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The Determination of Sex from Forcibly Removed Hairs

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ABSTRACT: The determination of sex from forcibly removed hairs in forensic science laboratories has, in the past, been based almost entirely on the presence or absence of the Y chromosome in the cells of the hair root sheath. Since the human male genotype is XY and the female is XX, a technique was devised that permits root sheath cells to be stained sequentially for the Y and then the X chromosome using quinacrine mustard. Following staining, the Y and the X chromosome fluorescence were observed, at pH 5.5 and 3.0, respectively, by epifluorescence. The X and Y chromosome counts obtained for a single hair root specimen were reported as a Y − X (Y minus X) score. The results reported show that specimens from males gave positive Y − X score while specimens from females gave negative Y − X scores. Results of an age study and blind trials were also reported.

KEYWORDS: pathology and biology, hair, chromosomes, human identification, sex chromatin, sex chromosomes, X chromosome, Y chromosome

In the forensic science laboratory, the determination of sex from forcibly removed hairs has in the past been based almost entirely on the presence or absence of the fluorescent Y chromosome following staining with either quinacrine mustard or its dihydrocloride derivative [*1-4*]. However, a high degree of variation has been shown to exist in the length of the fluorescent region on the human Y chromosome because of differences in the length of the long arms of the chromosome [*5*]. Further, it has been demonstrated that in some instances the Y chromosome may not exhibit any fluorescence following staining with the quinacrine dye [*6-8*]. Therefore, a false conclusion regarding the sex of the individual, as determined from the hair root, may be reached in cases where somatic cells from a normal male have a weakly fluorescent or nonfluorescent Y chromosome.

Since the human male genotype is XY and the female genotype is XX, this paper describes a technique that permits hair root sheath cells to be stained with quinacrine mustard, for first the Y and then the X chromosome, for the determination of sex from forcibly removed hairs.

Materials

The quinacrine mustard (QM) (Sigma No. Q 2000) was prepared as a 0.028% (w/v) solution in a buffer containing 8.64m*M* citric acid and 90.7m*M* dibasic sodium phosphate at pH 7.0.

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The QM staining solution was filtered and stored in an amber bottle and was stable for two weeks at 4°C. A modified McIlvaine buffer [9] consisting of 69m*M* citric acid and 182m*M* dibasic sodium phosphate, at pH 3.0 and pH 5.5, respectively, was used for X and Y chromosome differentiation [*10*]. Mounting buffers for X and Y chromosome differentiation were prepared as a 2:3 (v/v) mixture of glycerol and the corresponding pH 3.0 or pH 5.5 buffer. All buffers were stored at 4°C.

Number 1-1/2 (0.16 to 0.19 mm thickness; 22- by 50-mm) glass coverslips were used in the staining and the microscopic examination of the prepared slides.

Hair specimens used in this study were obtained from volunteer donors at the FBI Academy. These specimens, with the root present, were individually wrapped in weighing paper, and stored at room temperature. Unless otherwise stated, all specimens were examined within 24 h of collection.

Quinacrine-stained slides were examined using a Leitz Laborlux 12 microscope equipped with a Ploemopak 2.5 fluorescence vertical illuminator. The light source was a HBO 50-W ultra-high-pressure mercury lamp with a BP 390–490 exciting filter, 480 edge filter, and a LP 515 supression filter. All observations were made using a ×100 plan apochromatic oil-immersion objective and Leitz immersion oil (No. 513-522; refractive index of N_e^{23} = 1.5180). Photographs were taken using the Leitz Vario-Orthomat camera system and Kodak 200 Ektachrome color slide film push processed at 400 ASA.

Methods

The hair root bulb was placed on a microscope slide and soaked in four to five drops of 40% (v/v) acetic acid for approximately 5 min at which time enough acetic acid was removed as to leave a small amount remaining around the root bulb. With a No. 11 scalpel blade, the follicular cells were gently scraped from the root, pressed flat, and then gently spread to cover an area approximately 2 cm in diameter. The slide was then air-dried, fixed in methanol for 3 min, and again air-dried.

Chromosome staining was carried out by adding from four to five drops of the QM stain solution to the slide and covering with a 22- by 50-mm coverslip. After 10 min, the coverslip was removed and the slide rinsed with approximately 400 mL of distilled water. The slide was then immersed in the pH 5.5 (Y chromosome) differentiation buffer for 3 min and then mounted for examination using the pH 5.5 mounting buffer. The coverslip was positioned on the slide and the excess mounting buffer was removed by blotting along the edge of the coverslip. The slide was then examined and the results recorded as the number of Y chromosome positive cells observed when counting a total of 100 cells.

After examining the slide for Y chromosome fluorescence, the slide was then prepared for X chromosome identification by removing the coverslip and immersing the slide in the pH 3.0 (X chromosome) differentiation buffer for 3 min. Following differentiation, a 22- by 50-mm coverslip was positioned on the slide using the pH 3.0 mounting buffer and the excess buffer removed. The slide was then examined under oil at ×100 and the number of X chromosome positive cells per 100 count recorded.

The sex of an individual was determined based on the Y − X (Y minus X) score calculated from each hair specimen.

Results

The appearance of the X and Y chromosomes after QM staining of follicular cells from the hair root are shown in Figs. 1 and 2. In the nuclei from a male (genotype XY), the Y chromosome usually appears as a single bright spot (Fig 1*a*). However, in some cases the Y chromosome may appear in the duplex form (Fig. 1*b*). This duplex form of the Y chromosome may result from the mechanical treatment of the hair root during preparation of the follicular cell

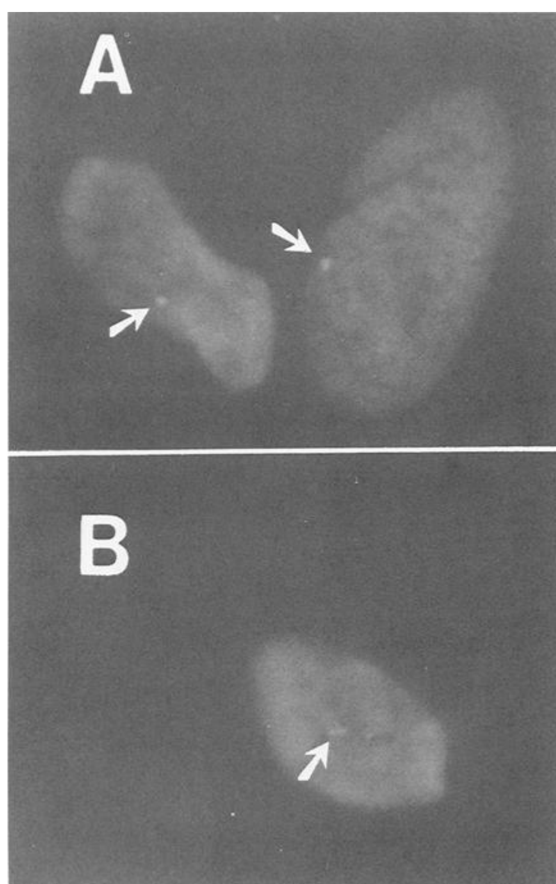

FIG. 1—*QM stained hair root follicular cells from a normal male showing:* (a) *condensed Y chromosome on periphery and* (b) *duplex form of Y chromosome.*

smear [*11*]. In the nuclei from a female (genotype XX), one of the two X chromosomes is inactive and condensed and usually appears at the edge of the nucleus as a large, weakly fluorescent ovoid body as shown in Fig. 2*a* and *b*.

In examining nuclei of follicular cells from hair obtained from known males, the Y chromosome was detectable in almost all of the nuclei observed. However, detection of the X chromosome, in nuclei of hair root follicular cells obtained from known females, was limited to those cells having a large flattened nucleus without signs of degeneration or pyknosis.

The frequencies of the X and Y chromosome fluorescence, observed in hair root sheath cells from 63 male and 64 female donors, are shown in Table 1. The X and Y chromosome frequencies were recorded as the number of cells, when counting a total of 100 cells, that exhibit X or Y chromosome fluorescence.

The data presented in Table 1 show that the mean Y chromosome frequency for the 63 specimens obtained from male donors was 42 ± 9 and ranged from 27 to 64. The corresponding mean X chromosome frequency obtained for these same specimens, following differentiation at pH 3.0, was 2 + 1 with a range from 0 to 6.

For the 64 specimens obtained from female donors, a mean Y chromosome frequency of 3 ±2, with a range of 0 to 8, was obtained. The corresponding mean X chromosome frequency, obtained following differentiation at pH 3.0, was 22 ± 4 and ranged from 11 to 30.

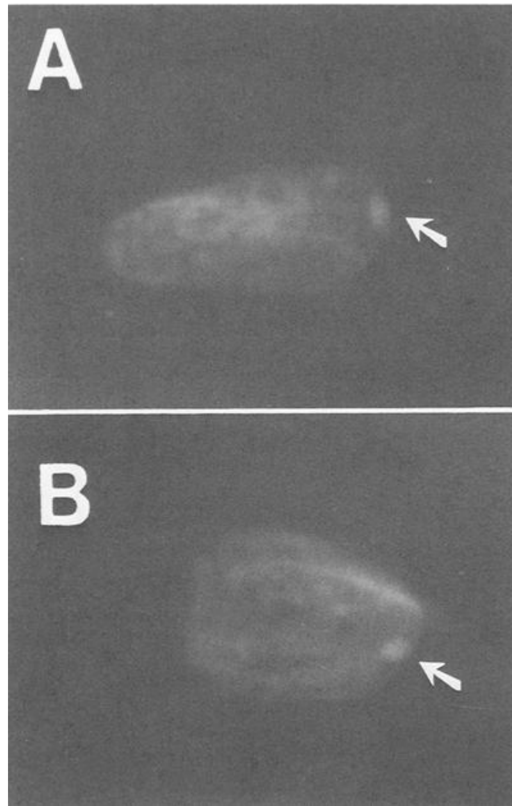

FIG. 2—*QM stained hair root follicular cells from a normal female showing:* (a) *and* (b) *condensed, inactive X chromosome on periphery.*

TABLE 1—*Summary of X and Y chromosome frequencies and corresponding Y − X scores following QM staining of hair root follicular cells from known male and female donors.*

	Y[a]	X[a]	Y − X
		Male (N=63)	
$\bar{x}$[b]	42	2	40
SD[c]	9	1	9
Range	27-64	0-6	25-67
		Female (N=64)	
$\bar{x}$	3	22	−19
SD	2	4	4
Range	0-8	11-30	−6-−27

[a]Chromatin count per 100 cells.
[b]Mean.
[c]±Standard deviation.

To determine the sex of an individual from the hair root, a Y − X transformation was made by subtracting the X chromosome frequency from the corresponding Y chromosome frequency. The frequency of the Y − X scores obtained from specimens from the 63 male and 64 female donors are shown in Fig. 3. As seen in this figure, complete separation was achieved between the Y − X scores determined from specimens from males and females. Consequently, the Y − X scores obtained for specimens from females were negative while the Y − X scores obtained for specimens from males were positive.

The frequencies of the Y − X scores, presented in Fig. 3, are also summarized in Table 1. For the specimens from 63 males, the mean Y − X score was 40 ± 9 with a range of 25 to 67. The mean Y − X score obtained for specimens from females was −19 ± 4 and ranged from −6 to −27.

Based on the Y − X scores calculated for these known specimens, the lower Y − X range for specimens from males was established as the mean Y − X score minus 2 standard deviations (that is, 40 − 18 = 22). The upper Y − X range for specimens from females was established as the mean Y − X score plus 2 standard deviations (that is, −19 + 8 = −11). Therefore, hair root specimens giving Y − X scores of 22 or greater were regarded as originating from a male, while those specimens giving Y − X values of −11 or less were regarded as female in origin. Those Y − X scores greater than −11 and less than 22 were regarded as inconclusive and therefore no conclusion could be drawn as to the gender of the specimen. By using a cutoff of two standard deviations, as described above, 97.72% of the specimens, in which the X and Y chromosomes could be detected, would be identified as originating from a male or female based on the Y − X score, and only 2.28% of the specimens would produce inconclusive Y − X scores.

To evaluate the reproducibility of this procedure, two examiners independently determined the Y − X scores for duplicate hair root specimens obtained from ten male and ten female donors. The results, presented in Table 2, show that 92% of the variance r^2 in the Y − X scores was accounted for by the consistency of the chromosome counts obtained by the two examiners.

The variation in Y−X scores obtained for multiple hair root specimens collected from three male and three female donors are shown in Tables 3 and 4, respectively. For the specimens collected from the 3 male donors, the mean Y − X scores were calculated to be 53 ± 4, 50 ±5, and 50 ± 1 and were well above the lower Y − X limit of 22 previously established for males. The mean Y − X scores calculated for the multiple specimens obtained from the 3 female donors were −19 ±2, −18 ±2, and −17 ±3. The Y − X scores obtained for these specimens were well below the upper Y − X limit of −11 previously established for females. From these results one sees that the variation in the Y − X scores, represented as the standard deviation

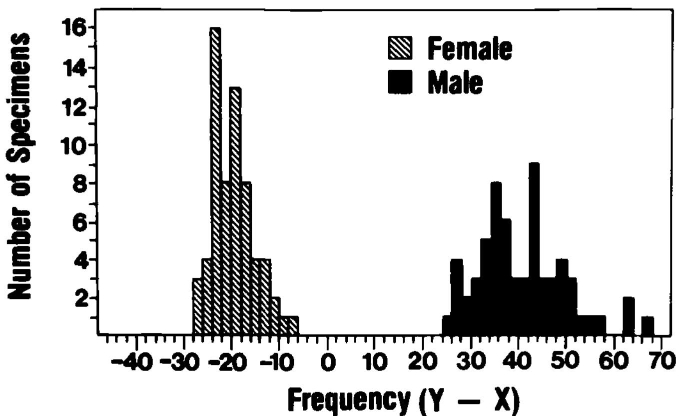

FIG. 3—*Distribution of Y−X scores obtained for hair root follicular cells from 63 male and 64 female donors.*

TABLE 2—*Comparison of Y − X scores determined for the same specimen by two independent examiners.*

	Examiner 1			Examiner 2		
	Y[a]	X[a]	Y − X	Y	X	Y − X
			Male (N=10)			
$\bar{x}$[b]	42	1	45	50	1	49
SD[c]	14	1	7	6	1	6
Range	33-56	0-3	32-56	43-57	0-2	40-57
			Female (N=10)			
$\bar{x}$	2	16	−14	4	20	−16
SD	2	7	8	1	4	4
Range	0-6	7-30	−4-−30	2-5	13-24	−9-−20

[a]Chromatin count per 100 cells.
[b]Mean.
[c]±Standard deviation.

from the mean, is rather small and that the Y − X scores for multiple specimens cluster very near the mean. Therefore, based on these results, one would not expect to see a large fluctuation in Y − X scores for multiple specimens from one individual.

Presented in Table 5 are the results of a blind study conducted on 22 hair root specimens. Of the specimens tested, 16 gave Y − X scores ranging from 31 to 57 with a mean of 46 ± 10. These specimens were correctly identified as originating from a male. The remaining specimens gave Y − X scores ranging from −17 to −21 with a mean of −18 ±2 and were correctly identified as originating from a female.

An age study was conducted with hair root specimens obtained from six male and six female donors. In this study, duplicate specimens were obtained from each donor. One specimen was QM stained on the day of collection, while the other was stored at room temperature for 46 to 149 days.

At different times during the storage period, the remaining specimen from each donor was analyzed and the Y − X score calculated. The initial and final Y − X scores obtained for each of these specimens are shown in Table 6. The Y − X scores determined for these specimens

TABLE 3—*Intraindividual variation in Y − X scores of hair root specimens from known male donors.*

Specimen	Y[a]	X[a]	Y − X
	M − 1 (N=6)		
$\bar{x}$[b]	53	0	53
SD[c]	4	1	4
Range	46-59	0-2	46-57
	M − 2 (N=6)		
$\bar{x}$	50	0	50
SD	5	0	5
Range	44-58	0-1	44-58
	M − 3 (N=6)		
$\bar{x}$	50	0	50
SD	1	0	1
Range	48-51	0-1	48-50

[a]Chromatin count per 100 cells.
[b]Mean.
[c]±Standard deviation.

TABLE 4—*Intraindividual variation in Y − X scores of hair root specimens from known female donors.*

Specimen	Y[a]	X[a]	Y − X
	F − 1 (N=6)		
$\bar{x}^b$	3	22	−19
SD[c]	1	1	2
Range	2-4	20-24	−17-−22
	F − 2 (N=8)		
$\bar{x}$	4	22	−18
SD	1	2	2
Range	3-5	20-25	−15-−20
	F − 3 (N=8)		
$\bar{x}$	4	21	−17
SD	1	2	3
Range	2-5	17-24	−12-−20

[a]Chromatin count per 100 cells.
[b]Mean.
[c]±Standard deviation.

TABLE 5—*"Blind" study conducted to determine sex from hair root specimens.*

	Y[a]	X[a]	Y − X	Identified
		Male (N=16)		
$\bar{x}^b$	47	1	46	
SD[c]	10	1	10	100%
Range	32-66	0-3	31-57	
		Female (N=6)		
$\bar{x}$	3	21	−18	
SD	2	2	2	100%
Range	1-6	18-23	−17-−21	

[a]Chromatin count per 100 cells.
[b]Mean.
[c]±Standard deviation.

TABLE 6—*Effects of storage at room temperature on the Y − X scores obtained for hair root specimens from known males.*

	Specimen Number (Storage Time, Days)[a]					
	1(46)	2(48)	3(55)	4(93)	5(149)	6(149)
Y[b]	69[c]/50[d]	49/43	48/54	29/33	43/51	39/27
X[b]	2/1	2/2	4/0	2/2	6/1	3/3
Y − X	67/49	47/41	44/54	27/31	37/50	36/24

[a]Each specimen was obtained from different individuals.
[b]Chromatin count per 100 cells.
[c]Prestorage count.
[d]Poststorage count.

ranged from Y − X = 49 at Day 46 to Y − X = 50 and Y − X = 24 at Day 149, and were consistent with the values previously established for males (Table 1).

In Table 7, the Y − X scores obtained for duplicate specimens from females, following storage at room temperature, are presented. All of the specimens gave Y − X values consistent with those from females following storage for 33 (Y − X = −17) to 119 days (Y − X = −21).

Discussion

In this study it was shown that follicular cells from the hair root could be stained sequentially for the Y and the X chromosome using quinacrine mustard. From this a Y − X score was calculated which permitted a precise determination of the donor's sex (male, Y − X ≥ 22; female, Y − X ≤ −11).

While one would normally expect Y chromosomes to be present in follicular cells obtained from known male donors (Table 1, 42 ±9), the appearance of a Y chromosome-like structure in specimens obtained from known female donors (Table 1, 3 ± 2) would not be consistent with the normal female genotype. Clearly, the "Y chromosomes" observed in these female specimens do not actually represent true Y chromosomes, but are probably a result of the bright centromers of the No. 3 or No. 13 chromosomes or the bright satellites of the D or G group chromosomes as suggested by Ju et al [*12*].

To eliminate the possibility of a false negative conclusion because of a nonfluorescent Y chromosome in a normal male or a false positive conclusion caused by the pseudo-Y chromosome in a normal female, the follicular cells from the hair root were stained for first the Y and then the X chromosomes. The resulting X and Y chromosome frequencies were then used to calculate the Y − X score which provides the examiner with a clear cutoff limit for specimens from males and females. Accordingly, those hair root specimens with Y − X scores greater than or equal to 22 were identified as originating from a male, while those specimens with Y − X scores less than or equal to −11 were identified as originating from a female. With Y − X scores between −11 and 22, no conclusions were drawn regarding the gender of the individual from which the hair specimen originated.

Studies of multiple specimens obtained from a single donor have demonstrated that one would not expect to see large fluctuations in the Y − X scores within an individual.

The reproducibility of this test was further demonstrated by the consistency of the chromosome counts, obtained from a single hair root specimen, by two independent examiners. In comparing these results, both examiners obtained a high or low Y − X score for the same specimen 92% of the time, while only 8% of the time did one examiner obtain a high Y − X score when the other obtained a low Y − X score. Despite this 8% variation, there were no incorrect conclusions made by either examiner as to the sex of the individuals based on the Y − X scores obtained from the hair root specimens.

TABLE 7—*Effects of storage at room temperature on the Y − X scores obtained for hair root specimens from known females.*

	Specimen Number (Storage Time, Days)[a]					
	1(33)	2(35)	3(35)	4(69)	5(92)	6(119)
Y[b]	4[c]/2[d]	1/3	1/2	4/4	4/3	3/0
X[b]	23/19	17/18	21/34	27/17	23/19	30/21
Y − X	−19/−17	−16/−15	−20/−32	−23/−13	−19/−16	−27/−21

[a]Each specimen was obtained from different individuals.
[b]Chromatin count per 100 cells.
[c]Prestorage count.
[d]Poststorage count.

Analyses of hair root specimens stored at room temperature indicated that the X and Y chromosomes in the follicular cells are stable since Y − X scores were not adversely affected. Although the sample number was small, these results suggested that the sex of an individual could be determined from hair roots maintained for at least 100 days. However, studies in this laboratory have indicated that mounting and storage of hair root specimens in Permount®, prior to QM staining, may result in a quenching of chromosome fluorescence.

The chromosome staining procedure described in this paper using quinacrine mustard has been shown to be a reliable method for the identification of the X and Y chromosomes in follicular cells of the hair root and can be successfully applied to the determination of the sex of an individual from forcibly removed hairs.

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