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Km(3) Identification by Enzyme-Linked Immunosorbent Assay (ELISA) as an Internal Control for Km(1) Activity Determined by Inhibition in Dried Bloodstains

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ABSTRACT: A stability study comparing the identification of kappa marker Km(1), using the classical inhibition of agglutination, and the identification of Km(3), using an automated enzyme-linked immunosorbent assay (ELISA) technique, was done.

Preliminary tests were performed to establish the specificity and sensitivity of the methods. Based on the results, the quantities of stain required to detect each marker were determined.

Blood samples from 24 staff donors of known phenotype were aged at room temperature and at 37°C in the dried stain and liquid forms. In addition, 192 stains from cases 1 to 7 months old and 76 staff-donor stains from 1½ to 10 years old were tested in dried stain form.

The known sensitivity of the ELISA technique was exploited by deliberately testing a decreased quantity of antigen. As control stains were aged beyond the detectable limits of sensitivity, results consistently showed an almost simultaneous success or failure to detect Km(1) and Km(3). This indicates that the interpretive criteria established for ELISA are sufficiently demanding to eliminate the danger of reporting false Km(−1) results but true Km(3) results.

KEYWORDS: pathology and biology, blood, genetic typing, bloodstains

The discovery of the kappa marker (Km) system was made in the early sixties by Ropartz et al. [*1*]. To date, only three antigens have been reported: Km(1), Km(2), and Km(3). These allotypes are found in the constant portion of kappa light chains in all immunoglobulin classes.

The three antigenic determinants are controlled by three co-dominant autosomal alleles—Km^1, $Km^{1,2}$, and Km^3. Studies of the amino acid sequences of kappa chains have shown that substitutions of amino acids at two positions, Positions 153 and 191, result in the homozygous and heterozygous phenotypes [*2*]. Studies have shown that the Km^1 allele is fairly rare [*2*]. In this laboratory, the Km(1) antigen is found in approximately 18% of the Ontario, Canada, population. In almost all Caucasian subjects, the Km(2) factor is found when the Km(1) factor is present since these two factors are transmitted together by the $Km^{1,2}$ allele. Subjects with the phenotype Km(−1,2,3) or Km(−1,2,−3) have not been reported [*2*]. It has not been possible to verify this fact in this laboratory as the antiserum for the Km(2) factor is not available commercially. The Km(3) factor, on the other hand, occurs frequently: approximately 95% of a Caucasian population possess Km(3) [*3*].

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The typing of kappa markers is commonly done by the classical inhibition of agglutination technique. The activity of the Km(1) factor in dried bloodstains has been well documented [*4*]. In this laboratory, this marker, together with the G1m(1), G1m(2), and G3m(10)/(11) markers, has been routinely detected for the past five years by using a microtube inhibition technique and SNagg antisera [*5*]. Identification of the G3m(10) or G3m(11) marker is used as an internal control for allotypic activity. Positive identification of G3m(10) or G3m(11) allows one to report the absence of the G1m(1) and G1m(2) markers, but does not act as a control for the absence of kappa marker activity.

Currently, 82% of the Km(1) results generated in this laboratory are not reported. The experimental absence of the Km(1) factor is insufficient evidence to report the genetic absence of that factor, unless direct identification of another kappa chain allele is used to serve as a control for kappa chain antigen activity. The most valid control for the genetic absence of Km(1) activity in a stain is the identification of its allele, Km(3). In our laboratory, the only SNagg anti-Km(3) available yielded poor results when tested against dried bloodstains by the classical inhibition technique [*6*]. An anti-Km(3) raised in rabbits was also tested using the inhibition technique; however, its low titer and high cost made it impractical to use on a routine basis [*6*]. The commercial availability of an anti-Km(3) raised in rabbits made the enzyme-linked immunosorbent assay (ELISA) a possible alternative. The ideal ELISA assay, of course, requires monoclonal antibodies, which are currently not available.

ELISA has been successful in several immunological applications [*6*]. Forensic studies using ELISA have been done on both the G1m(3) marker, using monoclonal antibodies, and on Km(3), using rabbit antiserum [*6–8*]. In this laboratory, Newall [*6*] has shown success in detecting the Km(3) factor in dried bloodstains using a manual ELISA technique. All that was required to detect the marker was a 1-mm piece of bloodstained yarn, as opposed to a 6-mm piece for Km(1) detection by inhibition.

A danger lies in using as an internal control the presence of one allele established by a sensitive technique [Km(3) by ELISA] as a basis for reporting the absence of another allele, which is tested for by a less sensitive technique [Km(1) by inhibition]. However, as neither rabbit nor monoclonal anti-Km(1) is commercially available, one cannot test for both alleles by ELISA. This study compared the relative stabilities of Km(3) identified using an automated ELISA technique and Km(1) identified using the classical inhibition technique.

Materials Used for ELISA

The following materials were used for the ELISA technique:

1. Antisera—anti-Km(3) raised in rabbits, obtained from the Department of Immunogenetics, Central Laboratory of The Netherlands, Amsterdam, The Netherlands.
2. Conjugate—goat $F(ab')_2$ anti-rabbit immunoglobulin (IgG) conjugated to alkaline phosphatase, obtained from Tago Inc., Burlingame, California.
3. Substrate—*para*-nitrophenylphosphate (PNPP), obtained from Sigma Chemical Co., St. Louis, Missouri.
4. Stain extract—5% ammonia and 0.1*M* phosphate buffer, pH 8.0; 13.18 g dibasic sodium phosphate (Na_2HPO_4); 0.99 g monobasic sodium phosphate (NaH_2PO_4); and 1 L distilled water.
5. Diluent—1% bovine serum albumin (BSA)/0.005% Tween 20 in phosphate-buffered saline (PBS), the BSA in lyophilized form, obtained from Boehringer Mannheim, Montreal, Canada.
6. Washing solution—0.0025% Tween 20 in PBS.
7. Alkaline phosphatase buffer—10% diethanolamine, pH 9.8, prepared by the method of Voller et al. [*9*].

8. 3*N* Sodium hydroxide (NaOH)—used to stop the enzyme reaction of alkaline phosphatase.

Materials Used for Inhibition

The following materials were used for the classical inhibition of agglutination technique:

1. Kappa marker sera—anti-Km(1) and anti-D-Km(1), obtained from Analytical Genetic Testing Center, Inc., Denver, Colorado.
2. Red blood cells—Group 0, R_1R_2, obtained from a donor, Centre of Forensic Sciences, Toronto, Canada.
3. Diluent—3% BSA in PBS with 0.1% sodium azide (NaN_3), the BSA as Fraction V, fatty acid free and lyopholized, obtained from Boehringer Mannheim, Montreal, Canada.

ELISA Method [for Km(3)]

Ten microlitres of 5% ammonia was added to a bloodstained yarn. The sample was then extracted further in 300 μL of phosphate buffer for ½ h at room temperature. Using a Nichiryo 8100 repeater, 100 μL of the extract was pipetted into prewashed distilled water (DH_2O) Immulon 2 (Dynatech) microtiter plates, according to a preestablished key, incubated at 37°C for ½ h, and then aspirated.

From this point on, all the washing and dispensing of reagents was done automatically using the Titertek microplate washer and Titertek Autodrop dispenser, respectively. Two hundred and fifty microlitres of 1% BSA in 0.005% Tween 20 was added to the plates. The plates were sealed with parafilm and incubated overnight at 4°C to complete the blocking phase. Blocking can be achieved at 37°C for 45 min. Alternative blocking proteins, such as casein, can also be used.

The next day the plates were washed three times in the wash solution described above. One hundred microlitres of antiserum, diluted 1/100 in diluent, was added and the plates were incubated for 1 h at 37°C. After the plates had been washed again as previously described, 100 μL of conjugate, diluted 1/1000 in diluent, was added and the plates were incubated for 2 h at 37°C. After another washing as previously described, 100 μL of PNPP in diethanolamine (2.5 mg/mL) was added and the plates were incubated for 45 min at 37°C. Seventy microlitres of 3*N* NaOH was immediately added. The optical density was assessed using a Minireader II photometer (Dynatech) at a wavelength of 405 nm.

Inhibition Method [for Km(1)]

Bloodstained yarns were extracted overnight at 4°C in 20 μL of anti-Km(1) diluted 1/130 in 3% BSA in PBS. After removal of the yarns, 20 μL of 0.01% R_1R_2 cells in 3% BSA in PBS, coated with anti-D-Km(1) reagent, was added. The mixture was rotated for 1½ h at 18°C and centrifuged for 1½ min at 1900 rpm. The entire contents of the tube were transferred to a slide and evaluated microscopically for agglutination at ×100 magnification.

Specificity and Sensitivity

A preliminary study was done to establish the quantity of stain required to detect each marker. The bloodstains used in this study were obtained from individuals who had been previously grouped for both markers by the Dutch Red Cross, and the results were confirmed in this laboratory. Blood samples were obtained by finger prick and prepared on 100% cotton cloth. The bloodstains used were two months old at the time they were

tested. Two-month-old stains were used because these stains would be equivalent to the age of actual case material stains routinely grouped in this laboratory.

As a dried stain, a 10-mm white cotton yarn contains approximately 0.5 μL of whole blood. Currently in this laboratory, approximately 0.3 μL of blood (a 6-mm bloodstained yarn) extracted in 20 μL of diluent is routinely used to detect the Km(1) marker by the inhibition technique. The original developmental work (Table 1) and repeated testing over seven years have established that this quantity is more than sufficient for accurate results.

To establish the minimum amount of bloodstain required for accurate determination of Km(3) by ELISA and to investigate the effects of gross overloading of antigen, a dilution series of known bloodstains from twelve donors of known phenotype, was tested (Table 2). Each sample was extracted in 300 μL of diluent, of which 100 μL per duplicate was used to obtain a result.

Although approximately 300 nL of blood is the minimum required amount for accurate determination of Km(1) by inhibition, as these results clearly show, approximately 80 nL of blood (a 5-mm bloodstained yarn) usually contains sufficient Km(3) antigen to be accurately detected by the ELISA technique as described. Consequently, this quantity was chosen for the remainder of the study. As has been emphasized, it is essential to desensitize a detection technique for an antigen, if its presence is to be used as an internal control for the absence of an antigen detected by a less sensitive method.

Aging Studies

Blood samples from 24 individuals were collected by venipuncture into heparinized tubes. The bloodstains were prepared on white 100% cotton cloth. These individuals had been previously grouped for both markers by the Dutch Red Cross, and those results were confirmed in this laboratory. The phenotypes of these individuals are recorded in Table 3. The aging studies were performed in five series.

Series I—Fresh Stains

One bloodstain per individual was prepared on Day 0, allowed to dry, and immediately frozen. These stains served as a control panel.

TABLE 1—*Effect of increasing the antigen concentration on Km(1) inhibition results from bloodstains of known phenotype, in number of individuals.*[a]

Known Phenotype	Km(1) Grouping Results	Length of Bloodstained Yarn, mm					
		1	2	4	6	8	10
Km(1,3)	Km(1)	0	0	1	5	5	5
	Km(inc)[b]	5	5	4	0	0	0
	Km(−1)	0	0	0	0	0	0
Km(1,−3)	Km(1)	0	0	1	2	2	2
	Km(inc)	2	2	1	0	0	0
	Km(−1)	0	0	0	0	0	0
Km(−1,3)	Km(−1)	5	5	5	5	5	5
	Km(1)	0	0	0	0	0	0
Total No. of individuals		12	12	12	12	12	12

[a]Note that each sample was extracted in 20 μL of diluent and that a 10 mm yarn contains approximately 0.5 μL of blood.

[b]The designation (inc) indicates that the typing results were considered inconclusive.

TABLE 2—*Effect of increasing the antigen concentration on Km(3) ELISA results from bloodstains of known phenotype, in number of individuals.*[a]

Known Phenotype	Km(3) Grouping Results	Length of Bloodstained Yarn, mm						
		1	3	5	7.5	15	30	90
Km(1,3)	Km(3)	1	2	3	4	4	4	4
	Km(inc)	4	3	2	1	1	1	1
	Km(−3)	0	0	0	0	0	0	0
Km(1,−3)	Km(−3)	2	2	2	2	2	2	2
	Km(3)	0	0	0	0	0	0	0
Km(−1,3)	Km(3)	2	4	5	5	5	5	5
	Km(inc)	3	1	0	0	0	0	0
	Km(−3)	0	0	0	0	0	0	0
Total No. of individuals		12	12	12	12	12	12	12

[a]Note that each sample was extracted in 300 μL of diluent, of which 100 μL per duplicate was used to obtain a result. Also a 10 mm yarn contains approximately 0.5 μL blood.

Series II—Aged Bloodstains

(*a*) Seven stains per individual were prepared on Day 0 and left at room temperature (RT). Every 24 h over a 7-day period one stain was frozen.

(*b*) Seven stains per individual were prepared on Day 0 and left to incubate at 37°C under humid conditions. Every 24 h over a 7-day period one stain was frozen.

(*c*) One stain per individual was prepared on Day 0, left to incubate at 37°C under humid conditions for 12 weeks, and then frozen.

Series III—Aged Liquid Blood

(*a*) Liquid blood was incubated at RT. Every 24 h over a 7-day period a stain was prepared, left to dry, and then frozen.

(*b*) Liquid blood was incubated at 37°C. Every 24 h for 7 days a stain was prepared, left to dry, and then frozen.

Series IV—Case Comparison Samples

A total of 192 stains were prepared from liquid comparison blood samples received in the laboratory. These stains were stored at RT and were from 1 to 7 months old when tested.

TABLE 3—*Phenotypes of the control panel.*

Phenotype	Total No. of Individuals
Km(1,3)	5
Km(−1,3)	17
Km(1,−3)	2
Total	24

Series V—Old Staff Bloodstains

Seventy-six bloodstains collected by finger prick were stored at RT and aged from 1½ to 10 years.

Interpretation of Results

Two known Km(3) and Km(−3) samples were run on each plate as controls. In addition, no-antigen controls were run on each plate. Each sample was repeated and the average optical density (OD) of four readings for each of the positive and negative controls was calculated. If the optical density readings for a replicated sample differed by 5% or more, the test was repeated.

The ratio of the average OD positive control to the average OD negative control was calculated. The average OD of stains from 22 of the known Km(3) donors tested repeatedly varied from 1.6 to 3.8 times the average OD of stains from known Km(−3) donors (Fig. 1). Therefore, all unknown samples that gave an average OD that was at least 2 times that of the negative samples were interpreted as being positive for the Km(3) marker. That is

$$\text{OD sample X} = \frac{2[\text{OD negative control } 1+2+3+4]}{4} = \text{Km(3)}$$

These stringent interpretive criteria were deliberately applied to take into consideration the known sensitivity of the ELISA technique.

Samples with a ratio between 1 and 2 were called inconclusive (inc). Samples were not called negative unless optimum bloodstain extraction in ammonia was observed and the average OD was below the highest single reading of the control negative stains run on the plate.

In addition, all samples were tested for Km(1) by inhibition to confirm that samples negative for Km(3) by ELISA were positive for Km(1) by inhibition and samples negative for Km(1) by inhibition were positive for Km(3) by ELISA.

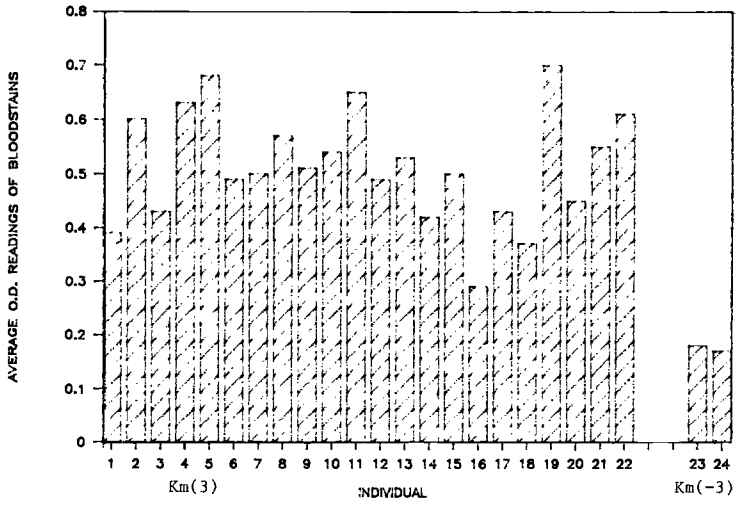

FIG. 1—*Quantitative variation in Km(3) expression in a population sample of 24 unrelated donors as demonstrated by a direct ELISA technique.*

Results

Tables 4 through 7 are a summary of the grouping results obtained from the aged bloodstains. Table 8 is the observed frequency of the phenotypes obtained from the combined results of stains from cases and from staff.

Discussion

Fresh stains (Series I) were grouped to serve as a comparison with aged bloodstains. Of a total of 24 samples grouped, 87.5% gave interpretable results for both markers, and of this total, 90.5% were positive for Km(3) and 76.2% were negative for Km(1). The Km(3) factor was identified in the absence of Km(1) in all but 1 stain.

In determining the stability of the Km(3) marker, the oldest stains in Series II and III were grouped first. If these stains yielded a total number of interpretable results in keeping with those for fresh stains, no further work was done within that series.

Stains incubated at room temperature for up to 7 days [Series II (*a*)] gave results in keeping with those for fresh stains (Table 4). The two Km(1,inc) results came from individuals whose fresh stains gave similar results. Both these individuals are phenotype Km(1,3). The one Km(−1,inc) stain came from an individual whose fresh stains gave the same result and who was Km(−1,3). It is possible that these individuals had lower quantities of kappa marker allotypes at the time they were bled and that, because of the stringent criteria applied to the positive identification of Km(3), the results were uninterpretable.

Old staff bloodstains (Series V) were stains of known phenotype from 1½ to 10 years old (Table 4). The 1985 stains were 17 months old at the time they were grouped, and 77.8% gave interpretable results for both markers, in comparison with a total of 87.5% interpretable results obtained from fresh stains. Stains ranging in age from 2 to 10 years were uninterpretable for the Km(3) marker in all cases. In 3 of the 76 stains the Km(1) factor was detected. These stains were 26 months old when grouped, and 2 were Km(1,3) and 1 was Km(1, −3). The Km(−3) stain failed to meet the criteria for calling a sample negative by 2.1%.

The results obtained from Km(1,3) stains aged at room temperature showed an almost simultaneous ability to detect Km(3) and Km(1). This ensures that the protocol set forth for detection of Km(3) by ELISA accounts for the sensitivity of this method, thus eliminating the danger of reporting false Km(−1) inhibition results based on Km(3) identification in stains aged at room temperature.

Stains incubated at 37°C under humid conditions for up to 7 days [Series II (*b*)] gave the same results as fresh stains (Table 5). The total percentage of interpretable results dropped, however, to 41.7% for stains aged for 12 weeks at 37°C under humid conditions [Series II (*c*)]. Two of the five Km(1,inc) results within this category came from stains of phenotype Km(1, −3). These two Km(−3) stains failed by 4.7% to meet the criteria for calling a sample negative. The other three Km(1,inc) results were from stains of phenotype Km(1,3). Of a total of five Km(1,3) stains grouped under these conditions, only 40% gave interpretable results for Km(3), whereas 100% gave interpretable results for Km(1). Again, as with fresh stains, the consistent failure to detect Km(3) before failure to detect Km(1) assures one that the protocol set forth for the detection of Km(3) safeguards against reporting false Km(−1) results in stains aged at 37°C under humid conditions.

Liquid blood aged at room temperature for 7 days [Series III (*a*)] gave results fairly consistent with those for fresh stains (Table 6). Two of the three Km(1,inc) results were from individuals of known phenotype Km(1,3). The third result, from an individual of known phenotype Km(1, −3), was inconclusive for Km(3) as the result failed to meet

TABLE 4—*Grouping results for fresh stains and stains aged at room temperature—Series I, II* (a), *and V.*

		Fresh Stains		7 Days at RT		17 Months (1985)		1975 to 1984	
Known Phenotype	Grouping Results	No.	%	No.	%	No.	%	No.	%
Km(1,3)	Km(1,3)	3	12.5	3	12.5	3	16.7	...	...
	Km(1,inc)	2	8.3	2	8.3	1	5.5	2[a]	2.6
	Km(1, −3)	0	0	0	0	0	0	0	0
	Km(inc,3)	0	0	0	0	0	0	0	0
	Km(−1,3)	0	0	0	0	0	0	0	0
	Km(−1, −3)	0	0	0	0	0	0	17	22.4
	Km(inc,inc)	0	0	0	0	0	0	1	1.3
Km(−1,3)	Km(−1,3)	16	66.7	16	66.7	9	50.0	0	0
	Km(−1,inc)	1	4.2	1	4.2	3	16.7	15	19.8
	Km(−1, −3)	0	0	0	0	0	0	33	43.4
	Km(1, −3)	0	0	0	0	0	0	0	0
Km(1, −3)	Km(1, −3)	2	8.3	2	8.3	2	11.1	0	0
	Km(1,inc)	0	0	0	0	0	0	1[a]	1.3
	Km(−1, −3)	0	0	0	0	0	0	7	9.2
	Km(1,3)	0	0	0	0	0	0	0	0
Total		24	100	24	100	18	100	76	100

[a]The stains were 26 months old at the time of grouping.

TABLE 5—*Grouping results for stains aged at 37°C and under humid conditions—Series II* (b) *and* (c).

Known Phenotype	Grouping Results	7 Days at 37°C		12 Weeks at 37°C	
		No.	%	No.	%
Km(1,3)	Km(1,3)	3	12.5	2	8.3
	Km(1,inc)	2	8.3	3	12.5
	Km(1,−3)	0	0	0	0
	Km(inc,3)	0	0	0	0
	Km(−1,3)	0	0	0	0
	Km(−1,−3)	0	0	0	0
	Km(inc,inc)	0	0	0	0
Km(−1,3)	Km(−1,3)	16	66.7	8	33.4
	Km(−1,inc)	1	4.2	9	37.5
	Km(−1,−3)	0	0	0	0
	Km(1,−3)	0	0	0	0
Km(1,−3)	Km(1,−3)	2	8.3	0	0
	Km(1,inc)	0	0	2	8.3
	Km(−1,−3)	0	0	0	0
	Km(1,3)	0	0	0	0
Total		24	100	24	100

the criteria for calling a sample negative by 2.0%. The total percentage of interpretable results for both markers was 83.3%.

Liquid blood aged at 37°C for 6 days [Series III (*b*)] gave a total percentage of interpretable results of 82.6% for both markers. However, the percentage of interpretable results for both markers was reduced to 68.4% for liquid blood aged at 37°C for 7 days. Two of the three Km(1,inc) results were from the same Km(1,3) individuals mentioned

TABLE 6—*Grouping results for liquid blood at room temperature and at 37°C—Series III* (a) *and* (b).

Known Phenotype	Grouping Results	7 Days at RT		6 Days at 37°C		7 Days at 37°C	
		No.	%	No.	%	No.	%
Km(1,3)	Km(1,3)	3	12.5	3	13.1	2	10.5
	Km(1,inc)	2	8.3	2	8.8	2	10.5
	Km(1,−3)	0	0	0	0	0	0
	Km(inc,3)	0	0	0	0	0	0
	Km(−1,3)	0	0	0	0	0	0
	Km(−1,−3)	0	0	0	0	0	0
	Km(inc,inc)	0	0	0	0	0	0
Km(−1,3)	Km(−1,3)	16	66.6	15	65.2	11	57.9
	Km(−1,inc)	1	4.2	1	4.3	3	15.8
	Km(−1,−3)	0	0	0	0	0	0
	Km(1,−3)	0	0	0	0	0	0
Km(1,−3)	Km(1,−3)	1	4.2	1	4.3	0	0
	Km(1,inc)	1	4.2	1	4.3	1	5.3
	Km(−1,−3)	0	0	0	0	0	0
	Km(1,3)	0	0	0	0	0	0
Total		24	100	23	100	19	100

TABLE 7—*Grouping results for liquid blood case controls—Series IV.*

Phenotype	Case Stains 1 to 7 Months Old	%
Km(1,3)	28	14.6
Km(−1,3)	129	67.2
Km(1,−3)	1	0.5
Km(1,inc)	11	5.7
Km(inc,3)	3	1.6
Km(−1,inc)	20	10.4
Total	192	100

TABLE 8—*Grouping results for frequency of phenotypes obtained from combined sample results—Series I and IV, in number of individuals.*

Phenotype	Case Stains	Staff Stains	Total	%
Km(1,3)	28	3	31	17.3
Km(−1,3)	129	16	145	81.0
Km(1,−3)	1	2	3	1.7
Total	158	21	179	100

previously. Again, Km(3) in heterozygous Km(1,3) individuals is consistently less stable or originally present in lower quantities than Km(3) in Km(−1,3) phenotypes. There was no marked change in stability for any phenotype for at least 6 days at 37°C.

It is important to address those stains in Series II and III of phenotype Km(1,−3) which, when grouped, were Km(1,inc). This by no means implies that these Km(3) inconclusive results could be falsely interpreted as Km(3) positive. It is only because of our stringent criteria for interpretation that these samples were reported as inconclusive. The variation between the average OD readings for the treated samples and the single highest OD reading for the Km(−3) controls only varied from 2.0 to 4.7%.

Table 7 illustrates the grouping results obtained from liquid blood case comparison stains aged at room temperature and ranging in age from 1 to 7 months. The total percentage of interpretable results for both markers was 82.3%. Eleven samples were Km(1,inc). Based on the statistic that 95% of Caucasian subjects possess Km(3) [*3*], it would be safe to assume that a major portion of these unknown samples are from individuals of phenotype Km(1,3).

The failure to detect Km(3) by ELISA over Km(1) by inhibition in stains of known phenotype Km(1,3) indicates that the interpretive criteria established for ELISA are sufficiently demanding to eliminate the danger of reporting false Km(−1) results but true Km(3) results.

In every instance, the percentage of interpretable Km(1) results exceeded those of Km(3). Aged stains had no demonstrable kappa chain marker activity. Although the percentage of inconclusive results was relatively high, it was because of the protocol. It is also important to note that no false positive or false negative grouping results for one allele were obtained from any of the stains of known phenotype, regardless of the treatment of that stain.

Table 8 deals with the frequency of the three different phenotypes, using combined sample results for staff stains and case stains (including fresh stains and liquid blood

comparison controls). Of a total of 179 conclusive results for both markers, 98.3% were positive for Km(3), and of the 34 samples positive for Km(1), 91.2% were positive for Km(3). Although the sample size of 179 is low, the observed frequencies are consistent with those from the literature [*2,3*]

Based on the data obtained in this study, all case material bloodstains in this laboratory are being tested for Km(3) by ELISA as a control for Km(1) marker activity determined by inhibition. This technique has been successfully used on a routine basis to analyze case material (approximately 3060 bloodstains) since 1987. This laboratory uses the Km(1) and Km(3) markers for court reporting purposes.

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