Dot blot ELISA on Nitrocellulose membranes (NC): a new method for typing Lewis and ABO of body fluids (saliva, semen, vaginal secretions, sweat and urine)

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1. INTRODUCTION

In forensic case work Lewis and ABO typing of nonblood body fluids is currently performed by absorption-inhibition and absorption-elution techniques (Pereira et al. 1976, Davie 1979, Pinet et al. 1980, Bäßler 1986). Recently various enzyme linked immunosorbent assays (ELISA) using microtiter plates for ABO and Lewis typing were described (Lang 1985, Katsumata et al. 1984, Bolton et al. 1986). These methods are superior in respect to their sensitivity, specifity and reproducibility, but they need a high volume of stain extract and an expensive technical equipment. The work reported here describes a very simple and easy to handle ELISA method on nitrocellulose membranes which overcomes the problemes alluded to.

2. MATERIALS AND METHODS

Washing buffer

0,01 M Tris/HCl buffered saline, pH 7,4 + 1 % Triton X-100 (v/v).

Antibody diluent and blocking solution

Washing buffer + 3 % bovine serum albumin (BSA).

Antisera

monoclonal Anti-A, Anti-B (Seraclone, Biotest, FRG)
monoclonal Anti-H (Fresenius, FRG)
monoclonal Anti-Le a, Anti-Le b (Biotest or Schwab, FRG)
anti-mouse IgM-alkaline Phosphatase Conjugate (Sigma)

Substrate stock solution for alk. phosphatase staining

50 mg 5-Bromo-4-chloro-3-indolylphosphate dissolved 1 ml Dimethylformamid (stored at 4°C).

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Samples

All samples were collected from donors of known ABO, Lewis and secretor type. Liquid samples (saliva) were used freshly - without any further pretreatment - with dilution steps from 10^{-2} to 5 x 10^{-6}. Stains of saliva, semen and urine were prepared on boiled cotton cloth. Sweat and vaginal secretions were collected on cellulose tissue. The stains were dried at room temperature and stored at -20°C until use.

Stain extraction

As reference for calculating the dilution factors we used for extraction 1 cm^2 of stained cloth equivalent to about 25 µl of neat liquid sample.
Extraction was carried out in serum samplers for about 2 h at room temperature with 250 µl (secretors) e.g. 50 µl extractant (nonsecretors), so that the neat extract was already diluted 1/10 and 1/2 respectively.

Extractants

Bidistilled water, 5 % ammonium, 6 M urea + 0,5 % BSA.

ELISA technique on Nitrocellulose (NC) (modified according to Pflug)

This is the same method as introduced for the highly sensitive detection of Gc-protein in bloodstains (Pflug 1985, Pflug 1986) after isoelectric focusing. The different steps are carried out as follows:

(1) 0,3 - 0,5 µl extract were applied with an Eppendorf Varipette on nitrocellulose membranes (0,2 µm - Schleicher und Schüll, FRG), dried at room temperature and fixed for one hour at 80°C. Prior to the application the position of each dot was marked with a pencil.

(2) blocking of free charges in the NC membranes with blocking solution by mechanical shaking for 1 h.

(3) Incubation with primary monoclonal antibody.
The optimized antibody dilutions were: Anti-A, Anti B 1/50 - 1/100, Anti-H 1/10, Anti Le a 1/2000, Anti-Le b 1/4000.
Each of the NC membranes was incubated with one of the different antibody dilutions on a glass plate covered with a plastic lid for about 50 minutes at room temperature (about 0,5 ml solution is sufficient for a NC membrane of the dimension 7 x 10 cm).

(4) washing of the membranes with washing buffer six times for about 5 minutes by mechanical shaking.

(5) Incubation with the secondary antibody. 1/400 diluted anti-mouse IgM alkaline phosphate conjugate was incubated with each of the NC's on a glass plate for 50 minutes at room temperature.

(6) washing of the membranes with washing buffer four times and further two times with washing buffer excluding Triton X-100 (each at least 5 minutes).

(7) Activity staining

To a molten solution (about 70°C) of 1,5 % agar the following solutions were added: 25 ml 0,25 M glycine/NaOH buffer, pH 10.4, 0,5 ml of 0,1 M $MgCL_2$ and 0,1 M $ZnCl_2$ and 0,1 ml of substrate stock solution. The mixture was poured on an agarose-coated polyester film (Gel-Fix for agarose, Serva, FRG), which produced a staining gel of about 240 x 70 x 2 mm.
After excess buffer was removed with filter paper the NC membranes were applied on the top of the staining agar and incubated at 37°C until dark blue dots appeared. For increasing the sensitivity, the incubation period was prolonged overnight until the agar and the membrane will be dried on the polyester sheet giving a stable document of the original results.

3. RESULTS

3.1 ABO grouping of liquid saliva and saliva stains

In tests with five secretor salivas (A Le Se, B Le Se, O Le Se, AB Le Se, AB le Se) using 0,3 µl amounts of liquid saliva with concentrations of 10^{-2} to 4 x 10^{-8} (fourfold dilution steps starting with 1/100), the limits for a minimum of detectable A, B and H activity varied from 10^{-4} (H-secretor) to 2,5 x 10^{-6} (A- and AB-secretors (Fig. 1, top No. 1 - 5). Liquid saliva of a nonsecretor (AB Le se) with double dilution steps from 1/4 to 1/2048 of pure saliva showed activity of A, B and H substances in a range of 1/32 - 1/128 (Fig. 1 top, No. 6). In comparison saliva stains from the five secretors were applied at concentrations of 2,5 x 10^{-2} to 5 x 10^{-5} by double dilution steps (Fig. 1 bottom, No. 1 - 5). The sensitivity was almost comparable with the equivalent liquid saliva, when 5 % ammonium was used for extraction. The saliva stain of the AB-nonsecretor gave no conclusive result.

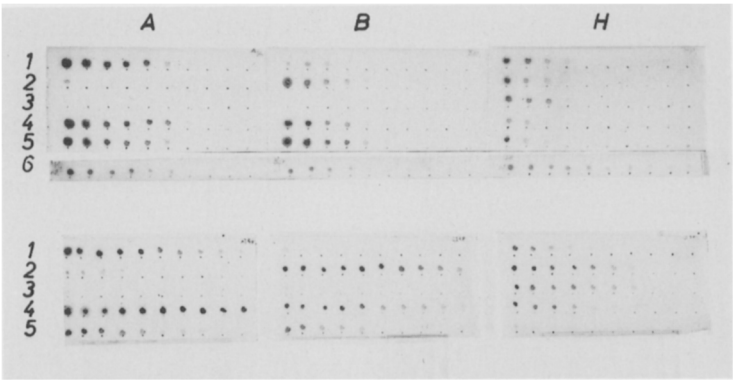

Fig. 1. ABO typing of liquid saliva (top) and saliva stains (bottom) on nitrocellulose membranes. Persons with the following ABO and Lewis status are shown (from 1 - 6): A Le Se, B Le Se, O Le Se, AB Le Se, AB le Se, AB Le se. Dilution steps for liquid saliva are fourfold, starting with 1/100 (Secretors). For saliva stains double dilution steps with an initial dilution of 1/40 are chosen. Nonsecretors are diluted 1/4 to 1/2048.

3.2 Lewis typing of liquid saliva and saliva stains

For testing Le a und Le b the same donors and the same dilution steps as described in 3.1 were used. The limits of activity for Le a and Le b varied from 5 x 10^{-4} to at least 10^{-5} (Fig. 2 top) for liquid saliva. Persons who are Le a-b+ by red cell typing showed considerably lower activity with anti-Le a than with anti-Le b. Individuals who are Le a+b- showed strong activity with anti-Le a and only weak activity with anti-Le b (Fig. 2 top, No. 6 and Fig. 2 bottom No. 6 - 8). A Le a-b- typed person (Fig. 2 top, No. 5) showed almost no activity. Compared with liquid saliva the saliva stains gave simil ar results in regard to sensitivity and activity distribution (Fig. 2, bottom).

3.3 ABO and Lewis typing of saliva-, semen-, vaginal secretions-, sweat- and urine stains

For each A, B, H, Le a and Le b typing we chose three different dilution steps with concentration ranges from 10^{-2} to 4 x 10^{-4} (saliva, semen and vaginal secretions) for secretors and 10^{0} to 10^{-1} for sweat and urine secretors and for all nonsecretors. Stain extraction was carried out with 5 % ammonium (saliva, vaginal secretions and sweat) and 6 M urea + 0,5 % BSA (semen and urine). The results we got with vaginal secretions and semen stains were comparable to those we discribed above for ABO and Lewis typing of saliva stains (Fig. 3). The ABO grouping of a nonsecretor (Fig. 3, vaginal secretions No. 5) was also possible. In addition ABO and Lewis typing of sweat and urine extracts showed also conclusive results with the exception of a nonsecretor person whose urine was only typable for Lewis but not for ABH substances (Fig. 3, urine No. 5).

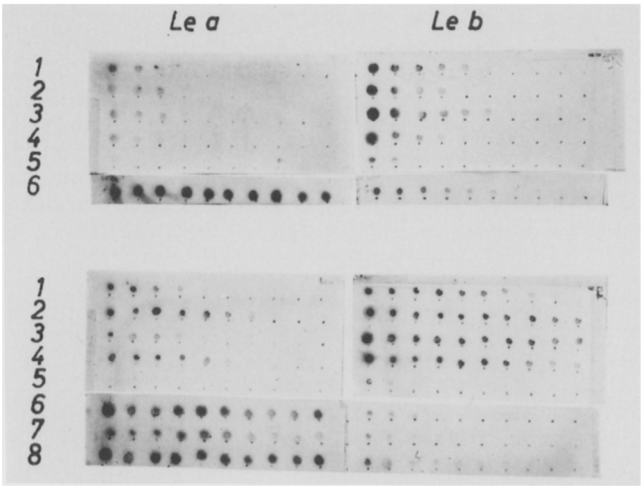

Fig. 2. Lewis typing of liquid saliva (top) and saliva stains (bottom) on nitrocellulose membranes. The ABO and Lewis status of the presented individuals and the dilution steps are the same as described in Figure 1. Two additional nonsecretor persons are added to the saliva stains (bottom, No. 7 and 8).

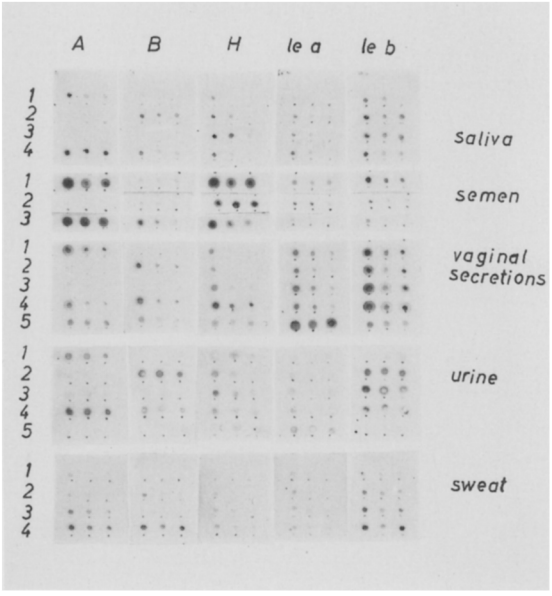

Fig. 3. ABO and Lewis typing of stains from saliva, semen, vaginal secretions, urine and sweat on nitrocellulose membranes. Persons with the following ABO and Lewis status are shown (from 1-n):
saliva: A Le Se, B Le Se, O Le Se, AB Le Se
semen: A Le Se, O Le Se, AB Le Se
vag.secretions: A Le Se, B Le Se, O Le Se, AB Le Se, AB Le se
urine: A Le Se, B Le Se, O Le Se, AB Le Se, AB Le se
sweat: A Le Se, B Le Se, A Le Se, AB Le Se
Dilution steps: 1/80, 1/320, 1/1280 for secretors (saliva, semen, vaginal secretions), 1/2, 1/4, 1/8 for secretors when typing urine and sweat and for nonsecretors.

4. CONCLUSIONS

The new method for ABO and Lewis typing of nonblood body fluids is very easy to handle and gives stable documents after activity staining. Furthermore the high sensitivity offers the possibility of analyzing even sweat and urine stains without the need of concentrating these extracts. Last but not least there is no need of an expensive equipment.

5. REFERENCES

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