min; 55°C 1 min; 72°C 1 min, followed by 72°C 30 min and 12°C until the samples were removed from the thermal cycler.

Fragment Analysis

Fragment size analysis was performed using GeneScan 2.1 software. Two microliters of the amplification products were mixed with 24 μL of Hi-Di Formamide (Applied Biosystems), 1 μL of the size standard TAMRA-350 (triplex reaction) or TAMRA-500 (tetraplex reaction) and subjected to capillary electrophoresis on the ABI 310 Genetic Analyzer (Applied Biosystems) using POP-4 (performance optimized polymer), filter set C and an injection time of 5 sec. The electrophoresis time was 24 min for the triplex reaction and 28 min for the tetraplex reaction.

Results and Discussion

In ordinary investigational procedures of sexual assault, a sample is collected from the victim and a cytological exam is carried out to search for spermatozoa for the purpose of confirming the occurrence of sexual intercourse (or the attempt of it). The impossibility of showing the presence of such cells is frequent; nevertheless, it does not exclude the presence of male DNA. In Brazil, the presence of spermatozoa is still very important in material collected from the victims of sexual assault.

Besides the technical problems inherent in the kind of material analyzed, the lack of spermatozoa in samples may be explained by several factors such as, among others, the long time period after the intercourse, penetration without ejaculation, or even the azoospermy or oligospermy of the aggressor. In these last two cases, there is much less seminal DNA as the spermatozoa represent the major source of DNA in ejaculates for potential genotyping analysis.

Autosomal STRs have been used for a long time for human identification and still are a valuable tool in forensic casework, where small amounts of DNA are available (17). Analysis of mixed samples containing male and female DNA presents certain challenges, even in sexual assault investigations where only one agressor is involved. At times, evidence samples from sexual assault cases contain relatively high quantities of female DNA, compared with male DNA. During analysis for autosomal STR loci, the profile from female DNA often masks the male DNA profile or competes for reagents such that no male profile is obtained (6). In cases where multiple males are involved, the autosomal STR profiles obtained often provide inconclusive results (5).

The study of Y-STRs has increased the chances of detecting low levels of male DNA mixed with a high content of female DNA. The DNA Commission of the International Society of Forensic Genetics supported the loci DYS19, DYS389I, DYS389II, DYS390, DYS391, DYS392, DYS393, DYS385I, and DYS385II, as being the Y-STR core set or minimal haplotype (min Ht) and the extended haplotype added by loci DYS438 and DYS439. Mutation rate was also studied (18).

In this study, 15 individuals were excluded because one presented a postvasectomy recanalization, five, although having consented to donate semen samples, did not consent to donate blood samples, and nine individuals did not return after surgery.

A great variation in the DNA concentration was observed in the prevasectomy semen samples. (Table 1). This may be due to the variation in the semen content of fertile men observed in different populations. The literature (19–21) refers to great variation of contents according to the age, length of sexual abstinence, season of the year, smoking habits, caffein intake, etc. The high DNA concentration in the semen of individuals 05, 60, and 67 can be explained by the presence of infection (Table 1).

Considering the ejaculate of vasectomized men, a great variation in DNA concentration was observed, as well. This was attributed to variation in the number of epithelial cells and/or leucocytes present in the semen. Such variation in the quantity of epithelial cells and/or leukocytes may be related to several factors such as the period of sexual abstinence and the presence of infections in the individuals. DNA concentration in these samples ranged from 0.9 to 96.5 μg/mL (Table 1).

Nonsperm cells in semen, including immature germ cells, leukocytes, and epithelial cells, are normally found in a concentration of $<15\%$ of the sperm material (22). The glands of the male genital tract such as the prostate and seminal vesicle are the major

TABLE 1—*DNA concentration (μg/mL) in blood, pre- and postvasectomy semen samples of 90 individuals.*

Samples	Blood DNA (μg/mL)	Semen 1 DNA (μg/mL)	Semen 2 DNA (μg/mL)
001	251.5	27.0	14.4
002	72.5	79.4	31.5
003	546.0	9.4	3.4
004	570.0	21.0	1.9
005	319.4	904.4	22.9
006	220.5	23.9	18.4
007	196.0	76.5	23.4
008	140.0	88.5	8.4
009	645.0	77.5	4.4
010	291.5	21.0	5.5
011	425.5	29.4	7.9
012	251.9	3.4	2.4
013	627.5	4.4	2.4
014	583.9	153.4	4.4
015	248.0	51.4	7.9
016	327.5	100.5	21.0
017	251.9	88.5	16.9
018	489.0	210.5	6.4
019	379.5	51.4	12.9
020	575.5	54.4	20.0
021	327.5	11.0	10.0
022	510.5	44.8	5.9
023	379.5	91.0	23.9
024	332.5	12.9	8.4
025	365.5	42.5	42.0
026	248.0	158.4	42.5
027	202.4	11.4	6.9
028	384.0	96.0	7.9
029	218.9	419.5	96.5
030	207.0	31.0	10.5
031	369.4	202.9	4.4
032	298.0	11.4	10.0
033	463.0	44.5	12.9
034	223.0	36.0	27.5
035	428.0	22.0	9.4
036	735.0	62.4	11.4
037	522.0	90.5	12.9
038	449.0	53.4	6.4
039	393.0	18.4	1.4
040	480.5	17.4	5.9
041	554.4	50.9	15.0
042	510.5	91.5	61.0
043	339.5	250.5	4.4
044	322.0	176.5	6.4
045	173.0	18.4	3.4
046	599.5	12.9	4.4
047	391.0	241.0	10.5
048	499.5	11.0	9.4
049	696.0	384.0	43.0
050	296.0	371.0	10.5
051	272.5	425.5	7.4
052	247.5	33.0	2.9
053	484.5	77.5	22.0
054	351.5	92.5	0.9
055	399.0	63.9	0.9
056	389.0	49.0	5.9
057	325.0	163.4	2.4
058	410.0	90.5	3.9
059	583.0	11.9	3.9
060	582.5	732.5	12.9
061	576.5	298.5	17.9
062	441.0	59.4	8.4
063	464.5	10.5	7.4
064	378.5	130.0	16.9
065	690.0	202.4	13.9
066	310.5	186.0	82.5
067	630.0	628.5	8.4
068	271.0	11.0	1.9
069	737.5	67.9	11.4
070	503.4	212.9	8.9
071	315.0	22.4	3.4
072	277.5	279.5	90.5
073	411.0	93.5	23.9
074	389.0	333.0	27.0
075	315.5	32.5	20.5
076	372.0	91.5	12.9
077	290.5	16.5	12.9
078	468.0	5.9	2.4
079	239.5	311.5	5.9
080	186.5	8.9	1.4
081	151.4	27.0	13.9
082	466.5	39.4	8.9
083	213.4	81.0	42.5
084	345.5	233.0	10.0
085	411.0	21.5	8.4
086	359.4	19.4	13.4
087	575.5	395.5	26.7
088	373.0	185.5	5.0
089	439.0	106.9	5.0
090	274.4	13.4	10.0
Mean	391.7	122.5	15.0
Maximum	737.5	904.4	96.5
Minimum	72.5	3.4	0.9
Standart deviation	145.88	160.75	17.75

source of epithelial cells in the semen. The prevalence and clinical significance of leukocytes, immature germ cells, and epithelial cells in semen is currently a subject of controversy (23). The World Health Organization proposed as normal the limit of 4.7×10^6 leukocytes/mL of ejaculate (15). This value for the upper limit of leukocytes per microliter of ejaculate should be considered as an approximation. Further studies are required to define the relationship between the number of white cells in the ejaculate and genital tract of inflammatory disorders.

DNA extraction and amplification were possible in all semen samples, even when there was an absence of spermatozoa.

The same alleles were observed, for each individual, from DNA extracted from pre- and postvasectomy semen and blood (Table 2). Although there was a lower concentration of DNA in the postvasectomy semen samples when compared to prevasectomy samples, they presented the same quality of the amplification profiles (complete haplotypes) (Figs. 1 and 2).

The primers for locus DYS391 consistently produced a nonspecific fragment around 247 bp length in the three samples (pre- and postvasectomy semen and blood). This fragment represents a sequence from the X chromosome homologous to the Y chromosome (24). It is out of the range of the alleles for this locus (275–295 bp) and, therefore, does not interfere in the identification result. The overlap of alleles from markers DYS392 and DYS391 does not interfere in the interpretation of the results because the primers were labeled with different colors.

The conditions for obtaining male DNA samples in rape cases are very much different than the ones developed in this experiment. In the majority of the rape cases, the aggressor's DNA is obtained from the vaginal fluid where the amount of female DNA is much higher. Another difficulty arises when multiple males are contributors and, therefore, we would expect to find two or more alleles for each marker. In both these conditions, we cannot exclude the possibility of the occurrence of allele dropout. Shewale et al. (5) demonstrated that Y-STRs were succesfully amplified when mixing male and female DNA at the ratio 1:800 (0.5:400 ng), showing that although there was a much higher con-

TABLE 2—*Y-STR haplotypes derived from 90 individuals.*

#	Samples	DYS393	DYS19	DYS391	DYS389I	DYS389II	DYS390	DYS392
hp001	S1, B, S2	12	14	10	13	30	23	11
hp002	S1, B, S2	13	14	11	13	29	24	13
hp003	S1, B, S2	13	14	10	13	29	24	13
hp004	S1, B, S2	13	14	11	14	31	24	11
hp005	S1, B, S2	13	15	10	13	31	24	11
hp006	S1, B, S2	13	15	11	14	32	24	13
hp007	S1, B, S2	12	15	9	13	29	23	11
hp008	S1, B, S2	12	13	10	13	30	24	11
hp009	S1, B, S2	13	14	11	13	29	24	13
hp010	S1, B, S2	13	15	11	13	31	21	11
hp011	S1, B, S2	12	15	10	12	29	23	11
hp012	S1, B, S2	13	14	10	13	29	23	13
hp013	S1, B, S2	12	14	10	13	29	24	13
hp014	S1, B, S2	12	14	11	13	29	24	14
hp015	S1, B, S2	13	15	10	13	29	24	13
hp016	S1, B, S2	13	14	10	13	29	24	13
hp017	S1, B, S2	14	15	10	13	32	22	11
hp018	S1, B, S2	13	14	10	13	29	24	13
hp019	S1, B, S2	13	14	11	13	30	25	13
hp020	S1, B, S2	14	15	10	12	29	22	11
hp021	S1, B, S2	13	14	11	12	29	25	12
hp022	S1, B, S2	13	14	11	13	29	24	13
hp023	S1, B, S2	12	14	10	13	30	24	11
hp024	S1, B, S2	13	14	11	13	29	24	13
hp025	S1, B, S2	13	14	10	13	29	24	13
hp026	S1, B, S2	13	14	11	14	30	25	13
hp027	S1, B, S2	12	14	10	14	30	25	11
hp028	S1, B, S2	13	13	10	13	30	23	11
hp029	S1, B, S2	14	15	10	13	30	21	11
hp030	S1, B, S2	12	14	10	14	29	23	13
hp031	S1, B, S2	13	14	11	13	29	24	13
hp032	S1, B, S2	13	14	10	12	28	24	13
hp033	S1, B, S2	12	14	10	13	29	23	11
hp034	S1, B, S2	14	16	10	13	31	23	14
hp035	S1, B, S2	12	16	11	13	29	23	11
hp036	S1, B, S2	13	13	10	13	30	24	11
hp037	S1, B, S2	15	15	10	13	31	21	11
hp038	S1, B, S2	13	14	10	13	29	24	13
hp039	S1, B, S2	13	14	11	13	29	25	13
hp040	S1, B, S2	13	14	11	13	29	25	13
hp041	S1, B, S2	13	14	10	12	28	23	11
hp042	S1, B, S2	15	15	10	14	31	23	12
hp043	S1, B, S2	12	15	10	12	28	23	11
hp044	S1, B, S2	13	13	10	13	30	23	11
hp045	S1, B, S2	13	15	11	13	29	25	13
hp046	S1, B, S2	13	14	12	14	30	23	13
hp047	S1, B, S2	13	14	11	13	30	23	13
hp048	S1, B, S2	12	14	10	13	29	24	13
hp049	S1, B, S2	14	14	10	12	27	25	13
hp050	S1, B, S2	13	14	12	13	29	24	13
hp051	S1, B, S2	14	15	10	14	31	22	11
hp052	S1, B, S2	12	15	11	12	28	25	13
hp053	S1, B, S2	11	14	11	13	29	25	13
hp054	S1, B, S2	13	14	11	13	29	24	13
hp055	S1, B, S2	12	14	10	13	29	24	11
hp056	S1, B, S2	13	14	10	13	30	23	13
hp057	S1, B, S2	12	15	9	13	30	23	11
hp058	S1, B, S2	13	14	10	13	30	25	13
hp059	S1, B, S2	13	14	11	13	28	24	13
hp060	S1, B, S2	13	13	10	13	31	24	11
hp061	S1, B, S2	13	13	9	14	30	24	11
hp062	S1, B, S2	14	14	10	12	29	22	11
hp063	S1, B, S2	14	15	10	13	29	23	12
hp064	S1, B, S2	14	16	10	13	30	24	11
hp065	S1, B, S2	13	14	11	14	31	24	13
hp066	S1, B, S2	13	17	11	13	30	24	11
hp067	S1, B, S2	13	14	11	13	29	24	13
hp068	S1, B, S2	14	15	9	13	30	21	11
hp069	S1, B, S2	13	14	10	13	29	25	13
hp070	S1, B, S2	13	14	10	14	31	23	13
hp071	S1, B, S2	13	14	10	12	28	23	13
hp072	S1, B, S2	13	14	10	12	28	24	13

TABLE 2— *Continued.*

#	Samples	DYS393	DYS19	DYS391	DYS389I	DYS389II	DYS390	DYS392
hp073	S1, B, S2	13	13	11	13	29	23	13
hp074	S1, B, S2	12	15	9	13	29	23	11
hp075	S1, B, S2	12	14	10	12	29	22	11
hp076	S1, B, S2	13	14	10	14	31	23	11
hp077	S1, B, S2	12	14	11	13	29	23	11
hp078	S1, B, S2	13	14	10	12	28	23	11
hp079	S1, B, S2	13	16	11	12	29	21	11
hp080	S1, B, S2	12	15	11	12	28	24	11
hp081	S1, B, S2	15	15	10	13	30	21	11
hp082	S1, B, S2	12	15	9	13	29	23	11
hp083	S1, B, S2	13	14	10	15	32	23	13
hp084	S1, B, S2	14	15	10	13	29	23	11
hp085	S1, B, S2	13	15	11	13	29	24	13
hp086	S1, B, S2	13	14	11	13	29	24	13
hp087	S1, B, S2	13	14	11	14	31	24	13
hp088	S1, B, S2	14	16	10	12	28	22	11
hp089	S1, B, S2	13	13	10	13	31	24	14
hp090	S1, B, S2	13	14	10	13	29	24	14

S1, prevasectomy semen; B, blood; S2, postvasectomy semen; STR, short tandem repeat.

centration of female DNA, this did not inhibit the amplification of the Y-STRs. In addition, these authors showed that the mixture of DNA, from two men who had distinct allele profiles for nine Y-STRs, was successful in the amplification of both haplotypes in mixtures of up to 1:30 (0.2:6.0 ng), although some variation in the degree of amplification had been observed for the minor compo-

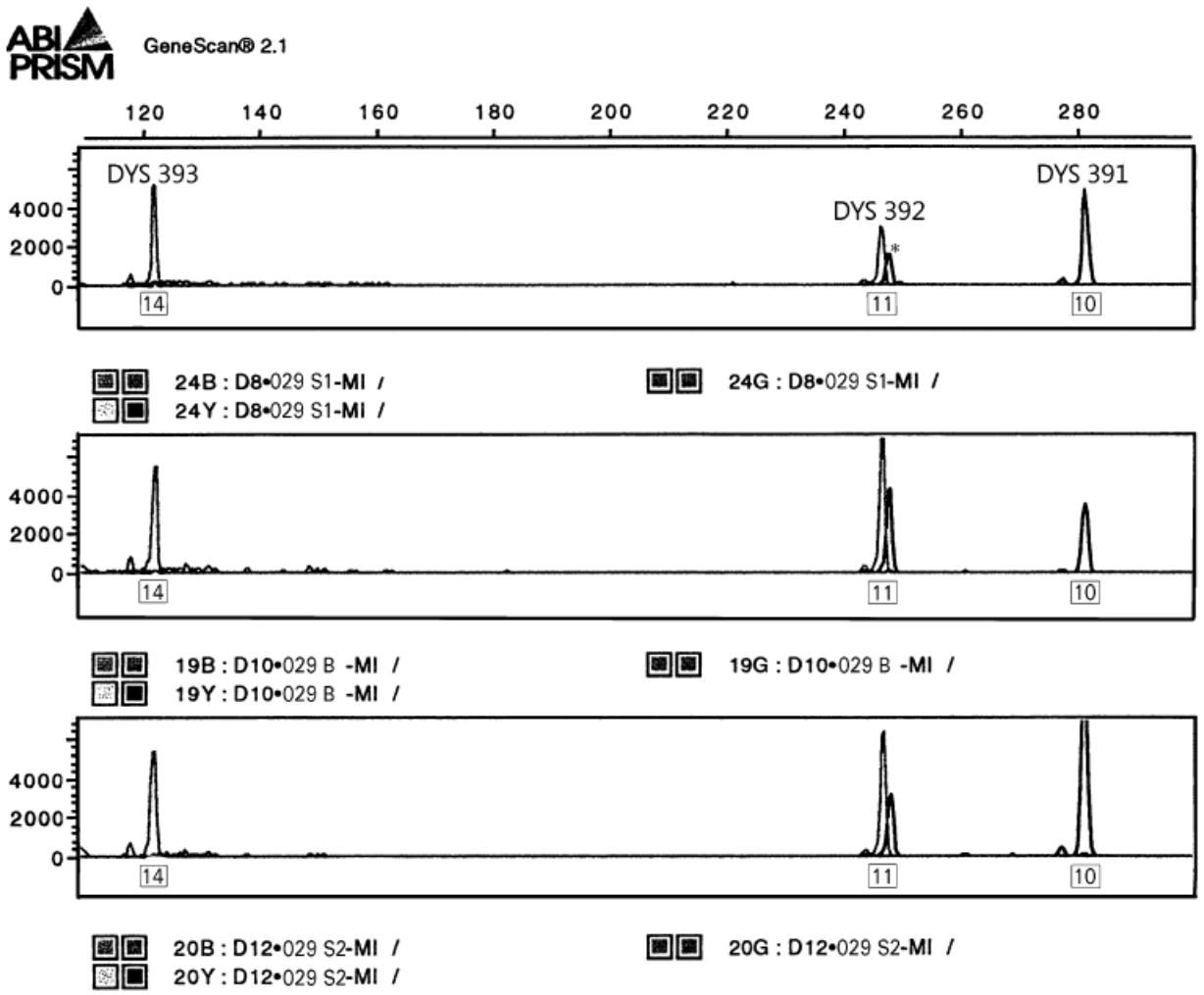

FIG. 1—*Y-short tandem repeat profile of sample 029 using Multiplex I. S1, prevasectomy semen; B, blood; S2, postvasectomy semen; *fragment amplified from the X chromosome.*

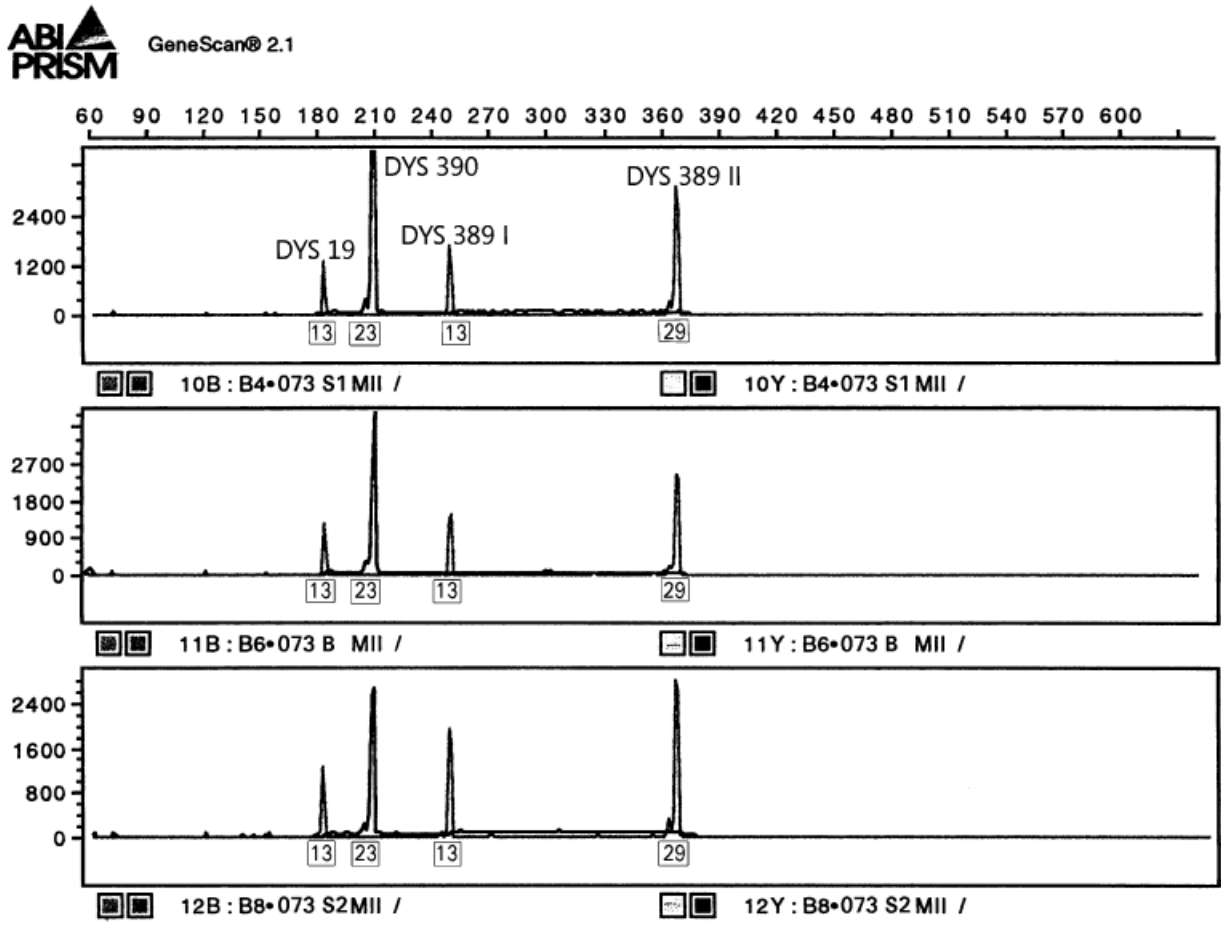

FIG. 2—*Y-short tandem repeat profile of sample 073 using Multiplex II. S1, prevasectomy semen; B, blood; S2, postvasectomy semen.*

nent. These data showed that our approach is useful in the DNA analysis of multiple rape case samples, wherein one of the aggressors is azoospermic.

This is the first Brazilian report which showed the value of Y-STRs in the identification of men through the study of semen of azoospermic individuals by comparing the results with respective blood samples. These data demonstrate the possibility of identification of azoospermic aggressors.

The study of the Y-STRs is, without a doubt, a very useful method for analyzing small quantities of DNA in mixture samples. The same methodology should be routinely used in investigating rape crimes.

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