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# HLA Typing of Dried Sperm

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**ABSTRACT:** HLA human histocompatibility leukocyte antigens -A and -B antigens are expressed on human spermatozoa. A micro- and a macro-technique are described to HLA-type dried sperm coming from unknown subjects. The high HLA genetic polymorphism allows a high degree of sperm individualization which may be of value to blame or discard suspects in forensic science cases.

**KEYWORDS:** pathology and biology, human leukocyte antigen, spermatozoa, criminal sex offenses, spermatozoa typing, HLA typing

Genetic markers, including ABO blood groups and the human histocompatibility leukocyte antigen (HLA)-linked enzyme $PGM_3$ have been found to be useful to individualize sperm samples [*1*]. The more polymorphic the genetic system used, the more sperm individualization capability is achieved. The major human histocompatibility leukocyte system (HLA) is the best known and most polymorphic genetic system in humans [*2*], and sperm HLA typing may prove to be useful in sperm samples identification. HLA-A and -B antigens have been shown to be expressed on human spermatozoa [*3,4*]. However, HLA-D expression on spermatozoa is controversial [*5-7*]. Techniques for detecting HLA-A and -B antigens (or, in general, sperm surface antigens) are cumbersome, and a clear improvement was accomplished by using complement mediated cytotoxicity followed by a double fluorescence staining method [*8*] to detect clearly live and dead spermatozoa.

In the present study we describe a radioactive microassay to detect HLA-A and -B antigens in dried sperm that may be useful in certain forensic science cases. HLA high polymorphism together with the possibility of detecting up to four different HLA antigens in sperm may give a relatively precise genetic information about unknown sperm sample donors.

## Materials and Methods

### *HLA Typing*

HLA typing was done with VIII HLA International Workshop and our local sera. Dr Vives (Hospital Clínico, Barcelona, Spain) kindly donated a monoclonal antibody against HLA-A25 and HLA-Aw32 [*9*]. A standard microlymphocytotoxicity technique was used for lym-

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phocyte typing [*10*], and dead and live lymphocytes were ascertained in fresh under phase contrast.

*Semen Samples*

Semen samples from HLA-typed donors were poured over clean cotton tissue and left to dry at room temperature in nonsterile conditions for up to two days. Seminal tissue "spots" from each sample were cut and placed in 25-mL tubes with Hanks' balanced salt solution (Flow, UK). Elution of seminal cellular debris and all other material (including dead but complete, recognizable spermatozoa and free heads and tails) was accomplished by tissue maceration against the tube wall with forceps or Pasteur pipettes or both. This eluate was concentrated up to five times more than the initial sperm sample volume using Minicon-B volume reducers (Amicon, UK) for less than 15 000 D molecular weight. The resulting samples were stored at −80 C until use.

*Lymphoblast Induction*

HLA-typed peripheral blood lymphocytes were isolated in Lymphoprep (Nyegaard and Co, Norway) [*11*], washed in Roswell Park Memorial Institute (RPMI) medium (Flow, UK) and resuspended up to $3 \times 10^6$ cells/mL in RPMI + 10% AB Rh+ serum, supplemented with glutamine (1%) and antibiotics (ampicillin + cloxacillin + gentamycin). Cultures were set up in 10-mL round bottom plastic tubes (Sterilin, UK). Phytohemagglutinin (PHA) (Wellcome, UK) was added 1/100 (v/v) and cultures were kept at 37°C in 5% carbon dioxide atmosphere for three days.

*Radioactive Microcompetition Assay*

Fifty microcuries of $^{51}$Cr-labelled sodium dichromate were added by each $10^6$ PHA-stimulated cells in pellet and placed 1 h in a stirring 37°C bath.

Two microlitres of specific HLA antisera, diluted to their end point as calculated in previous radioactive microassays on relevant lymphoblasts, were dispensed in each well of microtest U bottom plastic plates (Sterilin, UK) under 50 μL of sterilized liquid paraffin oil. Two microlitres of eluated-concentrated sperm was also added to each well and incubated at 4°C overnight. Two microlitres of relevant lymphoblasts (at $5 \times 10^6$ cells/mL) were subsequently placed in the wells and incubated a further 30 min at room temperature. Finally, 10 μL of nontoxic rabbit complement were added and after 1 h the reaction was stopped by addition of 250 μL RPMI + 10% AB Rh+ serum. Plates were spun down 5 min at 400 *g* and 150 μL of supernatants were carefully extracted from under the paraffin oil film in each well and counted in a gamma counter.

Cytotoxicity Index was calculated as:

$$\text{C.I.} = \frac{^{51}\text{Cr activity in test} - {}^{51}\text{Cr activity in negative control}}{^{51}\text{Cr activity in positive control} - {}^{51}\text{Cr activity in negative control}} \times 100$$

Commercial antilymphocyte serum (Behringwerke, West Germany) was used as positive control and AB Rh+ serum as negative control. Relevant specific HLA antisera were used in test wells.

*Macroabsorptions*

Other experiments were run in parallel with nonconcentrated dried sperm eluates. Pellets were mixed (v/v) with HLA antisera diluted to their end point previously calculated by the standard microlymphocytotoxicity test [*10*]. Mixtures were left 30 min rotating at room temperature and a further 18 h at 4°C. Absorbed antisera were obtained by centrifugation at 15 000 *g* during 5 min in a Beckman Microfuge and then used for standard HLA typing on normal lymphocytes [*10*].

## Results

*Radioactive Microcompetition Assay*

Several sera were preincubated with relevant and nonrelevant spermatozoa in the conditions described above, and subsequently used to type lymphoblasts of an already known HLA phenotype. In Table 1 data from five different experiments are shown. It is possible within each experiment to discriminate a positive and a negative C.I. when the sera are preabsorbed with nonrelevant or relevant (respectively) eluated concentrated sperm. Positive and negative C.I.s are within very different ranges. Variability of C.I.s in different experiments may be due to the extreme variability in HLA antisera strength and in cross-reactivity degree. This will probably be obviated when polymorphic monoclonal HLA antibodies are available (see below).

*Macroabsorptions*

If the radioactive microtechnique is not set up, and you want to know whether one or two particular HLA antigens are present in the sperm eluate (that is, when accused individuals exist, see below), more simple macroabsorptions may be used. In Table 2 positive or negative C.I.s are represented corresponding to preabsorptions with nonrelevant or relevant nonconcentrated sperm eluates. When a monoclonal HLA antibody is used, C.I. is very low after absorption with relevant spermatozoa eluates.

## Discussion

In the case that HLA-A and -B phenotypes of, say, two accused individuals are available, and completely different, one might look for the specific HLA antigens in the sperm eluate-concentrate. Lymphoblasts from the suspects might be used for the microcompetition assay; if one of them was indeed the sperm donor, the eluated concentrated sperm would inhibit his theoretically four (two HLA-A and two HLA-B) antigens when used in our assay. The non-sperm donor suspect could therefore be excluded from suspicion. If more suspects exist, two or three relevant and discriminatory HLA antigens should be looked for. Also, the least frequent HLA antigens in that particular population should be chosen when possible, and therefore lymphoblasts (from suspects or from others) bearing these chosen antigens should be prepared. When no suspects are available, we can try to HLA-type sperm with a chosen panel of lymphoblasts covering most HLA-A or HLA-B specifications or both. Even if only one HLA antigen could be detected in this way, the chance of exclusion obtained is very high. For example, if HLA-Aw24 is detected and its phenotype frequency in that particular population is 0.12, $1 - 0.12 = 0.88$ or 88% of that population's individuals (hence possible suspects) are excluded. If Aw31 is also demonstrated (phenotype frequency = 0.03), $1 - 2p_1p_2 = 0.9981$ or 99.8% of the individuals are excluded ($p_i$ = gene frequency of each allele, estimated as $p_i = 1 - \sqrt{1 - f_i}$). If three or four HLA antigens are found, the chance of exclusion is correspondingly higher, but it would not be of much practical interest. Appropriate formulas for these particular cases (with or without linkage disequilibria between HLA-A and -B antigens

TABLE 1—*HLA antisera cytotoxicity on lymphoblasts: effect of preabsorptions with sperm concentrated eluates (radioactive microassay).*[a]

| | Lymphoblasts Phenotypes | | |
|---|---|---|---|
| | A3 +ve B7 +ve (a) | B14 +ve Bw35 +ve (b) | A11 +ve (c) |
| a) Anti-HLA-A3 serum absorbed with sperm eluates: | | | |
| A3 +ve sperm (1) | 41 (−ve) | ... | ... |
| A3 −ve sperm (2) | 100 (+ve) | ... | ... |
| A3 −ve sperm (3) | 100 (+ve) | ... | ... |
| b) Anti-HLA-A11 serum absorbed with sperm eluates: | | | |
| A11 +ve sperm (3) | ... | ... | 68 (−ve) |
| A11 −ve sperm (2) | ... | ... | 93 (+ve) |
| c) Anti-HLA-B7 serum absorbed with sperm eluates: | | | |
| B7 +ve sperm (1) | 1 (−ve) | ... | ... |
| B7 −ve sperm (2) | 44 (+ve) | ... | ... |
| B7 −ve sperm (3) | 37 (+ve) | ... | ... |
| d) Anti-HLA-B14 serum absorbed with sperm eluates: | | | |
| B14 +ve sperm (2) | ... | 25 (−ve) | ... |
| B14 −ve sperm (3) | ... | 54 (+ve) | ... |
| e) Anti-HLA-Bw35 serum absorbed with sperm eluates: | | | |
| Bw35 +ve sperm (2) | ... | 3 (−ve) | ... |
| Bw35 −ve sperm (1) | ... | 17 (+ve) | ... |
| Bw35 −ve sperm (3) | ... | 15 (+ve) | ... |

[a]Radioactive microcompetition assay for different eluated concentrated dried sperm samples against relevant lymphoblasts with different HLA antisera.

Lymphoblasts HLA typing:

(a) A2,A3/B7,Bw60/Cw3/Bw6
(b) A2,Aw32/B14,Bw35/Cw4 Bw6
(c) A2,A11/Bw52,B18/Cw1,Cw5/Bw4,Bw6

Sperm donors HLA typing:

(1) A2,A3/B7,Bw60/Cw3/Bw6
(2) A2,Aw32/B14,Bw35/Cw4/Bw6
(3) A11,Aw23/Bw44,X/Cw4/Bw4

Antisera names and specificities:

a) Madrye 41B (anti-HLA-A3)
b) Madrye 33B (anti-HLA-A11)
c) Madrye 67B (anti-HLA-B7)
d) Madrye 28B (anti-HLA-B14+ B18)
e) Madrye 34B (anti-HLA-Bw35)

[*12*]) may be deduced. Chance of exclusion is much higher than using the ABO system for bloodstains (only 67.2% at most [*13*]).

Typing dried sperm has some limitations. Time between sperm ejaculation and analysis should be shortened as much as possible. The material available should be carefully collected to get enough sample either by tissue elution as described above or also by vaginal lavage with a buffered medium. Concentration procedures may probably overcome the problem of scanty sperm samples.

TABLE 2—*HLA antisera cytotoxicity on normal lymphocytes: effect of preabsorptions with sperm eluates (macroabsorption).*[a]

| | Lymphocytes Phenotypes | | | | | | |
|---|---|---|---|---|---|---|---|
| | A11 +ve (a) | A11 +ve (b) | A11 +ve (c) | Aw32 +ve (d) | Aw32 +ve (e) | A25 +ve (f) | A25 +ve (g) |
| a) Anti-HLA-A11 serum absorbed with dried sperm pellet of: | | | | | | | |
| A11 +ve sperm (1) | 35 | 43 | 18 | ... | ... | ... | ... |
| A11 −ve sperm (2) | 85 | 86 | 70 | ... | ... | ... | ... |
| b) Anti-HLA-A25+Aw32 serum absorbed with dried sperm pellet of: | | | | | | | |
| Aw32 +ve sperm (2) | ... | ... | ... | 9 | 0 | 0 | 13 |
| A25 +ve sperm (3) | ... | ... | ... | 9 | 0 | 0 | 2 |
| A25,Aw32 −ve sperm (1) | ... | ... | ... | 92 | 59 | 98 | 99 |

[a]Macroabsorptions for three different dried sperm pellets against relevant lymphocytes with two HLA antisera.

Lymphocytes HLA typing:

(a) A3,A11/Bw51,Bw60/Cw1,Cw3/Bw4,Bw6
(b) A11,Aw23/Bw44,X/Cw4/Bw4
(c) A2,A11/B18,Bw52/Cw1,Cw5/Bw4,Bw6
(d) A2,Aw32/B40,Bw51/Cw2/Bw4,Bw6
(e) A2,Aw32/B14,Bw35/Cw4/Bw6
(f) A2,A25/B7,Bw52/Bw4,Bw6
(g) Aw23,A25/B13,Bw44/Cw4,Cw6/Bw4

Sperm donors HLA typing:

(1) A11,Aw23/Bw44,X/Cw4/Bw4
(2) A2,Aw32/B14,Bw35/Cw4/Bw6
(3) Aw23,A25/B13,Bw44/Cw4,Cw6/Bw4

Antisera names and specificities:

a) Madryc 33B (anti-HLA-A11)
b) CATA-1 (monoclonal anti-HLA-A25+Aw32)

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