TECHNICAL NOTE

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Determination of ABO Blood Grouping from Human Oral Squamous Epithelium by the Highly Sensitive Immunohistochemical Staining Method EnVision+

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ABSTRACT: Using the highly sensitive immunohistochemical staining method EnVision+, which employs a dextran polymer reagent for the secondary antibody, the detection of the ABH antigens was attempted in the oral squamous epithelium. This new technique uses monoclonal antibody as a primary antibody and it takes about three hours for staining. The time is much shorter than conventional absorption-elution testing or absorption-inhibition testing for the determination of ABO blood grouping. Secretor saliva samples were stained at strong intensity by the antibody, which corresponded to its blood group and anti-H. On the one hand, nonsecretor saliva samples were stained at strong intensity only by the antibody that corresponded to its blood group, and at weak intensity only by anti-H. Since human oral squamous epithelium antigens were stained specifically by this method, we can examine the ABO blood group of saliva samples and perform cytodiagnosis at the same time.

Our research suggested that the EnVision+ Method is a useful technique for ABO blood grouping of saliva in forensic cases.

KEYWORDS: forensic science, ABO blood grouping, EnVision+, immunohistochemistry, oral squamous epithelium

While DNA profiling has become the principal technique for individualization of biological evidences, ABO blood grouping is still a useful test method in the initial stages of crime investigation. The ABO typing test does not consume time and resources, therefore, this type of test is the most popular blood group test in Japan. Absorption-elution tests and absorption-inhibition tests need to be reexamined for biological evidences such as blood stains or body fluid, and screening tests of suitable monoclonal antibodies for these tests are required. Gaensslen et al. (1) and Lincoln and Watts (2) have reported successful application of monoclonal antibodies to the absorption-elution and absorption-inhibition tests. On the other hand, Martin and Parkin (3) and Nagai et al. (4) indicated weak and nonspecific reactions in these applications. Kobayashi et al. (5) reported that displacing the solvent with various sera and proteins increased the titer and changed the specificity, making correct blood grouping from hair samples possible. The usefulness of the monoclonal antibody has been shown by Pedal (6) and Clausen and Hakomori (7) in the histological field. It falls in the transitional phase from polyclonal antibody to monoclonal antibody, and various techniques are being used to examine biological evidences in Japan. As a part of these trials, we examined ABO blood grouping from saliva samples. The usual saliva test is carried out by checking α-amylase by blue starch and then confirming oral squamous epithelium by cytodiagnosis. In this paper, we report a method of simultaneously cytodiagnosis and ABO blood grouping from saliva specimens by detecting ABH antigens on oral squamous epithelium using the highly sensitive immunohistochemical technique EnVision+, which employs the dextran polymer reagent for the secondary antibody.

Materials and Methods

Saliva

Human saliva was fixed in 4% Paraformaldehyde (4% Paraformaldehyde, 50 mM phosphate buffer pH 7.4) at room temperature for 30 min, then washed with PBS (20 mM phosphate buffer (pH 7.0), 150 mM NaCl, DAKO Corp., Lot No. 00128) three times. Ten μL of suspension is applied on a slide glass coated with MAS, and dried at 50°C for 30 min on a hot plate.

Reagents

The mouse monoclonal antibodies used as the primary antibody are shown in Table 1. All antibodies were diluted with Antibody Diluent with Background Reducing components (DAKO Corp., Lot No. 069-1). EnVision+ (DAKO Corp., Lot No. 099-2) was used as the secondary antibody. Coloration was performed by Liquid DAB Substrate-Chromogen System (DAKO Corp., Lot No. 069-3).

Immunohistochemical Stain

Paraformaldehyde-fixed saliva samples on a slide glass were immersed in distilled water for 20 min. The endogenous peroxidase

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TABLE 1—*Commercially-available antibodies used for immunostaining.*

Reagents (Trade Name)	Lot Number	Supplier
Monoclonal Antibodies		
Anti-A		
Anti-Blood Group Antigen A	0018C	DAKO Corp.
Monoclonal WAKO	HN210	Wako Junyaku
NOVACLONE	5906	Dominion
Gamma Clone	AM72-4	Kainos Corp
Neo Kokusai	029	Kokusai Shiyaku
Seraclone	111301	Biotest.AG
BCA	A8123	Cosmobio Corp.
Immucor	101260	Sanko Junyaku
Bioclone	BAA214A21	Ortho
Anti-B		
Anti-Blood Group Antigen B	0058B	DAKO Corp.
Monoclonal Wako	HP239	Wako Junyaku
NOVACLONE	06214	Dominion
Gamma Clone	BM124-3	Kainos Corp.
Neo Kokusai	029	Kokusai Shiyaku
Seraclone	161051	Biotest.AG
BCA	B8125	Cosmobio Corp.
Immucor	201270	Sanko Junyaku
Bioclone	BBB610A21	Ortho
Anti-H		
Anti-Blood Group Antigen H	0058B	DAKO Corp.
Gamma Clone	HM18-1	Kainos Corp.
Neo Kokusai	9176	Kokusai Shiyaku
Diagast	18000	Diagast

activity was blocked by 3% hydrogen peroxide for 10 min, followed by rinsing with distilled water for 10 min. After equilibrium in PBS for 10 min, incubation with: a) primary antibody, and b) EnVision+ reagent for 30 min were followed by rinsing twice with PBS. Coloration was performed by Chromogen solution for 5 min followed by rinsing with distilled water for 10 min. Nuclei were stained by Mayer hematoxylin solution. All procedures were carried out at room temperature.

Criterion

3+: Strongest intensity of staining,
2+: Stronger intensity of staining,
+: Intensity of staining is not strong, but epithelium is stained uniformly,
±: Partial staining, and
−: Absolutely no staining.

Results

Specificity and Dilution Test of Primary Antibodies

As shown in Table 2, DAKO, Monoclonal WAKO, NOVACLONE, Bioclone Anti-A diluted 30-fold, and Seraclone Anti-A diluted 10-fold showed strong intensities of staining against Type A squamous epithelium, but Type B and O cells showed no staining. Neo Kokusai and Immucor diluted ten-fold had strong intensities of staining against Type A squamouse epithelium, but Type B cell showed nonspecific staining. Gamma Clone and BCA had strong intensities of staining against Type A cells, but were nonspecific against Type B and O cells. Further dilution of DAKO,

TABLE 2—*Detection of A, B, H antigens in oral squamous epithelium by EnVision+ system.*

	Neat			×10			×30			×50			×100		
Reagents (Anti-A)	A	B	O	A	B	O	A	B	O	A	B	O	A	B	O
Anti-Blood Group Antigen A	3+	±	±	3+	±	±	3+	−	−	2+	−	−	+	−	−
Monoclonal WAKO	3+	±	±	3+	−	−	3+	−	−	+	±	±	+	±	±
NOVACLONE	3+	±	±	3+	−	−	3+	−	−	+	±	±	+	±	±
Gamma Clone	3+	+	+	2+	±	±	2+	−	−	NT	NT	NT	NT	NT	NT
Neo Kokusai	3+	+	+	3+	+	−	2+	+	+	+	±	±	+	±	±
Seraclone	3+	±	−	3+	−	−	2+	−	−	NT	NT	NT	NT	NT	NT
BCA	3+	+	+	2+	−	−	2+	±	−	NT	NT	NT	NT	NT	NT
Immucor	3+	±	+	3+	±	−	2+	−	−	NT	NT	NT	NT	NT	NT
Bioclone	3+	±	±	3+	±	±	3+	−	−	+	±	±	+	±	±
Reagents (Anti-B)															
Anti-Blood Group Antigen B	±	+	±	−	±	±	±	±	−	NT	NT	NT	NT	NT	NT
Monoclonal WAKO	±	3+	±	±	2+	±	−	+	−	NT	NT	NT	NT	NT	NT
NOVACLONE	±	2+	±	±	+	±	±	+	−	NT	NT	NT	NT	NT	NT
Gamma Clone	±	3+	±	−	3+	−	−	2+	−	NT	NT	NT	NT	NT	NT
Neo Kokusai	±	2+	±	±	2+	−	−	+	−	NT	NT	NT	NT	NT	NT
Seraclone	+	3+	±	±	2+	±	±	2+	±	NT	NT	NT	NT	NT	NT
BCA	±	2+	−	±	+	±	−	+	−	NT	NT	NT	NT	NT	NT
Immucor	±	+	±	±	+	−	±	±	−	NT	NT	NT	NT	NT	NT
Bioclone	+	3+	±	±	2+	±	±	2+	−	NT	NT	NT	NT	NT	NT
Reagents (Anti-H)															
Anti-Blood Group Antigen H	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	2+	2+	2+
Gamma Clone	±	±	±	±	±	±	NT	NT	NT	NT	NT	NT	NT	NT	NT
Neo Kokusai	+	+	±	±	±	±	NT	NT	NT	NT	NT	NT	NT	NT	NT
Diagast	+	+	+	+	+	+	NT	NT	NT	NT	NT	NT	NT	NT	NT

NT: not tested.

Monoclonal WAKO, NOVACLONE, Bioclone, and three sources except DAKO showed remarkable decreases in intensity of staining against Type A cells, while Type B and O cells showed nonspecific staining. DAKO showed a slight decrease in intensity of staining, and Type B and O cells showed no staining. Tenfold Gamma Clone stained Type B squamous epithelium with strong intensity, while Type A and O cells were not stained. Neat Monoclonal WAKO, Seraclone, and Bioclone showed strong intensities of staining against Type B cells, but were nonspecific against Type A and O cells. Neat DAKO and Immucor showed slightly intense staining against Type B cells. The more detailed dilution test for Gamma Clone anti-B indicated that the optimal dilution level is from five to tenfold. Four commercial sources of anti-H were examined, but only DAKO showed a strong intensity of staining against H antigen. The three other sources showed no vivid staining.

The above result shows that DAKO's anti-A, anti-H, and Gamma Clone's anti-B were the most suitable primary antibody, for this method.

ABO Blood Grouping from the Saliva

Twenty saliva samples (Type-A secretor, four samples; Type-A nonsecretor, two samples; Type-B secretor, four samples; Type-B nonsecretor, two samples; Type-O secretor, three samples; Type-O nonsecretor, two samples; Type-AB secretor, one sample; Type-AB nonsecretor, two samples), in which the ABO blood group had already been examined by the absorption-inhibition test and the hemagglutination test, were stained by this method. As shown in Table 3, the blood groups of all samples were determined correctly. Figure 1 shows typical results of secretor and nonsecretor samples. Secretor samples were stained with strong intensity against antigen B and H. But nonsecretor samples were stained in a strong intensity against only anti-B.

Discussion

Immunohistochemical methods make it possible to identify the antigens from formalin-fixed and paraffin-embedded tissue sections. The peroxidase-anti peroxisidase (PAP) method by Stern-

TABLE 3—*Results of blood grouping from oral squamous epithelium.*

Blood Groups of Donor	Anti-A	Anti-B	Anti-H
A secretor	3+	−	3+
B secretor	−	3+	3+
O secretor	−	−	3+
AB secretor	3+	3+	3+
B secretor	−	3+	3+
B nonsecretor	−	3+	±
A nonsecretor	3+	−	±
A secretor	3+	−	3+
A secretor	3+	−	3+
B secretor	−	3+	3+
A nonsecretor	3+	−	±
B nonsecretor	−	+	−
O nonsecretor	−	−	±
O secretor	−	−	3+
O secretor	−	−	3+
B secretor	−	3+	3+
AB nonsecretor	3+	3+	±
O nonsecretor	−	−	±
A secretor	3+	−	3+
AB nonsecretor	3+	3+	±

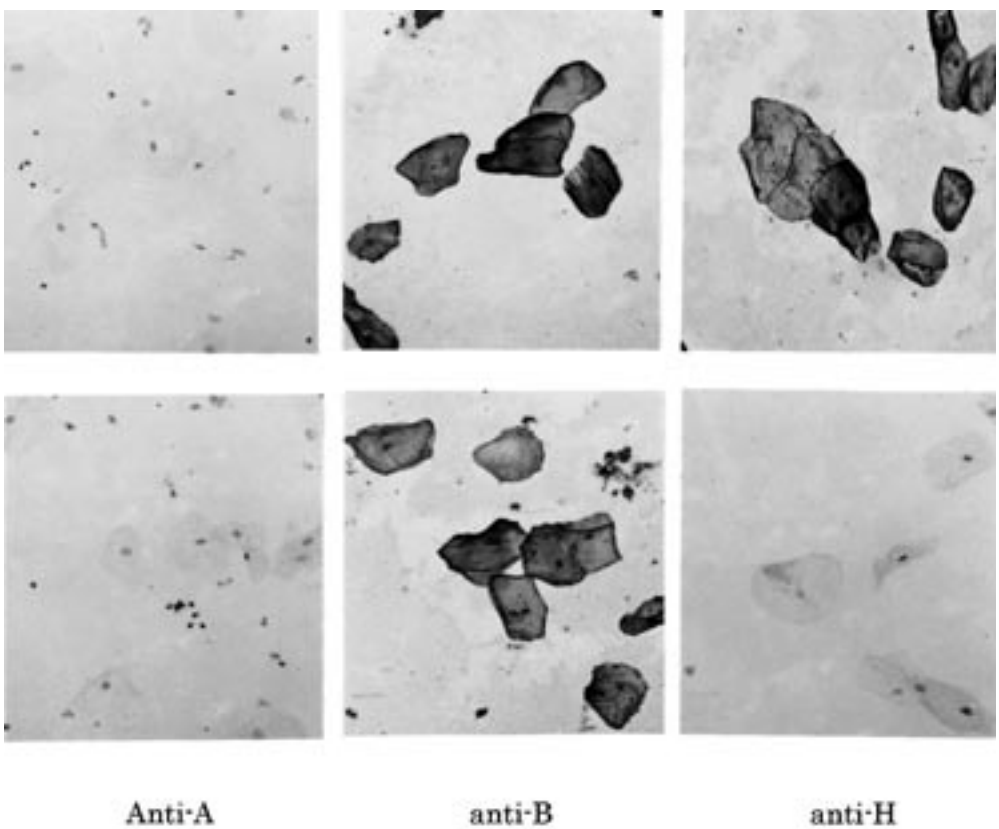

FIG. 1—*Examples of immunohistochemical staining with monoclonal antibody. B secretor (upper side) and nonsecretor (bottom side) saliva were stained by the EnVision + system.*

berger et al. (8), the ABC method that uses avidin-biotin strong units by Hus et al. (9), and the LSAB method, which shows increased sensitivity by direct labeled streptavidin with peroxidase (10) are used mainly in this field. Since Yamamoto et al. reported on nucleotide sequences of the cDNA of three major alleles (A^1, B, O^1) of the ABO blood system (11,12), ABO genotypes have been determined by restriction fragment length polymorphism (RFLP) (11,13–15), allele-specific PCR amplification (ASPA) (16,17), single-strand conformation polymorphism (SSCP) (13), or amplified product length polymorphism (APLP) techniques (18). Of course, DNA analysis is a powerful technique for biological evidences, although these techniques consume time and resources. Therefore, we studied the more simple and quick method. The absorption-inhibition test and absorption-elution test carried out by monoclonal antibodies showed weak and nonspecific reactions (3,4), therefore, these applications were reevaluated. More accurate and specific methods are being developed. Although the ELISA method (19) is also used for the determination of ABO blood grouping from saliva materials, this technique detects soluble antigens from saliva by diluting saliva with a buffer solution. In this method, a cell portion collects at the bottom of the tube after centrifugation, and is usually disposed of. However, if the determination of ABO blood grouping from this pellet is possible by an immunohistochemical method, the results will become more accurate. The recent highly-sensitive immunohistochemical staining reagent from Dako Japan, EnVision+, was reported (20) to employ polymeric conjugates comprising a high number of enzyme molecules bound to a polymer backbone together with the detection antibody molecules. Since there is as yet no kit for this method, various factors such as sample preparation, dilution degree of primary antibody, and selection of appropriate monoclonal antibody were examined. Our results showed that preparation of the saliva material in 4% paraformaldehyde (pH7.4) for 30 min., and three times washing with PBS (pH7.0) is sufficient. In commercial sources of anti-A for diagnosis of blood group, Monoclonal Wako, Navaclone and Bioclone, which are diluted 30-fold, seem to be usable as the primary antibody, but further dilution of these antibodies leads to nonspecific staining on Type B and O cells. The staining intensity is generally low on the anti-B antibody, and only Gamma Clone has a strong intensity of staining against Type B cells. This antibody should be used at the dilution degree of five to ten-fold, which is a rather high concentration. Although DAKO anti-H showed a strong intensity of staining against antigen-H at the dilution degree of fifty-fold, there were no other strong intensities of staining. Differences among the monoclonal antibodies in intensity of staining dependent on immunogens are unclear, as reported by Kobayashi et al. (5). It is most important to check the reactivity of available antibodies and to select the highest sensitivity and specificity antibody for this method. The blood groups of known saliva samples were correctly detected by staining intensity. Secretor samples had a strong intensity of staining against anti-H, but nonsecretor samples showed markedly decreased staining. Hence, discrimination of secretors and nonsecretors is possible. The above results show that the EnVision+ method can detect ABH antigens form oral squamous epithelium; therefore, ABO blood grouping and cytodiagnosis can be performed at the same time, and we can thus save much time in the examination of saliva material. Since this method uses pellets that are normally disposed of in routine work, the supernatants can still be examined by the ELISA method, and collating both results offers a more reliable examination of biological evidences.

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