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Detection and Use of Salivary Hemagglutinins for Forensic Blood Grouping

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ABSTRACT: A sensitive method for the detection of anti-A and anti-B hemagglutinins in fresh saliva has been developed. The method utilizes a bromelin treated erythrocyte suspension as indicator cells and includes a simple procedure to concentrate these hemagglutinins. Antiserum directed against immunoglobulin A enhances the hemagglutination assay. We find that these salivary hemagglutinins are present in over 90% of the population and that their titer remains stable over a period of two months. These hemagglutinins can be used to blood type the donor of a saliva sample and can be used in a confirmatory test that complements the commonly used absorption-inhibition test which is used to detect salivary blood group agglutinogens. In preliminary studies we have determined that hemagglutinins can be successfully isolated and analyzed from dried saliva stains.

KEYWORDS: forensic science, genetic typing, saliva, hemagglutinins, blood typing

Saliva was first analyzed for the presence of anti-A and anti-B hemagglutinins in 1928, when Yosida examined 58 different saliva samples and determined the ABO blood group of each donor [*1*]. Since then many investigators have confirmed the presence of hemagglutinins in saliva that have specificity for the A and B erythrocyte antigens [*2-8*]. Although these hemagglutinins provide information concerning the ABO blood group of the individual from whom the saliva has been obtained, salivary hemagglutinins have not been used for forensic science purposes primarily as a result of their low concentration and the lack of a sufficiently sensitive procedure to detect and study them.

The ability to blood type an individual by analysis of his or her saliva is important, especially when cigarette butts, envelope flaps, or other articles with saliva stains are available. Physical evidence of this type can reveal information of considerable value in distinguishing between suspects of different blood groups. Forensic serologists currently rely on detection and identification of salivary hemagglutinogens to derive blood group information. Unfortunately, the identification of these antigenic markers can be made difficult by many factors, including (1) limited amount of sample, (2) contamination, and (3) exposure to various envi-

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ronmental factors such as high humidity and temperatures that may adversely affect the analysis. Scott and Corry performed a study on saliva samples which yielded false-positive ABO grouping results using the absorption-inhibition and absorption-elution methods [9]. They concluded that this phenomenon can be caused by the presence in saliva of large quantities of blood group active microorganisms.

This paper describes a procedure that permits the detection of specific ABO hemagglutinins in fresh saliva and thus can serve as a complementary test for current procedures which detect only hemagglutinogens. This procedure is applicable to dried stains as well.

Material and Methods

Collection of Samples

Two hundred volunteers who had been blood typed in the ABO system donated saliva. Saliva was used fresh or frozen at $-20°C$ for 24 h and then tested. Donors varied with respect to age, sex, and ethnic origin. Whole saliva was obtained by having donors chew paraffin to increase saliva production. Collection took place at least 2 h after eating. The subjects salivated into a clear plastic cup, and the saliva was then transferred into a glass test tube for analysis.

Hemagglutination Assay

We used four stock solutions for the hemagglutination assay:

1. Phosphate buffered saline (PBS), pH 7.4, is prepared by addition of 1.15-g sodium phosphate, dibasic (Na_2HPO_4), 0.20-g potassium phosphate, monobasic (KH_2PO_4), and 8-g sodium chloride (NaCl) to 1 L of distilled water.
2. Bromelin solution is prepared by mixing 0.1 g of bromelin with 10 mL of PBS, treating the mixture briefly with a homogenizer and then centrifuging for 5 min at $1000 \times g$ to remove particulates. The supernatant is stored at $-20°C$.
3. Albumin, polyvinyl pyrrolidone, and dextran-PBS used in the test cell preparation is prepared by adding 0.5 g of bovine serum albumin, 0.5 g of polyvinyl pyrrolidone, and 0.5 g of dextran to 100 mL of phosphate buffered saline.
4. Albumin PBS consists of 0.5-g bovine serum albumin in 100-mL PBS. PVP-Dextran PBS consists of 0.5 g of dextran and 0.5 g of polyvinyl pyrrolidone in PBS.

Sample Preparation—Frozen saliva samples are allowed to thaw for 10 min at room temperature. One volume of toluene is then added to two volumes of saliva, mixed for 30 s on a vortex mixer and centrifuged at $450 \times g$ for 5 min. Three layers become visible. The top layer is cloudy and contains mucous, the middle layer is less cloudy, and the lower layer contains amorphous debris. The middle layer is carefully separated for agglutination tests, and the other layers are discarded.

Preparation of Test Cells—Erythrocytes to be used as test cells are washed in two volumes of PBS and centrifuged at $450 \times g$ for 2 min to form a pellet of cells. This procedure is repeated two additional times. The cells are then suspended in bromelin solution (1:2) and maintained at 37°C for 1 h. The cells are again washed three times with PBS as described above, and a 0.5% volume/volume cell suspension is prepared in a mixture of albumin, polyvinyl pyrrolidone, and dextran phosphate buffered saline.

Hemagglutination Assay—The chemically treated cells are prepared in varying concentrations of albumin, dextran, and PVP (Table 1) and a 50-μL aliquot is added to 150 μL of toluene treated saliva. The mixture is allowed to stand for 2 h at 4°C. Hemagglutination is then read visually with a $\times 15$ hand magnifying lens. Red cell buttons that have smooth edges and run slowly down the vertical surface of the test tube are scored as negative or "0."

TABLE 1—*Results of agglutination tests using fresh saliva obtained from 50 individuals having Blood Group O, chemically treated Type A_1 and B cells, and various concentrations of albumin, dextran, and polyvinyl pyrrolidone.*

	Concentration of Albumin		Concentration of PVP Dextran and Albumin[a]				Concentration of PVP and Dextran
Titer	10%	20%	5%	10%	15%	20%	10%
2	3	3	4	4	4	4	4
4	2	3	3	4	4	4	4
8	1	2	2	4	3	3	3
16	0	1	0	3	3	3	2
32	0	0	0	3	3	3	1
64	0	0	0	2	2	2	0
128	0	0	0	1	1	1	0
256	0	0	0	0	0	0	0

[a]Total polymer concentration after mixing equal amounts of PVP, Dextran, and Albumin (weight/volume). See Table 2 for a description of grading. Titer refers to salivary dilution factor.

Cell clusters that have rough edges and remain attached to the bottom of the test tube even when it is inverted are scored positive. Clusters that slide down the test tube as a single mass or a small number of clumps are also scored as positive, but to a lesser degree. Table 2 describes the grading of the hemagglutination assay.

Type O test cells remain unagglutinated regardless of the type of saliva under study and serve as negative controls for nonspecific agglutination.

Protein Determinations—The total concentration of protein in toluene extracted and untreated saliva was determined using a standard Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA, Catalog No. 500-0001). A secretory immunoglobulin A (IgA) protein standard purchased from Cappel Laboratories (Division of Biological Corporation of America, West Chester, PA, Catalog No. 6001-0010) was used to prepare standard protein concentrations ranging from 0.2 to 1.4 mg/mL. Protein concentrations in saliva samples were determined using a visible wavelength spectrophotometer by comparing optical density readings at 595 nm for known and unknown samples.

Stability Studies—Liquid saliva samples held in sealed glass tubes were aged at −20, 25, and 56°C for periods ranging from 1 h to 14 weeks. Hemagglutination studies were then conducted as described above.

Hemagglutinins—Salivary hemagglutinins referred to throughout this paper are only those which specifically react with A and B erythrocyte cell surface agglutinogens.

TABLE 2—*Hemagglutination reaction grading chart.*

Score	Description
4	button of cells remains in one clump after being dislodged from the wall of the test tube; background is clear
3	button breaks into four to six large clumps; background is clear
2	button breaks into smaller sized clumps; background may become slightly cloudy
1	button breaks into numerous tiny clumps; background becomes cloudy; agglutination is read microscopically
0	no visible agglutinates are observed microscopically

Results

Preparation of Erythrocyte Suspensions

Several erythrocyte test cell suspensions were prepared using various mixtures of albumin, dextran, and PVP to establish the optimum combination and concentration of polymers needed to render the most sensitive hemagglutination reactions. Table 1 gives the results of agglutination studies using albumin, dextran, and PVP at various concentrations. These observations were made using enzyme treated A_1 and B cells and saliva obtained from individuals known to have Type O blood. The results indicate that hemagglutination is optimal when both polymers are present and are in agreement with the findings of McNeil and Trentelman, who found that optimum agglutination takes place when a concentration of 10 to 20% of a colloidal suspension is used [*10*]. The combination of albumin, PVP, and dextran enhanced the hemagglutination reaction and increased assay sensitivity approximately 16-fold. These polymers were therefore included in the test-cell suspension used in the hemagglutinin assay.

Protein Concentration of Saliva and Saliva Extracts

Ten saliva samples were assayed for protein concentration using the spectrophotometric assay described above. The average for these ten samples was 1.67 mg/mL. Extraction of ten saliva stains with isotonic saline for a period of 1 h resulted in an average extract protein concentration of 1.6 mg/mL, indicating an extraction efficiency of 96%.

Addition of Anti-Human IgA

To enhance further the sensitivity of the hemagglutination reaction, goat anti-human secretory IgA antiserum (Cappel Laboratories, Catalog No. 0101-0011) was added to the hemagglutinin-erythrocyte suspension. This noticeably enhanced the reaction and rendered it easily visible.

Variation in Hemagglutinin Titer

Eighty saliva samples were collected, including twenty-five of blood group A, twenty-five of group B, 25 of group O, and five of group AB, at biweekly intervals over a period of two months. The samples were tested to determine if any variation in hemagglutinin titer could be detected within the same individual's saliva over a two-month period. Seven volunteers were tested three times daily over a period of two weeks to determine if any variation in hemagglutinin titer occurs during the course of a single day. No significant variation in titer was found, suggesting that secretion of ABO hemagglutinins in saliva is a stable phenomenon. These findings are in agreement with those of Prokop and Uhlenbruck [*11*]. Saliva collected from Type O individuals consistently yielded agglutination reactions at higher titers than saliva samples from Type A or B individuals.

Effect of Temperature on Hemagglutination Reaction

Most of our hemagglutinin studies were performed at 4°C. In 1982 Hardman found that the sensitivity of hemagglutinin reactions involving IgA antibodies was enhanced when conducted at lower temperatures. We collected saliva samples from 30 volunteers over a 2-h period. The samples were divided into three aliquots and each maintained at a different temperature (4, 25, and 56°C). Thirty such samples were incubated with a 10% albumin-dextran phosphate-buffered saline erythrocyte suspension. The agglutination reaction was much stronger and easier to observe when the incubation was conducted at 4°C rather than

at 25 or 56°C (Table 3). In addition, the agglutination reaction occurred much more rapidly at 4°C than at room temperature. The reactions could usually be read within 15 min when the incubation temperature was 4°C.

Hemagglutinin Frequency in Saliva

A frequency study was conducted to determine the percentage of the population secreting salivary anti-A and anti-B hemagglutinins. It appears that specific salivary hemagglutinins exist in over 90% of the population (Table 4). These results are in agreement with those of Otten [*3*], Bell and Fortwengler [*5*], and Saneshige and Woodfield [*12*], all of whom detected hemagglutinins in over 90% of the samples tested.

Preliminary studies with saliva stains indicate that approximately 800 μL of saliva (1.28 mg of total salivary protein) are needed to ensure a high rate of anti-A and anti-B hemagglutinin detection.

Stability of Hemagglutinins

Fresh saliva samples were examined to determine hemagglutinin stability over time at different temperatures. Table 5 summarizes these findings. Hemagglutinins appear to be stable for up to two months when stored at −20°C. Unfortunately, they can be detected for only about one day when maintained as stains at room temperature. Our studies on salivary stains indicate that it is essential to refrigerate or freeze such evidentiary material and to analyze it as quickly as possible.

TABLE 3—*Results of agglutination tests at various incubation temperatures.*

Temperature, °C	Number of Samples	Number Typed	Agglutination Result[a]
56	30	4	2
25	30	26	3
4	30	28	4

[a]Average of three determinations per sample. See Table 2 for a description of grading.

TABLE 4—*Population frequency of salivary hemagglutinins.*[a,b]

Blood Type	Number of Samples	Hemagglutinins Detected		% Positive
		Anti-A	Anti-B	
A	58	0	53	91.4
B	47	44	0	93.6
O	90	88	89	99.0
AB	5	0	0	0

[a]Samples that yielded hemagglutination grades of 1 through 4 using Table 2 were considered positive.

[b]Salivary hemagglutinins referred to describe only those which specifically react with A and B cell surface agglutinogens.

TABLE 5—*Hemagglutinin stability in fluid saliva with respect to aging and temperature.*[a]

Blood Type	Number of Samples	Temperature, °C	2 h	6 h	12 h	24 h	1 Week[b]	2 Weeks	6 Weeks	8 Weeks	10 Weeks	12 Weeks	14 Weeks
A	25	−20	4	4	4	4	4	4	4	4	3	0	0
		25	4	4	3	3	1	0	0	0	0	0	0
		56	2	0	0	0	0	0	0	0	0	0	0
B	25	−20	4	4	4	4	4	4	4	4	3	1	0
		25	4	3	3	2	1	0	0	0	0	0	0
		56	2	1	0	0	0	0	0	0	0	0	0
O	25	−20	4	4	4	4	4	4	4	4	3	2	1
		25	4	4	3	3	2	1	0	0	0	0	0
		56	3	2	1	0	0	0	0	0	0	0	0
AB	25	−20	0	0	0	0	0	0	0	0	0	0	0
		25	0	0	0	0	0	0	0	0	0	0	0
		56	0	0	0	0	0	0	0	0	0	0	0

[a]Grading from 0 to 4 reflects the strength of agglutination. These numbers represent the most frequently observed agglutination levels. See Table 2.

Discussion

Salivary anti-A and anti-B hemagglutinins are potentially important since it is possible to determine an individual's ABO blood group by analysis of these antibodies. Forensic serologists have thus far been unable to exploit these molecules for the purpose of blood grouping for several reasons: low concentration of IgA molecules in saliva; lack of a sufficiently sensitive detection method; lack of knowledge concerning hemagglutinin stability in wet and dried states; and lack of a data base with which to interpret observations.

We have attempted to address some of these problems by first developing a sensitive assay system for the detection of salivary hemagglutinins. Two hundred fresh saliva samples were tested using a chemically treated erythrocyte test cell suspension. Erythrocytes were first treated with bromelin, a proteolytic enzyme, and then suspended in a solution containing albumin, PVP, and dextran in PBS (20% weight/volume). This concentration was chosen because it was found that the hemagglutination reaction was strongest under these conditions. Bromelin treatment may alter erythrocytes by removing polar groups and thus reducing the zeta potential [*13*]. It has been reported [*14*] that proteolytic enzymes can reduce the cell's zeta potential by approximately 50% and can cause changes in the erythrocyte surface structure that promote antibody binding. This may result from removal of steric impediments to antibody attachment and reduced hydration in the immediate vicinity of the antigenic determinants.

It has been suggested that polymers enhance hemagglutination by increasing the dielectric constant of the medium and thereby reducing the zeta potential of the erythrocytes [*15*]. Alternatively, they may act by polymer bridging that facilitates erythrocyte interactions and thus enhances the rate and extent of the overall hemagglutination process. Van Oss et al. have stated that polymers such as dextran change the surface morphology of erythrocytes and cause spiculation (blebbing) of the cell surface [*14*]. Our attempts to verify this alteration in morphology by scanning electron microscopy (SEM) have thus far failed. It is possible that surface changes do indeed occur but the morphological evidence is minimal.

The problem of relatively low IgA concentrations in saliva was addressed by using a toluene extraction method similar to that of Hummel and Schoch [*4*]. When toluene is mixed with saliva, three layers form, and the middle layer is enriched with IgA. Toluene treatment is known to remove mucin from tissues such as saliva [*12*] and may serve to separate hemagglutinins from mucin in collected samples. In addition, we found that we could enhance the sensitivity of the hemagglutination assay by addition of goat anti-human IgA antiserum to the hemagglutinin-erythrocyte suspension. This reagent presumably binds to erythrocyte-bound IgA molecules, thereby producing complexes that are readily visible to the naked eye. We have observed that hemagglutination often does not occur when anti-human IgA is not included in the assay mixture.

After a sufficiently sensitive hemagglutinin procedure was developed, 200 saliva samples were collected from different volunteers and tested. A hemagglutinin detection rate of over 90% indicates that a large majority of this population secretes salivary hemagglutinins in sufficient quantity to make their detection possible using the enhanced assay system. This fact is important to those wishing to make use of this system for forensic science analysis.

We have been able to blood type individuals successfully by studying salivary hemagglutinins in fluid saliva. However, if this system is to be of significant forensic science value, the assay must work with dried stains as well. We are currently using this enhanced assay system to study the feasibility of blood grouping salivary hemagglutinins in saliva stains. Our preliminary results indicate that hemagglutinins can be successfully isolated and analyzed from stains. The major drawback appears to be the lack of long-term stability of the salivary hemagglutinins, and thus we are trying to develop methods to preserve their structure and function.

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