TECHNICAL NOTE

Shigetaka Matsuzawa,[1] *M.D.; Yumi Kobayashi,*[1] *Phar. B.; Ryo Kobayashi,*[1] *B.S.; and Hiroko Suzuki,*[1] *M.D.*

Determination of ABH Secretor Status by an Electronic Quantitation Method

REFERENCE: Matsuzawa, S., Kobayashi, Y., Kobayashi, R., and Suzuki, H., **"Determination of ABH Secretor Status by an Electronic Quantitation Method,"** *Journal of Forensic Sciences*, JFSCA, Vol. 30, No. 3, July 1985, pp. 898-903.

ABSTRACT: Blood Group A and B substances in secretor (Se) and nonsecretor (se) salivas were tested by means of an electronic data processing-hemagglutinin-inhibition test (EDP-HAIT) with immunoglobulin M (IgM) isohemagglutinins. Besides a difference in quantity, the blood group substances in Se saliva showed high binding efficiencies compared with those in se saliva. EDP-HAIT with IgG isohemagglutinins proved no difference in the binding efficiencies of Se and se salivas. The determination of secretor status by EDP-HAIT with IgM isohemagglutinin was accurate because the conclusion was obtained based on two different quantitative results. Secretor status of some salivas in gargled water could be determined by comparing the binding efficiencies.

KEYWORDS: pathology and biology, genetic typing, saliva

Secretor status of ABH blood group substances have been classified in most instances by the hemagglutinin-inhibition test (HAIT) of salivas. However, some saliva samples showed the intermediate results of both secretor (Se) and nonsecretor (se) [*1,2*]. Most of these cases were successfully classified by testing erythrocytes [*3*] or salivas [*4*] with anti-Lewis (Le) antibodies. However, repeated tests of the saliva samples collected at different times are required to classify clearly the secretor status of rare cases.

In the studies by Le Pendu et al [*5,6*], the binding efficiencies of Se and se salivas toward anti-A or anti-B agglutinin reagents were compared by radioimmunoassay, and they concluded that mean values of the immunologic efficiencies of A and B substances in se salivas were low compared with those in Se salivas.

We have developed a new quantitation method called the electronic data processing (EDP)-hemagglutination test for isohemagglutinins [*7*]. Kimura et al [*8*] quantitated immunoglobulin M (IgM) and IgG isohemagglutinins by this method, and they found the differences in shape and steepness of the calibration curves.

In the present study, the secretor status of salivas has been classified by EDP-HAIT with IgM or IgG isohemagglutinins. EDP-HAIT with the IgM isohemagglutinins clearly differen-

Received for publication 16 Aug. 1984; accepted for publication 19 Nov. 1984.

[1]Professor, forensic scientist, forensic scientist, and graduate student, respectively, Department of Forensic Medicine, School of Medicine, Juntendo University, Tokyo, Japan.

tiates Se and se salivas by comparing the binding efficiencies of blood group substances, as well as by the quantity differences of these substances. Secretor status was also determined from gargled water by comparing the binding efficiencies. Practical availability of EDP-HAIT is discussed.

Materials and Methods

Saliva

After a thorough mouth rinsing, saliva was collected with a tube, which was heated for 20 min in a boiling water bath. After cooling the tube was stored at −70°C, and it was immersed in tepid water to thaw the saliva before use. Insoluble substances were removed by centrifugation for 30 min at 1500 *g*. Gargled water from some donors was similarly treated.

Hemagglutinin Reagents

Both anti-A and anti-B reagents containing IgM type agglutinins were the serum pools from Group B and Group A healthy adults, respectively. No content of IgG hemagglutinin was proved by the dithiothreitol test [*9*]. IgG anti-A and anti-B agglutinins were prepared by the dithiothreitol treatment of blood grouping reagents supplied from Tokyo Standard Serum Laboratory (Matsumoto, Japan).

Semi-Quantitative Hemagglutinin-Inhibition Test

The saliva was serially diluted doublefold with phosphate buffered saline (PBS) (pH 7.2) and each dilution was mixed with an equal volume of titer 1:8 hemagglutinin reagent. After absorption for 1 h at room temperature and overnight at 5°C, 1% erythrocyte suspension (25 μL) was added to an equal volume of the mixture of agglutinin and saliva, and allowed to stand for 30 min with gentle shaking five to six times at intervals of 5 min. Maximum dilution to cause complete inhibition of hemagglutination was determined by the naked eye. Classification of the secretor status for this study was performed by this method. Donors secreting relatively small quantities of blood group substances were additionally examined by anti-Le^a hemagglutination.

Electronic Data Processing-Hemagglutinin-Inhibition Test

A saliva specimen was serially diluted with PBS, and 200 μL of each dilution in the well of an acryl plate was mixed with an equal quantity of isohemagglutinin reagent of optimum potency. It means an approximate titer of 1:2 by the naked eye result determination, or more precisely, the agglutinin activity caused a 30 to 40% increase of the mean particle volume (MPV) compared with the suspension of nonagglutinated erythrocytes. After absorption for 1 h at room temperature and overnight at 5°C, 100 μL of 0.5% indicator erythrocyte suspension in PBS was added to the mixture, which was rotated at 120 revolution/min for 10 min by a slide test rotator to cause agglutination. Immediately after the rotation process, 100 μL of 1% glutaraldehyde in PBS was added and additional rotations were continued for 5 min. Within 2 h thereafter, 50 μL of thus fixed erythrocyte suspension was taken by a micropipet and gently mixed with 10 mL of a diluting medium ("Celluent," Sysmex Co. Kakogawa, Hyogo, Japan) in a small beaker. This mixture was measured within 1 h by a continuous MPV analyzing system (Sysmex) consisted of a cell counter (type CC-108), a data processor, and a printer. From the mean of five times measured values, a MPV decreasing curve was drawn; and after excluding the upper and lower 20% of its slanted portion, the steepness of the remaining 60% was determined by the calculation of a value in the formula:

$$y = a \log_{10} x + b$$

where y is the MPV value; x is saliva concentration (the inverse of dilution); a and b are the regression coefficient and the y intercept, respectively. The a shows slope or binding affinity.

When the curve was so steep as to include only one measuring spot, steepness was not calculated. In such cases, the saliva tested was determined to be Se; and, the a value was obtained from 90% of the slanted portion. For determining the saliva dilution to neutralize hemagglutinin by EDP-HAIT, the point for neutralizing 50% of the hemagglutinin activities was calculated from the MPV decreasing curve.

Results

Figure 1 shows two typical curves of A · Se and A · se salivas obtained by EDP-HAIT with an IgM anti-A agglutinin reagent. The MPV of agglutinated erythrocytes decreased according to the increase of saliva concentrations. Significant inhibition occurred at the presence of 1:1000 or more diluted A · Se saliva, whereas a similar degree of inhibiting effect was obtained by less diluted A · se saliva. It was also found that the curve of A · Se saliva was steep compared with that of A · se saliva. These results indicate that the ABH secretor status of salivas can be classified by either diluting the ratio to neutralize hemagglutinin or immunologic binding efficiency shown by the steepness of the MPV decreasing curve.

As in Fig. 2, the absorption experiment using IgG anti-A hemagglutinin indicated that the steepness of the curves of A · Se and A · se salivas were very similar to each other, although the hemagglutinin neutralizing activity of A · Se saliva was higher than that of se saliva. Use of blood grouping reagents containing both IgM and IgG hemagglutinins showed the intermediate grades of steepness. An absorption study with IgM and IgG anti-B agglutinins and Group B salivas showed the results equal to those for Group A salivas.

Tables 1 and 2 show the compared results of blood Groups A and B activities of saliva samples determined by three values obtained with IgM hemagglutinins: (1) maximum saliva dilution to cause complete inhibition determined by the broadly used semiquantitative HAIT, (2) saliva dilution to show 50% agglutinin-inhibition determined by EDP-HAIT and, (3) steepness of the MPV decreasing curve determined by EDP-HAIT. The saliva dilutions that cause 50% neutralization of hemagglutinin were exceedingly sensitive relative to the semiquantitative HAIT, which determined the degree of inhibition by complete absorption. Clear differentiation of Se and se salivas was sometimes difficult by means of semiquantitative HAIT only, because the hemagglutinin-neutralizing degrees of the weak Se specimen and the se specimen with relatively strong blood group activity were close to each other, as in the results of Group A salivas in Table 1.

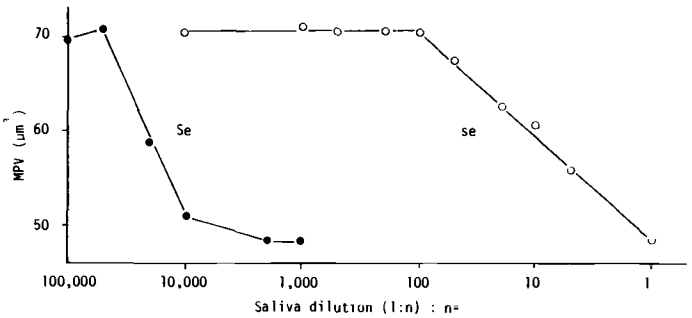

FIG. 1—*MPV decreasing curves by EDP-HAIT of A · Se and A · se salivas with an IgM anti-A hemagglutinin reagent.*

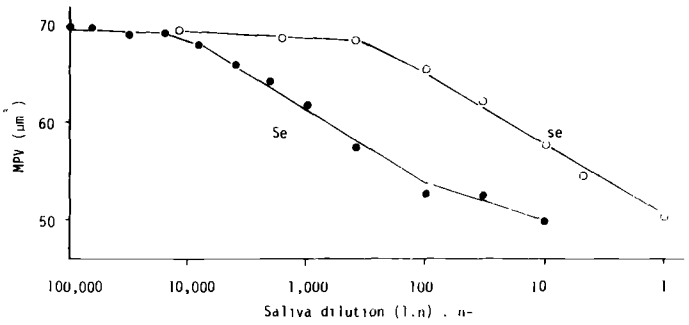

FIG. 2—*MPV decreasing curves by EDP-HAIT of A · Se and A · se salivas with an IgG anti-A hemagglutinin reagent.*

TABLE 1—*Compared results of secretor status determination methods: semiquantitative HAIT, 50% absorption of agglutinin by EDP-HAIT, and steepness of absorption curve by EDP-HAIT for Group A salivas.*

Secretor Status	Sample Number	Saliva Dilution to Cause Hemagglutinin-Inhibition by: Semiquantitative HAIT	Saliva Dilution to Cause Hemagglutinin-Inhibition by: 50% Absorption by EDP-HAIT	Steepness of Absorption Curve by EDP-HAIT
Se	7679	>512	11 000	−9.11
Se	8044	512	26 000	−10.92
Se	8047	512	23 000	−10.50
Se	8004	512	9 100	−8.59
Se	8075	256	24 000	−10.31
Se	8185	128	20 000	−9.83
Se	8043	64	7 100	−9.40
Se	7676	64	5 300	−10.50
Se	7601	16	1 000	−8.50
se	7759	8	110	−3.57
se	7660	2	50	−2.49
se	8064	2	19	−4.43
se	7504	1	33	−3.31
se	7669	<1	8.3	−3.82
se	7617	<1	17	−4.35
se	7703	<1	10	−5.92
se	8022	<1	12	−4.46
Se[a]	8044	ND[b]	ND	−9.83
Se[a]	7675	ND	ND	−10.42
se[a]	7660	ND	ND	NI[b]

[a]Gargled water.
[b]ND = not determined and NI = no detectable inhibition.

Secretor status could be classified by either one of two results obtained by EDP-HAIT: quantitatively determined saliva dilution to neutralize 50% of the hemagglutinin or the binding efficiencies shown by the steepness. The *a* values of A · Se and B · Se salivas were above 8 and 5, respectively; whereas below 6 and 4 for A · se and B · se, respectively. The results of semiquantitative HAIT and EDP-HAIT seemed to have a close association, whereas no correlation was found between the *a* values and the intragroup diversities of these hemagglutinin-inhibiting degrees among the salivas belonging to either Se or se.

TABLE 2—*Compared results of secretor status determination methods: semiquantitative HAIT, 50% absorption of agglutinin by EDP-HAIT, and steepness of absorption curve by EDP-HAIT for Group B salivas.*

Secretor Status	Sample Number	Saliva Dilution to Cause Hemagglutinin-Inhibition by		Steepness of Absorption Curve by EDP-HAIT
		Semiquantitative HAIT	50% Absorption by EDP-HAIT	
Se	8032	512	42 000	−8.27
Se	8034	256	23 000	−7.64
Se	8050	256	23 000	−5.51
Se	8062	256	7 700	−6.85
Se	8063	256	5 300	−6.46
Se	7653	128	16 000	−6.25
Se	8081	64	12 000	−7.11
Se	8040	64	7 100	−6.15
Se	7663	64	4 200	−9.12
Se	9010	32	4 800	−6.74
se	8178	8	630	−3.15
se	7603	8	588	−3.68
se	7628	2	400	−3.63
se	7792	2	23	−2.47
se	7748	<1	22	−3.90
se	8027	<1	6.7	−3.88
Se[a]	8061	ND[b]	ND	−7.52
se[a]	7603	ND	ND	−3.15

[a]Gargled water.
[b]ND = not determined.

Gargled water samples from three Se and two se individuals were tested by EDP-HAIT. One se saliva in the gargled water could not be classified because the degree of inhibition was too weak to determine quantitatively the steepness of absorption curve. The remaining three Se and one se salivas had their secretor status determined by the *a* values.

Discussion

Determination of the secretor status by EDP-HAIT with IgM isohemagglutinins is reliable because saliva samples are classified based on two results different from each other in nature. Individual differences of result determination among examiners can be much reduced by the use of the automated system. Owing to high sensitivity, EDP-HAIT can detect very weak blood group activities of se salivas that show no immunologic capacities by the semiquantitative HAIT. In spite of the studies of Le Pendu et al [5,6], it is unclear whether or not the radioimmunoassay is available to determine the binding efficiencies of individual saliva samples, because EDP-HAIT and radioimmunoassay differed from each other at the modes of immunologic reactions, and because they did not show the results of individual saliva samples.

Classification of secretor status by the steepness determination of the MPV decreasing curve is applicable to the forensic medical examinations of various body fluids, for example, urine, semen, and vaginal secretions. Secretor status determination of seminal blood group substances in vaginal contents may be an effective tool for sex crime examinations. Secretor status determination from gargled water may be useful for testing babies or diseased persons from whom sufficient volumes of clean and nondiluted salivas cannot be obtained.

We are exploring the method for testing the binding efficiencies of the extracts from the stains of various external secretions.

References

[1] Sekimoto, H., "Secretor and Non-Secretor Status of Human Saliva," *Nagasaki Medical Journal*, Vol. 18, No. 5, May 1940, pp. 526-542 (in Japanese).

[2] Matsuzawa, S., "On the Blood Group Substances in Human Salivas," *Japanese Journal of Legal Medicine*, Vol. 8, No. 5, Sept./Nov. 1954, pp. 423-442 (in Japanese).

[3] Mourant, A. E., "A 'New' Human Blood Group Antigen of Frequent Occurrence," *Nature*, Vol. 158, No. 4007, Aug. 1946, pp. 237-238.

[4] Ueyama, R., "On the New Antigen 'T' in Human Non-Secretor Salivas," *Hanzaigaku Zasshi (Japanese Journal of Criminology)*, Vol. 13, No. 1, Jan./Feb. 1939, pp. 51-64 (in Japanese).

[5] Le Pendu, J., Lemieux, R. U., Dalix, A. M., Lambert, F., and Oriol, R., "Competition Between ABO and Le Gene Specified Enzymes, 1. A Lewis Related Difference in the Amount of A Antigen in Saliva of A_1 and A_2 Secretors," *Vox Sanguinis*, Vol. 45, No. 5, Nov. 1983, pp. 349-358.

[6] Le Pendu, J., Oriol, R., Lambert, F., Dalix, A. M., and Lemieux, R. U., "Competition Between ABO and Le Gene Specified Enzymes, II. Quantitative Analysis of A and B Antigens in Saliva of ABH Nonsecretors," *Vox Sanguinis*, Vol. 45, No. 6, Dec. 1983, pp. 421-425.

[7] Matsuzawa, S., Bannai, Y., Kimura, H., and Kitamura, H., "Quantitation of Isohemagglutinins by a Particle Volume Distribution Analyzing Technique," *Japanese Journal of Legal Medicine*, Vol. 36, No. 3, May/June 1982, pp. 436-440.

[8] Kimura, H., Bannai, Y., and Kobayashi, R., "The Ratio Estimation of IgM and IgG Isohemagglutinins by a Particle Volume Distribution Analyzing Method," *Proceedings of Japan Academy*, Vol. 58, No. 7, Series B, Sept. 1982, pp. 232-236.

[9] Pirofsky, B. and Rosmer, E. R., "DTT Test: A New Method to Differentiate IgM and IgG Erythrocyte Antibodies," *Vox Sanguinis*, Vol. 27, No. 5, Nov. 1974, pp. 480-488.

Address requests for reprints or additional information to
Shigetaka Matsuzawa
Department of Forensic Medicine
Juntendo University
Hongo 2-1, Bunkyo-ku
Tokyo, Japan, 113