The intra- and inter-assay variation of the indirect mixed antiglobulin reaction test: is a quality control suitable?

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The test most commonly used to detect sperm antibodies is the mixed antiglobulin reaction (MAR), standardized by the World Health Organization. The indirect MAR test detects soluble sperm antibodies in seminal plasma by using healthy donor spermatozoa as antigen. In this study we systematically investigated the influence of donor spermatozoa and the source of sperm antibodies upon the results of the indirect MAR test, and calculated the intra- and inter-assay variations. Using one individual seminal plasma and the same donor semen, results of the indirect MAR test are highly reproducible (low intra-assay variation). Two dimensions of inter-assay variation must be considered: (i) serial ejaculates of an individual donor may be used at different times; (ii) different donors may be applied to identical antibody sources. Donor spermatozoa strongly influenced the results of the indirect MAR test. Using multivariate statistical tests, highly significant main effects between the different donors ($P < 0.001$) and specific reciprocal effects between donor spermatozoa and seminal plasma samples ($P < 0.001$) were observed. The high inter-assay variation of the indirect MAR test will lead to incorrect results. There is urgent need of a reliable and reproducible test for sperm antibody detection to improve quality control of the methods.

Key words: antisperm antibody/donor spermatozoa/intra-, inter-assay variation/mixed antiglobulin reaction

Introduction

Since the important role of antisperm antibodies in infertility was recognized, numerous different methods for the detection of these antibodies have been introduced. A reliable and reproducible method, however, is not yet available.

The test most commonly used is the mixed antiglobulin reaction (MAR), which was firstly described by Jager et al. (1978). A standardization was recommended by the World Health Organization task force group (WHO, 1987). The MAR test may be performed using one of two modifications: a direct or an indirect variation. In the direct MAR test, the antibodies attached to motile spermatozoa are detected. The indirect MAR test is used if no spermatozoa or no motile spermatozoa can be found in the semen. In this case the soluble sperm antibodies in seminal plasma are detected by using healthy donor spermatozoa as the antigen.

Each laboratory performs the indirect test using different donor spermatozoa, and even in one laboratory different donors are used for the test. The comparability of the results is therefore poor. This is not only a problem of structure and process quality, but individual interactions between seminal antisperm antibodies and donor sperm antigen structure seem to influence the test results. Focus was placed on antibodies in the seminal plasma rather than sera because sperm antibodies in sera are not thought to be related to infertility.

In this study we systematically investigated the influences of donor spermatozoa and antibody sources upon the indirect MAR test and resultant differences in intra- and inter-assay variation.

Materials and methods

Patients

Semen samples from 12 patients who were referred to our clinic for infertility investigations were obtained by masturbation after 3–6 days’ abstinence. The semen was centrifuged for 15 min at 3000 g at room temperature. The seminal plasma was then stored at −20°C until assayed. The soluble sperm antibodies in seminal plasma were determined by means of the indirect MAR test (see below).

Donor sperm preparation:

Spermatozoa from five donors, obtained by masturbation after 3–6 days’ abstinence, showing normal sperm parameters according to the WHO recommendations (WHO, 1987) and the absence of sperm antibodies, were isolated by a swim-up preparation. Firstly, 1 ml of semen was diluted 1:5 with in-vitro fertilization (IVF) buffer (MediCult a/s, Copenhagen, Denmark) and thereafter centrifuged at 300 g at room temperature for 10 min. The sperm pellets were resuspended in 5 ml IVF buffer and washed again. Two ml of IVF medium were then carefully placed above the final pellet, after which the tube was inclined at an angle of 45° and incubated for 1 h at 37°C. The supernatant containing the enriched fraction of motile spermatozoa was aspirated and centrifuged again for 10 min at 500 g at room temperature. Finally, the cell concentration was adjusted to $20 \times 10^6$ ml$^{-1}$ with IVF medium.

Direct (SpermMar®) test for sperm antibodies

Prior to the experiments, the donor ejaculates were tested with direct SpermMar® test to exclude the presence of sperm antibodies in the donor seminal plasma. For the detection of sperm antibody, the reagent kit SpermMar® IgG Test from FertiPro N.V. (Sint-Martens-Latem, Belgium) was used. The test was performed according to technical data given by the manufacturer. Briefly, the direct SpermMAR® test was performed on a microscopic slide by mixing 10 μl of fresh semen and 10 μl suspension, containing latex particles
coated with human immunoglobulin (IgG). In the next step, 10 µl of monospecific antihuman IgG antiserum anti Fab’-fragments were added. A cover-slip was applied to the mixture and after 3 min 100 motile spermatozoa were evaluated. The percentage of spermatozoa with attached latex particles represented the test result.

**Indirect MAR test**

The soluble antibodies of the patients’ seminal plasma samples were tested using the indirect MAR test (Hinting et al., 1988). Firstly, 100 µl seminal plasma were diluted 1:4 with 300 µl IVF buffer; 100 µl of this dilution was then carefully mixed with 100 µl of the donor sperm suspension and incubated for 30 min at 37°C. The mixture was then processed as in the direct MAR test (see above).

**Intra-assay variation**

The indirect MAR test assay was run seven times with seven sperm antibody positive samples of seminal plasma using identical donor spermatozoa, originating from one individual ejaculate. Control seminal plasma samples without sperm antibodies were assayed under the same conditions.

**Inter-assay variation**

**Day-to-day variability of donor samples**

Four seminal plasma samples containing antibodies and three control samples were diluted at 1:4 with IVF buffer and tested five times using spermatozoa from one donor over a period of 5 weeks. Each test was assayed in triplicate.

**Comparison between different donors**

Seminal plasma samples of eight patients containing different antibody concentrations and four control samples were diluted at 1:4 with IVF buffer and were tested with five different donor spermatozoa. Each test was assayed in triplicate.

**Statistical methods**

The multivariate tests were performed using the SPSS 6.1.3 for windows programme (SPSS Inc., Chicago, IL, USA). Tables were carried out with EXCEL 7.0 (Microsoft).

**Results**

The indirect MAR test using identical donor spermatozoa and identical seminal plasmas as antibody sources was run seven times. The intra-assay variation was calculated (Table I, last column). The results of the indirect MAR test performed using one individual seminal plasma and semen from the same donor are thus highly reproducible.

The use of serial ejaculates from the same donor, as it is applied in the routine laboratory, represent the first dimension of inter-assay variation. The day-to-day variability of the test performed in this manner is illustrated in Table II. Each test was done in triplicate, which produced similar small variation as in the first experiment. However, the differences in the results of the MAR test over subsequent weeks were significantly larger (P < 0.005) and highly significant main effects between the different seminal plasma samples were observed (P < 0.001). There was also a significant reciprocal effect between donor samples and patient seminal plasma samples, when calculated with multivariate statistical tests (P < 0.001).

The use of different donor spermatozoa for the indirect MAR test represents the second dimension of inter-assay variation. The results of the MAR test performed with different donor spermatozoa are summarized in Table III. Eight seminal plasma samples containing sperm antibodies were tested with five different donors. Each value in Table III represents again a mean of triplicate measurements. Using multivariate statistical tests, highly significant main effects between the different donors and seminal plasma samples were observed (P < 0.001), but also a highly significant specific reciprocal effect between donor spermatozoa and seminal plasma samples (P < 0.001).

For example, the seminal plasma sample 1 had a high MAR test result if donor 2 was used (74.7%). With donor 3, however, the test result was significantly lower (45%). On the other hand, in seminal plasma sample 4, a low test result was observed if donor 2 was used (14%); however, the test result was significantly higher (41%) when using donor 3.
result of 31.3% was obtained using the spermatozoa from donor 2, while it was distinctly higher with the spermatozoa of donor 3 (66.7%).

The results for the controls (seminal plasma samples without antibodies) were zero in the indirect MAR test, as well as in the intra-assay and the two inter-assay experiments.

Discussion

Quality control for the detection of sperm antibodies by means of the MAR test is hampered by the fact that 'standard' spermatozoa are not available. Thus no gold standard exists.

By repeating the tests using identical antigens and antibodies, internal comparisons can be performed. As shown in our study, the intra-assay variation of the MAR test is low and independent of individual donors.

However, in the calculation of inter-assay variations two dimensions have to be considered. The first dimension is represented by the use of serial ejaculates from one individual donor at different times. This test condition leads to high variations of the indirect MAR test results, making it doubtful that this comparison is useful for quality control.

The second dimension of inter-assay variation is the application of different donor sperm samples to the patient’s antibody source. This would be unavoidable if external comparisons between different laboratories were to be made. In our study, we found that the donor sperm samples strongly influence the results of the indirect MAR test. Although the ‘positive’ or ‘negative’ test results showed a relationship between the different donors, the results could be different for a given patient sample according to the donor. The variation of values was significantly greater than the intra-assay variation or the inter-assay variations using a single donor providing serial ejaculates. Therefore, from a biological standpoint, as well as a statistical one, the results are entirely satisfactory: small variation when using the same donor and much larger, interactive variation when using different donors.

The differences obtained in the indirect MAR test results (Table III) using different donors may lead to distinct clinical treatments of infertile couples. In the literature, the usual recommendation for couples with a positive MAR test is to perform intracytoplasmic sperm injection (ICSI). However it was indicated (Ombelet et al., 1997) that intrauterine insemina-

<table>
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<tr>
<th>Donor</th>
<th>Seminal plasma</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>Seminal plasma mean</th>
<th>Mean of deviations in %</th>
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<td>34.08</td>
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<td>25.88</td>
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Variation of the indirect MAR test

sperm antibody detection. To reach this goal, it will be necessary to isolate intact sperm surface antigens in order to guarantee the presence of the whole immunologically active structure.

References


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