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EVALUATION OF THE DETECTION RATE OF HEMOPHILIA CARRIERS

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ABSTRACT

The detection rate of hemophilia A carriers was evaluated on the basis of AHF-activity and AHF-like antigen determination. Statistical tolerance regions were constructed containing 50 and 95% of the AHF-activity and AHF-like antigen determinations of individuals of the normal or carrier group. Using these regions 18 out of 22 obligatory carriers are outside the 95% tolerance region of the normals. On the basis of AHF-activity alone only 8 out of 22 obligatory carriers are outside the 95% tolerance interval for the normals.

For individual counselling for possible or potential carriers, with a prior chance (i.e. genetically determined) on carriership, the probabilities of carriership are calculated with the theorem of Bayes.

INTRODUCTION

Detection of hemophilia A carriers was based on AHF activity assays alone till 1971; the discrimination between normals and obligatory carriers was not absolute as can be judged from the range of activities in the two groups, e.g. for normals 0.40 U - 1.60 U and for carriers from 0.18 U - 1.26 U/ml (1), although the mean values differed significantly. These results could fit the Lyon hypothesis, i.e. the random inactivation of one of the two X-

chromosomes in females early in embryonic development (2). Because of the proposed random nature of this mechanism one would expect to find equal numbers of inactivated normal and mutant-gene-bearing X-chromosomes in the total carrier population, resulting in an average AHF activity level of half that of normal for the carriers, as is the case.

Theoretically the AHF activities in carriers should range from $<$0.01 U, in the case of inactivation of all normal X-chromosomes, to the highest normal value when all X-chromosomes carrying the hemophilia gene are inactivated.

It was generally felt that if the product of the mutant gene could be detected and quantitatively assayed carrier detection would improve considerably. For G-6-PD deficiency it was demonstrated that in heterozygotes the peripheral blood contains two populations of erythrocytes, one normal and one enzyme-deficient (3); fibroblasts derived from heterozygotes for electrophoretic variants of G-6-PD were found to contain either the wildtype gene-product or the mutant gene-product (4). In a hemolysate of blood from such heterozygotes both gene-products can be demonstrated by simple electrophoresis.

The quantitation of AHF-like antigen using electrophoretic technics with a precipitating antibody against purified human AHF by Zimmerman et al (5), and the successful application of the comparison of AHF-procoagulant and antigen plasma levels for carrier detection (6), gave way to the speculations that hemophilia A is due to a structural mutation and that the antigen - assay is measuring both the normal and the mutant gene-product (7).

The detection rate in Zimmerman's (6) study is 92 percent of the obligatory carriers and this study extended by Bennett and Ratnoff (8) yields a detection rate of 95 percent.

It appeared subsequently that investigators were not quite as succesful

in detection of hemophilia carriers, although the discrimination between normals and carriers was found to be considerably improved as compared to that based on the AHF-activity assay alone. Furthermore evidence is accumulating that AHF-like antigen is more directly related to the primary gene-product of the Von Willebrand locus (9, 10, 11, 12, 13, 14) than to AHF itself, thereby diminishing the likelihood that there is a sound theoretical basis behind the empirical way in which carrier detection is being carried out.

We decided to evaluate the detection rate of hemophilia A carriers, using a different statistical approach, from that used by Zimmerman, Bennett and Ratnoff (6,8). Their approach is based on confidence region around the regression line of AHF-like antigen on AHF-activity for the normal group. Two disadvantages can be mentioned with respect to this approach:

1) The carriers are not treated as a group contrary to the normals.

2) In regression analysis the 2 parameters are asymmetrically treated. For these reasons we choose for the characterization of the tolerance regions for the normal as well as the carrier group. These regions characterize the area in which 50 and 95% of the individuals can be expected for the normal or carrier group respectively. For individual counselling for possible or potential carriers with a prior chance (i.e. genetically determined) on carriership the probabilities of carriership were deduced with the theorem of Bayes.

MATERIALS AND METHODS

A. Coagulation methods

The samples were collected in Utrecht by mixing 9 parts of blood into one part of sodium citrate solution (0.129 M). After centrifugation at 6000 g for 30 min. the supernatant plasma was frozen at $-70^{\circ}C$.

AHF-activity was assayed according to Veltkamp (15), a one-stage kaolin activation technic, using coagulometers (Depex, De Bilt, Netherlands) to read clotting times. Hyland standard (batch no 3403T001A1: 1 U AHF - activity per ml) was used as reference plasma. The substrate plasma obtained from a severely affected hemophiliac was used diluted 1/1 (v/v) with citrated Michaelis buffer as described elsewhere (16).

AHF-like antigen was assayed by electroimmunodiffusion with a commercially available antiserum to purified human AHF prepared in rabbits and absorbed with normal plasma or plasma fractions (Nordic Immunological Laboratories, P.O.B. 22, Tilburg, The Netherlands). The method used is in principle the same as that before (12) with the exception that the temperature during electrophoresis is kept at +10°C by using the agar-electrophoresis apparatus of Wieme (17), and a sample size of 7 instead of 8 µl was shown to improve the sharpness of precipitin lines (rockets). One unit of AHF-like antigen is defined as the amount present in 1 ml of pooled normal plasma. Pooled normal plasma was prepared from 40 healthy subjects. All blood samples were drawn on a 1/10 vol of a 0,129 M citrate solution. The plasma separated by centrifugation at 6000 g for 30 min was stored at -70°C.

To evaluate the influence of storage on AHF-activity a total of 20 samples were assayed fresh and after two weeks of storage at -70°C. A median of 1.03 U/ml was found before storage and 1.06 U/ml after storage. Before storage a 10th and 90th percentile of 0.58 and 1.71 u/ml respectively were found. After storage these percentiles were respectively 0.54 and 1.75 U/ml.

The determinations of AHF-activity and AHF-like antigen an plasma samples of carriers and normals were performed within two weeks of storage at -70°C

B. Carriers of classic hemophilia

The following were considered as obligatory carriers of classic hemophilia: daughters of hemophiliacs, mothers with more than one hemophilic son and mothers with one hemophilic son and other hemophilic relatives. Possible and potential carriers were sisters of hemophiliacs (genetic chance on carriership $g = 0.5$), maternal first cousins of hemophiliacs ($g = 0.25$), mothers of a single sporadic case of hemophilia ($g = 0.66$) and their daughters ($g = 0.33$).

Blood from 22 obligatory and 19 possible carriers was drawn on three different occasions with at least a one week interval. The results of AHF-activity and AHF-like antigen assays on the three samples were averaged, after taking the logarithms.

Control, presumably normal subjects were 30 female laboratory and hospital personnel with no family or personal histories of bleeding disorders.

C. Statistical methods

For the reference group of normals and obligatory carriers calculations were carried out on the logarithms of both parameters, because these logarithms show better fulfillment of the assumption of normal distributions.

The following notation is used:

$u = {}^{10}\log$ AHF-activity, $v = {}^{10}\log$ AHF-like antigen.

In graphs with u and v on the x-axis and y-axis respectively 50 and 95% tolerance ellipses were constructed for the normals and for the obligatory carriers using equation (3.11) of Guttman (18), see Figure 1. These ellipses are expected to contain respectively 50 and 95% of the (u, v)- observations of individuals of the normal- or carrier group. They are for each group a kind of 'normal region'.

In the case of individual counselling for a possible or potential carrier, laboratory tests lead to a pair of (u, v)-observations. Corres-

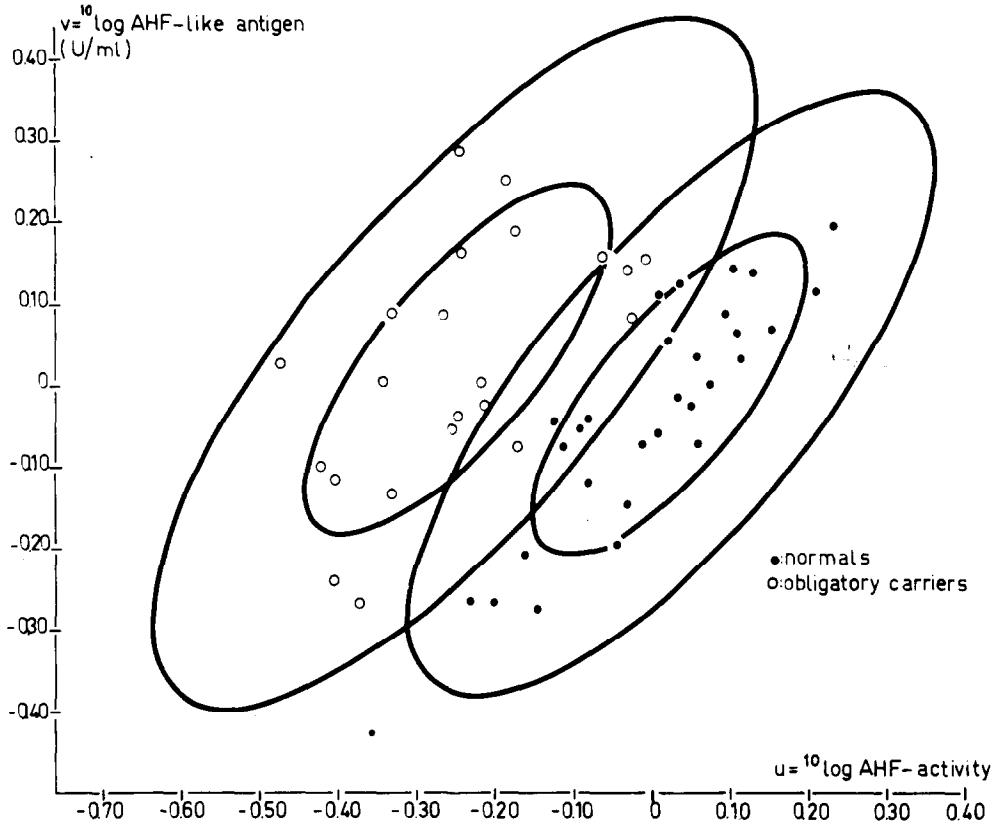

FIG. 1

Results of AHF-activity and AHF-like antigen assays in 22 obligatory carriers of hemophilia and 30 normal women. The equation of the tolerance ellipses in the u - v plane are given by:
the

$$131.158\ (u-\bar{u})^2 - 180.846\ (u-\bar{u})\ (v-\bar{v}) + 180.147\ (v-\bar{v})^2 = \frac{2(N^2-1)}{N(N-2)}\ F_{2,N-2;\alpha}$$

with:

	$\bar{u}$	$\bar{v}$	N	$F_\alpha = 0.50$	$F_\alpha = 0.95$
normals	- 0.0065	- 0.0390	30	0.81	2.95
carriers	- 0.2483	0.0262	22	0.72	3.49

The inner ellipses contain 50%, the outer ellipses contain 95% of the combinations of normal and carrier values for AHF-activity (u) and AHF-like antigen (v).

TABLE I

Results of Application of Bayes Rule on Possible and Potential Carriers

No	AHF-activity U/ml			AHF-like antigen U/ml			^{10}log AHF-act.	^{10}log AHF-like ant.	Probability on carrier-ship		Conclusion
	a	b	c	a	b	c	$\bar{u}$	$\bar{v}$	g prior	P(c) posterior	
1	0,50	0,67	0,70	0,98	0,91	0,83	- 0,2100	- 0,0436	.25	.72	unclassifiable
2	0,53	0,62	0,54	1,30	1,12	1,32	- 0,2503	0,0946	.66	1,00	true carrier
3	0,78	1,06	0,90	0,70	1,01	0,99	- 0,0428	- 0,0517	.33	.01	normal
4	0,60	0,60	0,65	0,80	0,84	0,80	- 0,2103	- 0,0899	.33	.50	unclassifiable
5	1,10	1,13	1,25	1,20	0,87	1,04	0,0638	0,0119	.50	.00	normal
6	0,85	0,87	0,90	0,85	0,98	0,75	- 0,0589	- 0,0681	.25	.00	normal
7	0,79	1,01	0,89	1,10	0,70	0,66	- 0,0496	- 0,0980	.50	.00	normal
8	1,39	0,66	0,57	0,94	0,76	0,64	- 0,0939	- 0,1133	.50	.01	normal
9	0,91	0,78	0,65	0,53	0,51	0,54	- 0,1120	- 0,2786	.50	.00	normal
10	0,36	0,87	0,44	0,85	2,35	1,29	- 0,2869	0,1370	.50	1,00	true carrier
11	1,00	1,05	0,94	1,93	1,28	1,68	- 0,0019	0,2060	.50	.81	unclassifiable
12	0,38	0,44	0,46	0,70	0,72	0,79	- 0,3713	- 0,1333	.50	1.00	true carrier
13	0,72	1,05	1,24	0,93	1,02	1,36	- 0,0094	0,0369	.50	.04	normal
14	0,85	1,20	1,21	1,93	1,32	1,85	0,0305	0,2244	.50	.68	unclassifiable
15	0,33	0,29	0,89	0,80	0,66	2,35	- 0,3566	0,0312	.66	1,00	true carrier
16	0,70	1,00	0,61	0,75	0,80	0,62	- 0,1232	- 0,1432	.66	.03	normal
17	0,60	0,59	0,92	1,65	1,24	1,50	- 0,1624	- 0,1623	.66	1.00	true carrier
18	0,81	1,25	1,59	1,40	1,58	1,70	0,0689	0,1917	.66	.28	unclassifiable
19	0,95	1,15	0,93	0,80	0,83	0,90	0,0023	- 0,0745	.33	.00	normal

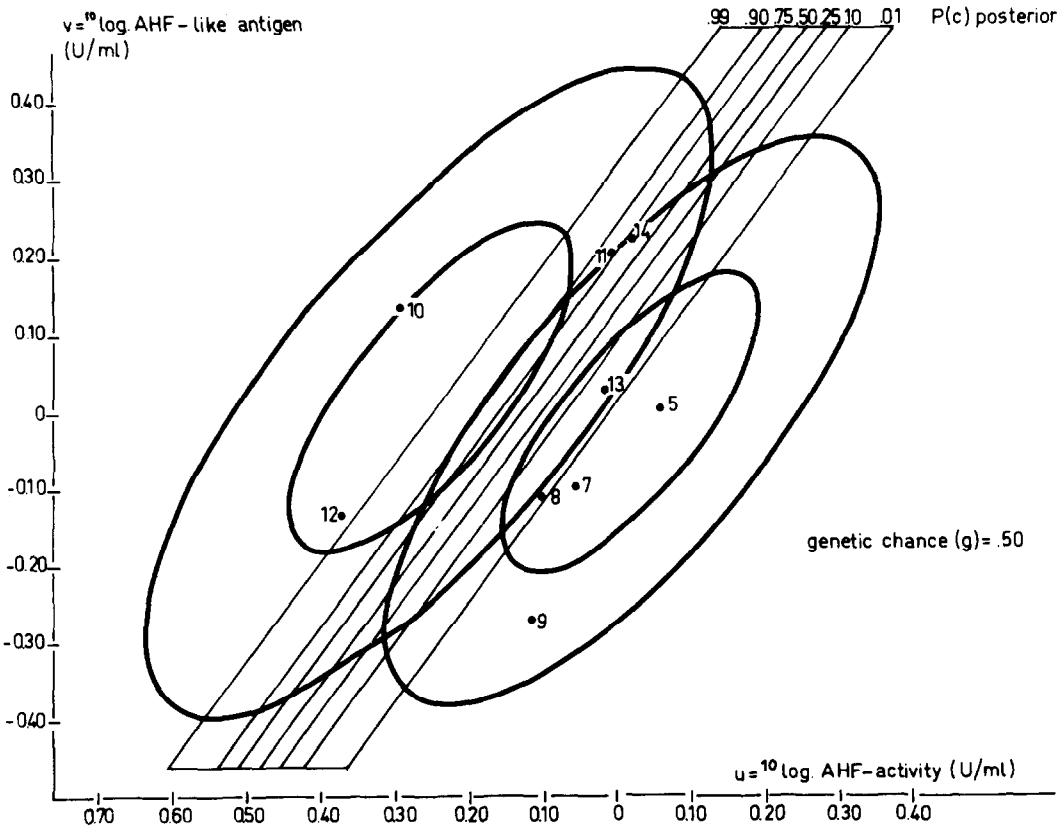

FIG.2

Nomogram to read the posterior probability on carriership (P(c)) for a prior probability (g), i.e. a genetic chance of 0.5.

ponding to the observations of this individual a probability of carriership or, complementary, of normality can be deduced with the theorem of Bayes.

Assuming normal distributions with equal covariance matrices for both reference sets, see also Figure 1, this theorem leads to the following expression for the posterior probability (i.e. with known values for u and v) on carriership

$$P(c) = \frac{g}{g + (1 - g) \exp (t(u, v))} \qquad (1)$$

in which t (u, v) is a linear function in u and v, Fisher's linear discriminant function, while g is the prior probability (i.e. genetically deter-

mined) on carriership. For fixed values of g nomograms can be constructed in which the lines indicate points of equal posterior probability, see Figure 2.

RESULTS AND DISCUSSION

The evaluation of the detection rate of hemophilia carriers was performed on frozen samples as we could not detect a difference in AHF-activity between fresh and frozen samples.

Our statistical approach is different from that used by Zimmerman, Bennett and Ratnoff (6, 8). Their approach is based on confidence region around the regression line of AHF-like antigen on AHF-activity for the normal group. Our approach is based on tolerance regions containing 50 and 95% of the AHF-activity and AHF-like antigen determinations of the normal or carrier group. These regions characterize the area in which 50 and 95% of the individuals can be expected for the normal or carrier group respectively.

The results (Fig. 1) indicate that 18 out of 22 (i.e. 82%) of the obligatory carriers are outside the 95% tolerance region of the normal group. Furthermore we found 23 of the 30 normals (i.e. 77%) to be outside the 95% tolerance region of the carrier group. A nearly identical study carried out in Leiden with 20 obligatory carriers and 33 normals yielded 14 carriers (i.e. 70%) outside the 95% tolerance region of the normals and 17 normals (i.e. 52%) outside the 95% tolerance region of the carriers (19).

It is interesting to note that on the basis of the AHF-activity alone only 8 out of 22 obligatory carriers are outside the 95% tolerance interval for the normals, indicating that combination of AHF-activity and AHF-like antigen assay results in a considerable improvement of the detection rate of hemophilia A carriers.

Our results are not as good as those described by Zimmerman, Bennett

and Ratnoff, but this may be due, at least in part, to the difference in statistical approach.

For individual counselling for possible or potential carriers with a prior chance (i.e. genetically determined) on carriership the probabilities of carriership were deduced with the theorem of Bayes. On the basis of our observations (Fig. 1) the following expression needed for the application of equation (1) is calculated

$$t(u, v) = 4.61 + 37.65\, u - 28.95\, v \quad (2)$$

In Table I the calculated posterior probabilities are given for possible and potential carriers with different genetic chances on carriership.

Example of the calculation for the posterior probability of case no. 1 Substitution of its observations $u = -0.210$ and $v = -0.044$ in equation (2) gives $t(u, v) = -2.02$. Substitution of this t-value together with the prior probability $g = 0.25$ in equation (1) gives

$$P(c) = \frac{\frac{1}{4}}{\frac{1}{4} + \frac{3}{4} \exp(-2.02)} = 0.72$$

For medical practice this probabilistic diagnosis might be translated into 'quite certain carrier' for high posteriors, 'quite certain normal' for low posteriors, and 'no conclusive evidence' or 'unclassifiable' for intermediate values. Above or below which level the posteriors will be considered as high or low enough for these conclusions is a subjective matter. Levels of 0.95 and 0.05 respectively seem reasonable to us in the present context. Using these criteria in Table I, 5 out of 19 women might be considered as 'true carrier', 9 others as 'normal', whereas in 5 cases no decision can be reached. The score in those women using oral contraceptives ($n = 6$) is that 4 are normal and 2 are unclassifiable. The fact that no true carriers were detected in those women using oral contraceptives might suggest an influence

of oral contraceptives on AHF activity, if not 3 out of 4 obligatory carriers using oral contraceptives were outside the 95% normal range. Furthermore the number of possible carriers using oral contraceptives is too small to warrant a conclusion anyway.

The fixed genetic chances (g) nomograms can be constructed. An example is given in Fig. 2 for a genetic chance of 0,5

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