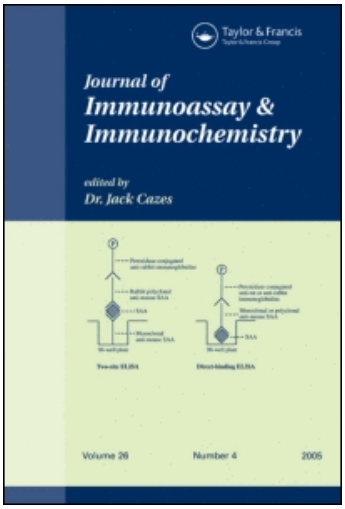

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A Simple Dipstick Immunoassay for Detection of A and B Antigens

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A SIMPLE DIPSTICK IMMUNOASSAY FOR DETECTION OF A AND B ANTIGENS

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(KEY WORDS: Dipstick Immunoassay, A&B Antigens, Bloodstains, Absorption Elution, Reverse Grouping).

ABSTRACT

ABH antigens, by virtue of their stability and widespread distribution are of unique significance in forensic examination. The conventional methods of typing often lack sensitivity and specificity. They fail to provide dependable results when samples are of minute size and exposed to harsh climatic conditions. We have developed a simple, rapid and highly sensitive solid phase double antibody dipstick immunoassay for detection of A&B antigens by using A&B antibodies (Human) immobilized on nitrocellulose membrane (NCM) strips. The bound antigens have been probed by enzyme labelled second antibodies (Mouse monoclonals). The dipstick assay successfully detected A&B antigens in stains containing as little as 100ng of dried blood. Bloodstains as old as two years could be correctly typed by this method. The assay has the added advantage of simplicity and rapidity. A and B antigens found in tissues, saliva, urine, or sweat can also successfully be detected by the assay. Contaminated bloodstains that conventional methods failed to detect were also identified by this assay.

INTRODUCTION

Absorption elution, mixed agglutination, absorption inhibition and reverse grouping are some of the routine methods for detection of ABH antigens in forensic analysis (1-4). Antigens in body fluid stains are often damaged by exposure to inclement climatic conditions thereby making their detection difficult by conventional methods. Bacterial contamination of blood stains can give false positive results due to the presence of blood group mimicking substances in bacterial cell walls (5).

Enzyme immunoassays have also been described for typing of these antigens (6). Antigen (blood extract) immobilised direct ELISAs require more antigen (7) and inaccurate results may occur due to the interference of endogenous peroxidase activity present in the sample (8). Sandwich immunoassays are of limited forensic relevance because of their complex protocols requiring expertise and instrumentation (9). To overcome these drawbacks a simple dipstick immunoassay (DSA) using a nitrocellulose support and two different kinds of antibodies is described here for simultaneous typing of A and B antigens. The sensitivity, specificity and suitability of DSA is assessed by comparing the results with conventional methods.

MATERIALS AND METHODS

Mouse monoclonal anti-A (MabA) and anti-B (MabB) were obtained from Dakopatts, Denmark and human antibodies against A and B substances procured from Stanzen Diagnostics, India. Bovine Serum Albumin (BSA) was purchased from Sigma Chemicals Co., USA and Nitrocellulose Membranes (NCM) of 0.22um pore size and polycarbonate sticks were supplied by Micro Device Pvt. Ltd., India.

Preparation of Dipsticks

Three NCM strips each of 7 x 7mm size were glued to a 7 x 70mm sized polycarbonate stick closely adjoining to each other on one end using adhesive (Quickfix) (FIG.1). The first strip was coated with anti-A, the second with anti-B, and the third strip, serving as a control, with BSA.

Peroxidase Labelling

MabA and MabB were conjugated with horse radish peroxidase by the glutaraldehyde method (10).

Antibody Coating

A 1:20 dilution of polyclonal human anti-A and anti-B were prepared in carbonate buffer, 50m mol/L, pH 9.6 and 10ul each of this antibody solution was immobilized on the respective strips by incubating in a moisture chamber at 37^{o}C for 2 hours. The excess of

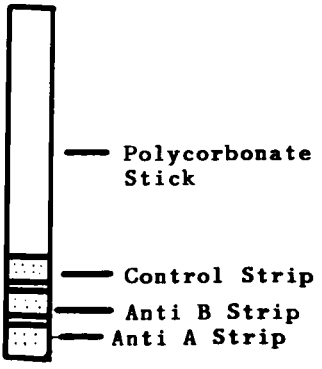

FIGURE 1 : Schematic Diagram of Dipstick

antibody was removed by aspiration and repeated washing with several changes of phosphate buffered saline, 10m mol/L, pH 7.2 containing 0.02% Tween20 (PBS-T).

Blocking

After immobilization of the antibodies on the membrane strips, the dipstick was incubated with 2% BSA in phosphate buffered saline 10m mol/L, pH 7.2 (PBS) for one hour at 37°C to block the non-specific binding sites on the membrane strips.

Dipstick Immunoassay (DSA)

A dipstick was incubated at 37°C for one hour with 1.0ml of stain or tissue extract in a 3ml test tube. The mouth of the tube was covered with parafilm, and the tube constantly rocked. After incubation, the

extract was decanted and the dipstick washed thoroughly with PBS-T. The dipstick thereafter was incubated for one hour at 37°C with one ml of equal parts of 1:1000 dilutions of enzyme labelled MabA and MabB. After being washed with PBS-T, the dipstick was immersed for 10 min in a substrate solution containing 0.1% chloronaphthol and 0.002% hydrogen peroxide in PBS. Development of a purple colour indicates a positive reaction.

Absorption Elution Method (AEM)

The modified absorption elution method of Howard and Martin was carried out to detect the A and B antigens (11). The blood stained fibres were attached, using adhesive (Quickfix), to the bottom of a microplate well instead of the cellulose acetate sheet.

Reverse Grouping Method (RGM)

The reverse grouping method of Mudd, J.L. (4) was performed in V bottom microplates.

Sample Preparation

Blood samples from volunteers whose blood group was determined by a slide agglutination method, were collected on cotton cloth by fingerpricking. Approximately 500ul of saliva, sweat and urine were also collected from every volunteer of known blood group and secretor status and stains were prepared

on cotton cloth. The donors selected for collection of body fluids represented individuals of both sexes, ranging from six months to 70 years.

Skin cells from the edge of heels of volunteers were also collected to study the blood group antigens in tissues.

Body fluid stains from a 3mm square of cotton were extracted in 500ul of normal saline containing 0.001% Tween-20 for typing by DSA and RGM while for detection by AEM, blood stained fibres teased out from these stains were used.

All the experiments were conducted in a blinded manner to eliminate subjective bias.

Sensitivity Study

From the dried blood stains of known group a small quantity of blood was scraped and the following dilutions were prepared in normal saline: 10ng/ml, 100ng/ml, 500ng/ml, 1000ng/ml, 2500ng/ml, 3000ng/ml. DSA, AEM, and RGM were used to detect A and B substances in all the above dilutions.

Suitability of Assay for Stored Stains

Bloodstains of known type were stored for 1, 6, 12 and 24 months before testing by the three methods. A hundred stains stored for each time period

were tested. Blood extraction from the stain was carried out as explained elsewhere (12).

Tissue Typing

100mg of skin tissue was dispersed by homogenization and subjected to alcoholic extraction as described by Lee, et al. (13). Ten donors of each group (A, B, AB and O) were selected for the study.

Typing of other Body Fluids

100 samples constituting equal numbers of A, B, AB and O groups each of saliva, sweat and urine were studied.

Study of Contaminated Stains

60 microbially contaminated blood stains were selected for the study.

RESULTS

Typical ABH typing results of DSA are shown in FIG.2. A minimum of 100ng of dried blood was needed for detection of A and B antigens by DSA whereas AEM and RGM required 2.5ug and 3.5ug respectively.

Bloodstains stored for two years were correctly typed by DSA, compared with only 1 month and 6 months respectively for RGM and AEM (Table 1).

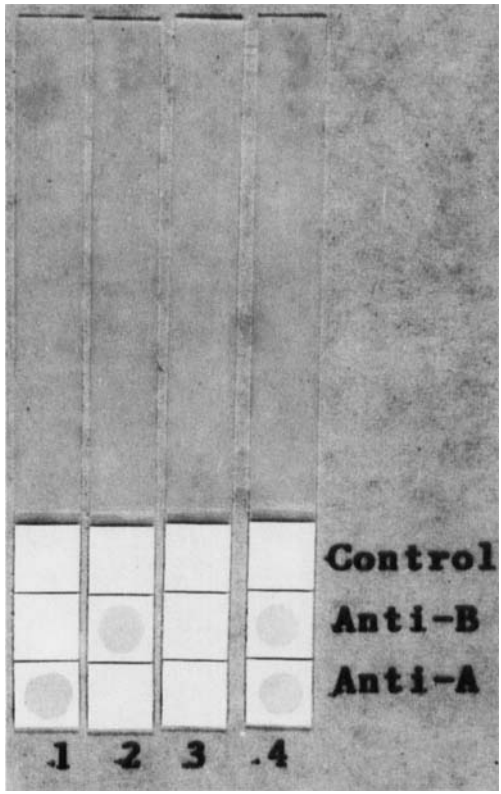

FIGURE 2 : Typical ABH grouping results of DSA with bloodstains : 1) A group; (2) B group; 3) O group; (4) AB group.

DSA detected antigens in AB stains with similar sensitivity to that of A and B stains, whereas RGM and AEM showed a slight decline in the detectability of antigens in AB stains (Table 2). The sex and age of the individual from whom the stain was derived did not influence the detection ability of the methods.

TABLE 1

ANALYSIS OF ABH ANTIGENS IN OLD STAINS BY DIFFERENT METHODS

S. No.	Age of stains	No. of stains	%of correct grouping results		
			R G M	A E M	D S A
1.	Fresh	120	100	100	100
2.	1 month	120	100	100	100
3.	6 months	120	72	100	100
4.	12 months	120	41	94	100
5.	18 months	120	11	94	100
6.	24 months	120	5	86	100

TABLE 2

DETECTION OF ANTIGENS IN AB STAINS AND A & B STAINS

S. No.	Type of stain	No. of stains	No.of correctly detected stains		
			R G M	A E M	D S A
1.	A	20	20	20	20
2.	B	20	20	20	20
3.	AB	20	16	18	20
	Total	60	56	58	60

TABLE 3

SUITABILITY OF DIPSTICK ASSAY IN TYPING OF CONTAMINATED BLOODSTAINS

S. No.	Method	No.of stains tested	No.of correct results
1.	D S A	60	60
2.	A E M	60	36
3.	R G M	60	28

Saliva, urine, sweat and tissue extracts were corectly detected by DSA in all the samples. Neither RGM nor AEM were reliable when used with these materials.

In the contamination study Aspergillus niger, Aspergillus nidulus, Aspergillus flavus, Penicillium notatum were observed in all the stains included in the study. Table 3 shows the results of typing of contaminated bloodstains by different methods. All the contaminated stains tested in the study could be accurately detected by DSA, whereas the results obtained by RGM and AEM were not reliable.

DISCUSSION

The DSA test is simple, rapid and sensitive. The need of indicator cells with a short shelf-life and the complicated procedures of AEM and RGM are also eliminated. The high affinity of proteins for nitrocellulose membrane is exploited for charging of dipsticks with antibodies (14). Membrane bound antigens and antibodies showed significant stability over a long period (15).

Polyclonal antibodies to A and B substances used to coat matrix in the assay are mainly of IgM type possessing specificities to a wide range of determinants that enables them to capture even a minute amount of antigen present in the degraded samples. A panel of enzyme labelled monoclonals were used in the assay to ensure binding with even a single determinant of the bound antigen thereby generating a signal.

From the results obtained on comparison of sensitivity of DSA, AEM & RGM, it is evident that DSA has superior performance with bodyfluids, and with old stains. The dipstick assay is based on direct detection of antigens which are highly stable glycolipids (16), whereas RGM detects agglutinins which are highly labile (17). Although AEM is also

a direct antigen detection method, it requires relatively large quantity of antigen for producing reliable results. Due to the multivalence and high avidity of the capturing antibodies and the enzyme amplification step used in the assay, blood group antigens present in as little as 100ng of dried blood could be successfully detected by the dipstick assay.

AB group individuals have poor expression of antigens due to competition between A and B transferases for H antigen precursor (18) and the volume of AB blood required for successful typing by AEM and RGM is relatively large. Similarly, in infants there is a decrease in the blood group antigen expression (19). In both of these situations, the DSA successfully detected A and B antigens.

The degradation of constituent molecules of body fluids in stains over the time is a well known phenomenon (20).

A and B antigens in 2 year old stains were enough to generate a strong positive signal in DSA. The rapid decline in the detectability of RGM is due to the fact that agglutinins are rapidly denatured with the passage of time (21). Low concentration of agglutinins and variations in their titres in the population (22) may be another reason for the poor

efficiency of methods based upon agglutinins detection.

Biological samples exposed to inclement environmental conditions became contaminated with microorganisms (23), and blood group mimicking substances present in these may have contributed to false results by causing agglutination of indicator cells on testing with AEM and RGM. The DSA is capable of discriminating between blood group antigens and microbial pseudoagglutinogens, thereby providing reliable results.

RGM is not feasible for the analysis of blood group antigens in tissues as agglutinins are absent in them. RGM is also not practicable for typing body fluids other than blood, although agglutinins are reported to be present in saliva (24). Here again, the dipstick has a high success rate.

Due to high stability of antibodies coated to nitrocellulose membranes, the dipsticks can be stored at $4^{o}C$ for several months (25), and appears to be suitable for blood grouping in field conditions.

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