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# Cytochemical Detection of ABH Antigens in Human Body Fluids

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**Summary.** The use of the peroxidase-anti-peroxidase (PAP) technique has been described previously for the detection of cellular antigens and in particular ABO antigens from tissue samples (Pedal and Hülle 1984; Pedal and Baedeker 1985; Pedal et al. 1985). In this survey, the PAP method has been employed to study the detection of ABO antigens in cells from body fluids of particular interest to forensic science, namely buccal cells and vaginal cells. Also tested, but in a limited number, were mixtures of body fluids and semen samples. No false reactions were obtained from buccal cells, all samples corresponding to the ABO blood type of the donor. Preliminary results from vaginal cells, vaginal/buccal cell mixtures, and semen were encouraging but must be treated with caution due to the limited number tested. Vaginal smears contaminated with semen showed varying degrees of nonspecificity.

**Key words:** Immunohistochemistry – Peroxidase-anti-peroxidase (PAP) method – ABO blood groups, body fluids

**Zusammenfassung.** Die Anwendung der indirekten Immunoperoxidasetechnik (PAP) ist zuvor im Zusammenhang mit der Bestimmung zellulärer Antigene, insbesondere ABO-Antigene aus Gewebeproben, beschrieben worden (Pedal und Hülle 1984; Pedal und Baedeker 1985; Pedal et al. 1985). In der vorliegenden Untersuchung wurde die PAP-Technik angewendet zur Bestimmung von ABO-Antigenen an Zellen und in Körperflüssigkeiten. Untersucht wurden Mundschleimhautzellen, Vaginalzellen, letztere von speziellem Interesse für die forensische Praxis, und in begrenzter Anzahl Mischungen aus Körperflüssigkeiten und Spermaproben. Es fanden sich keine falsch positiven Ergebnisse bei der Untersuchung von Mundschleimhautzellen; die Resultate korrespondierten mit den ABO-Merkmalen aus

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dem Blut der Spender. Vorläufige Untersuchungen von Vaginalzellen, von Mischungen aus Vaginal- und Mundschleimhautzellen und von Spermaproben verliefen erfolgversprechend, müssen aber aufgrund der nur begrenzten Anzahl der Untersuchungen vorsichtig interpretiert werden. Vaginalabstriche, kontaminiert mit Sperma, zeigten unterschiedlich starke, unspezifische Reaktionen.

**Schlüsselwörter:** Immunhistochemie – Peroxidase-Anti-Peroxidase (PAP) Technik – ABO-Antigene, Körperflüssigkeiten

## Introduction

The present methods of detection of A, B, and H antigens in body fluids (absorption/elution, absorption/inhibition, and Holzer inhibition tests) and the subsequent interpretation of the results have several drawbacks. The result is dependent on the secretor status of the individual and in mixtures of body fluids, as is very often the case in sexual assault, the masking of one group by another can occur. The ability to detect the antigens also depends on the amount of sample present in a mixture and the presence of contaminating or modifying bacteria (Pereira and Martin 1976). The detection methods used at present rely mainly on the presence of the soluble antigens in body fluids which cannot be separated in a mixture.

If the cellular ABH antigens could be detected and attributed to a particular constituent of a mixture of body fluids and/or bacteria, this would prove to be a great advantage in the interpretation of results.

The development of the peroxidase-anti-peroxidase (PAP) technique for the detection of cellular antigens by Sternberger et al. (1970), provided an excellent method for the detection of A, B, and H antigens, which has been used by several authors. Recently, Pedal et al. reported successful results from paraffin sections from autopsy material (1984), from decomposed tissue (1985), and from placental tissue (1985) using a modification of this technique.

This method with various modifications has been applied not for the identification of tissue sections but of the individual cells which can be found in body fluids (Brinkmann et al. 1985). As a preliminary investigation a blind trial was carried out on buccal cells taken from saliva samples for the presence or absence of A, B, and H antigens. In later experiments we have looked at cells from other body fluids and mixtures of cells from individuals of different blood groups to test whether it is possible to obtain specific reactions for the individual cell components in the presence of other contaminants.

## Materials and Method

### *Materials*

Buccal cells from 50 donors were collected on sterile cotton-wool swabs and smears made on microscope slides. Blood samples were also collected from these donors and typed by the ABO and Lewis systems for control purposes.

A limited number of vaginal swabs and semen samples have also been collected from donors of known ABO and Lewis type and were prepared in the same manner. Vaginal/buccal cell mixtures were made by mixing equal volumes of extracts of the swabs (aqua dest).

After drying at room temperature the buccal and vaginal smears were heat-fixed prior to staining, whereas it was found that with the seminal smears the use of Methanol or Merckofix-Spray (Merck) produced better results. For each sample tested, three microscope slides were prepared, one for each of the A, B, and H antigens.
The following commercially available reagents were used:

Normal swine serum – Flow laboratories
Monoclonal mouse, anti-A- and -B (Seraclone) – Biotest
Ulex europaeus anti-H – Medac
Rabbit-anti-mouse IgM – Bionetics
Anti-Ulex-europaeus – Medac
Swine-anti-rabbit-immunoglubulins – Dakopatts
Peroxidase-anti-peroxidase (rabbit) – Dakopatts
$H_2O_2$ and 3-Amino-9-Ethylcarbazole – from PAP Kit made by Ortho-Diagnostika

*Method*

The peroxidase-anti-peroxidase (PAP) staining technique was carried out as follows:

The samples were rehydrated by carefully washing in Tris-saline buffer (0.5 M, pH 7.6). The microscope slide was covered by normal swine serum and left at room temperature for 10 min.

After washing in Tris-saline buffer, monoclonal anti-A, -B, and Ulex anti-H were added to the appropriate samples for 30 min. Anti-A and anti-B were diluted 1:5, and the anti-H 1:2000. Hereafter, washing with ice-cold Tris-saline buffer was carried out between each stage, and each stage was of 30 min duration. Rabbit anti-mouse IgM was added to the anti-A- and anti-B-treated samples (dilution 1:500) and anti-Ulex (UEA) added to the Ulex anti-H-treated sample (dilution 1:100).

All three microscope slides were treated with swine anti-rabbit immunoglobulin (1:100) followed by the addition of the PAP-complex at a dilution of 1:100

Visualization of the PAP-complex was achieved by treating the samples with a mixture of 1% $H_2O_2$ and 3-amino-9-ethylcarbazole to give a red coloration when positive. Counter staining with hematoxylin was used for better discrimination of PAP-negative cells, and the sam-

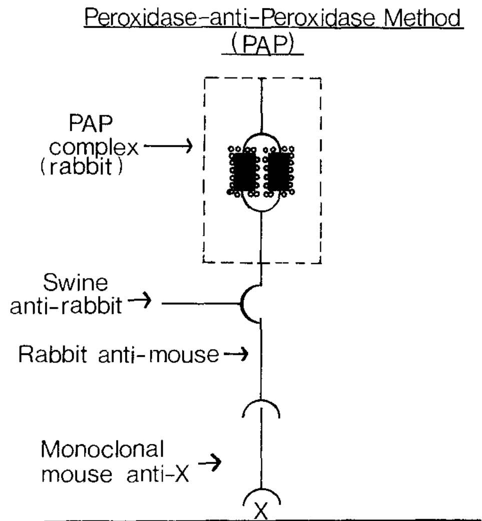


**Fig. 1.** Diagrammatic representation of the peroxidase-anti-peroxidase reaction (courtesy of J. Burns, *Techniques in Immunocytochemistry,* edited by Bullock and Petrusz)

ples were embedded in Eukitt. The slides were microscopically examined for the presence of the PAP-positive staining (for details see Fig. 1).

The testing of the 50 buccal smears was carried out as a blind trial, the individual blood groups being unknown to the operator.

## Results

The 50 samples of buccal cells included all ABO blood types, secretor and non-secretor types. All 50 samples gave the correct result irrespective of the secretor status (Table 1a), but we found a distinction between strong positive and weak positive results. These results (Table 1b) show a definite correlation to the Lewis blood group (secretor status). Five of the Le(a+b−) samples (i.e., non-secretor) showed weak positive reactions although two gave strong positive results. All of the Le(a−b+) samples (i.e., secretors) gave strong positive results (Fig. 2a). The distinction between a positive result (Fig. 2b) and a negative result (Fig. 2c) can be seen easily, and that the use of a counter stain is necessary to visualize the negative reacting cells, but does not hinder the detection of a positive reaction.

An equivalent number of vaginal smears has not yet been carried out due to unavailability of the requisite number of donors, although we have at present tested some 20 samples from donors of known blood group. Unfortunately, in this random sample only one B and no AB donors were found, and this cannot be considered a representative sample. Although the results were not so clear-cut as those of buccals cells, some positive interpretations can be drawn which will be discussed later on. Nevertheless, all the results showed good correlation to the blood group of the donor.

Combinations of vaginal cells and buccal cells, made by mixing equal volumes of the appropriate homogeneous body fluids of known ABO/Le groups, were also tested using this method, and it was found that in these cases some 50% of the cells stained positive and approximately 50% were negative (Fig. 2d). It was, however, not possible to distinguish the two cell populations microscopically.

Semen samples showed an obvious microscopic distinction between positive and negative staining although it was difficult to locate the source of the staining (Fig. 2e, f). The significance of these results will be discussed later on.

**Table 1. a** Results of ABO grouping of 50 buccal cell samples using the PAP technique. **b** Correlation of the results from **a** with Lewis typing

| **a** PAP blind trial $n$ = 50 | | | | **b** PAP blind trial – Lewis | | | |
|---|---|---|---|---|---|---|---|
| Blood | $n$ | % | Correct | Blood | $n$ | Strong + | Weak + |
| $A_1$ | 15 | 30 | 15 | a− b+ | 37 | 37 | – |
| $A_2$ | 4 | 8 | 4 | a+ b− | 7 | 2 | 5 |
| B | 7 | 14 | 7 | a− b− | 4 | 4 | – |
| 0 | 23 | 46 | 23 | Unknown | 2 | 2 | – |
| AB | 1 | 2 | 1 | | | | |

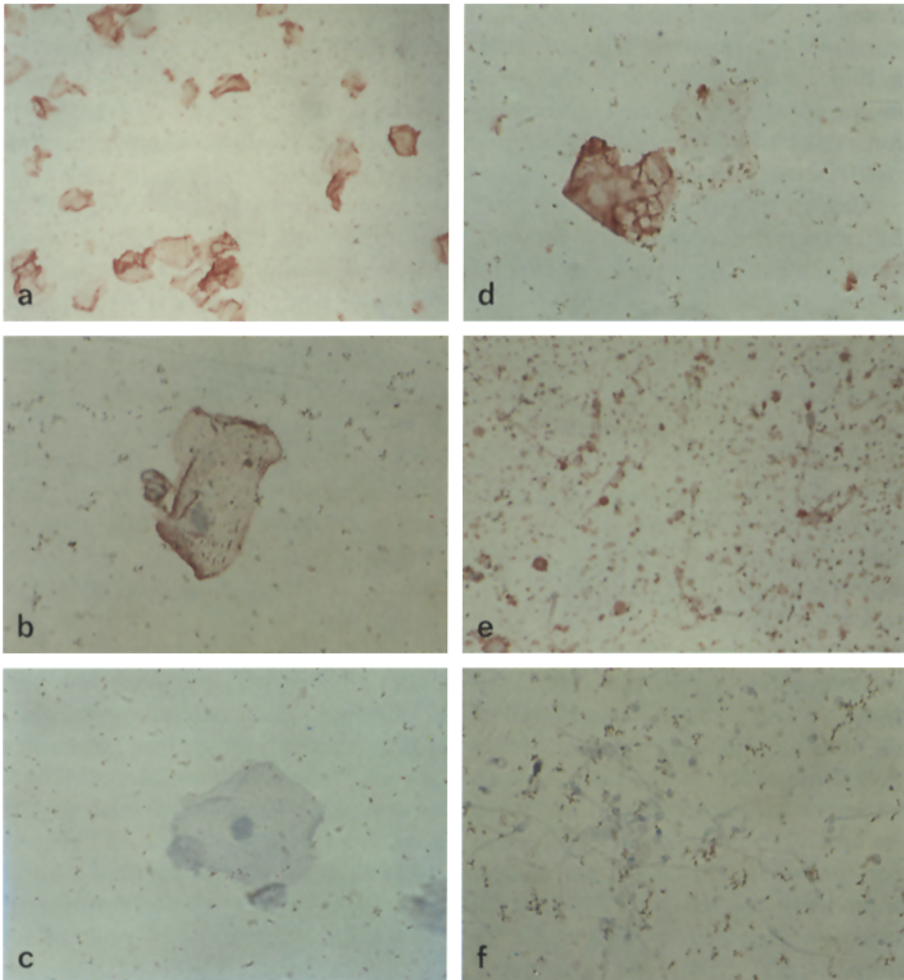


**Fig. 2 a–f.** Results obtained from buccal cells using the PAP technique. **a** Group B cells with anti-B. **b** Magnification of single cell: group A with anti-A. **c** Magnification of single cell: group A with anti-B. **d** Mixture of group A vaginal cells and group B buccal cells with anti-A. **e** Group A semen with anti-A. **f** Group A semen with anti-B

All samples tested so far, which showed a definite positive reaction for A or B, have also shown positive reaction for H. In the case of group A, this was independent of the $A_1$, $A_2$ subtype of the donor.

## Discussion

The results from the blind trial using buccal cells, as shown in Table 1a and 1b, seem to indicate that A, B, and H antigens can be reliably detected on the cell membrane. Positive staining with PAP was obtained on the sample corresponding to the blood group of the donor, and in all cases with H. This continuous

presence of H reaction is difficult to evaluate in terms of specific or non-specific reactions. It is possible that, although a distinction between $A_1$ and $A_2$ subtypes on blood cells can be made, there is sufficient H (precursor) substance present on epithelial cells and/or the sensitivity of the method, allows no such distinction. The absence of a negative control makes this difficult to evaluate. Further experiments must be made to resolve this problem.

The reactions for A and B antigens have been shown to be specific, and from the results of the blind trial they are apparently independent of the secretor status. This method, therefore, seems to have an advantage over conventional elution or inhibition techniques and mixed cell agglutination (Pereira et al. 1969) in that the reaction can be directly attributed to specific cellular structures. In a mixture of two cell populations (e.g., body fluids) it would be possible to assign a particular blood group to a particular cell structure, allowing a more precise definition of the constituents.

In cases of bacterial contamination (Scott and Corry 1980) and the presence of acquired B (Jenkins et al. 1972), the occurrence of aberrant B reactions (Davies et al. 1984) and in general problems involved in the typing of body fluids (Pereira and Martin 1976), it is at least theoretically possible that this false reaction could be attributed to a particular structure using this method.

Uncontaminated vaginal cells gave results which showed a good correlation with the blood group of the donor. The results did not always show a strong positive reaction for the corresponding antigen, but no false positives were found. Again H was always positive. The presence or absence of vaginal flora did not seem to affect the quality of the results although only a limited number have been tested so far.

Seminal samples taken direct from male donors also showed correlation with the blood group of the donor, as determined by absorption-elution and absorption-inhibition tests, but very weak or no staining of the spermatozoa membrane was observed. Instead, the red colouration appeared to be concentrated in extracellular constituents, and it is hypothesized that this could be precipitated antigen/antibody complexes formed by the soluble ABO antigens.

Analysis of mixtures of vaginal constituents and spermatozoa from post-coital swabs, have shown some degree of success although the results were not as clear-cut due mainly to the presence of this "clumping" which appeared to some extent to adhere to the cell membranes.

Our results indicate that A, B, and H antigens can be demonstrated on buccal and vaginal cells. At present, there is no reason to doubt the specificity of the A and B reaction, but the quality of the H reaction must be investigated further. Our preliminary investigations into the universal problem of mixtures of body fluids, as found in sexual assault cases, have shown encouraging results although disappointingly no activity could be demonstrated on the spermatozoa themselves. However, it is hoped that this could provide a basis for an improvement or modification of the existing technique to provide the extra information, which is at present lacking, for the characterization and identification of body fluid mixtures.

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