

Comparison of a Direct and Indirect ELISA for Quantitating Antisperm Antibody in Semen

DONA M. LYNCH AND STEPHEN E. HOWE

A direct and an indirect quantitative ELISA for antisperm antibody were compared using the spermatozoa and cell-free seminal fluid of 66 infertile males. The normal concentration of sperm binding immunoglobulin was ≤ 1.5 fg Ig per spermatozoon for the indirect seminal plasma assay and ≤ 1.5 fg Ig per spermatozoon by the direct assay. Of the 66 infertile males, 21% (14/66) had elevated levels of antisperm antibody in their seminal plasma and 26% (17/66) had elevated levels bound directly to their spermatozoa. The direct correlation between the results of these assays was 94%. A simple linear regression analysis between the indirect and direct measurements of antisperm antibody resulted in a correlation coefficient of $r = 0.907$. There was no statistically significant difference between results from the direct and indirect methods of the patients as a group. However, there was evidence of autospecificity in a small percentage of males who had elevated levels of antisperm antibody by the direct assay that was not detected by the indirect assay using pooled donor spermatozoa.

Key words: antisperm antibody, ELISA, immunologic infertility, seminal fluid.

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Elevated levels of free and *in vivo* bound antisperm antibodies in semen have been demonstrated in patients with suspected immune infertility. Functional tests such as the gelatin agglutination test

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(Kibrick et al, 1952) and sperm immobilization test (Isojima et al, 1968), as well as radioimmunoassay (Haas et al, 1980; Czuppon and Mettler, 1983) and enzyme linked immunosorbent assay (ELISA) (Ackerman et al, 1981; Witkin et al, 1981; Alexander and Bearwood, 1984; Howe and Lynch, 1986) have been utilized to measure free antisperm antibodies in serum and seminal fluid. Additional assays, including the Immunobead[®], mixed agglutination reaction, cytotoxicity and hemagglutination inhibition assays have been developed to detect both free and bound antisperm antibody (Jager et al, 1978; Mathur et al, 1981; Hendry et al, 1982; Bronson et al, 1984; Clarke et al, 1985; Meinertz and Hjort, 1986). Advantages of an indirect assay for unbound antisperm antibody include the ability to perform titration studies and assays on patients with oligozoospermia and poor motility as well as ease of specimen handling and assay control. The disadvantages of indirect assays lie in the potential for false negative results when the antisperm antibody has specificity to an antigen not present on the target spermatozoa or when the antibody has a high *in vivo* binding affinity that leaves little unbound antibody free in the fluid phase. The

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significance of these potential problems in indirect antisperm antibody assays is evaluated in this report by performing both direct and indirect quantitative ELISA's for antisperm antibody using semen specimens from 66 infertile males.

Materials and Methods

Controls and Patients

Seminal fluid from 52 healthy men whose fertility was established within the year were used to determine the normal range of the direct and indirect ELISA procedures. The patients consisted of 66 males who were referred for evaluation of a possible immune component of their infertility. All patients had a history of infertility of greater than 1 year's duration. Fresh semen specimens were submitted and a routine semen analysis was performed. The specimens were centrifuged and separated from the cellular elements and frozen as cells and seminal plasma within 2 hours of receipt. All specimens were maintained at -70°C .

Preparation of Spermatozoa

Pooled target spermatozoa were prepared for evaluation as detailed previously (Howe and Lynch, 1986). For the direct assay, individual sperm specimens were prepared as for the indirect assay with the following exception. The frozen cells were quickly thawed and resuspended in 10 ml of PBS-EGTA (8.0 g NaCl, 1.4 g NaH_2PO_4 , 0.7 ml 10 N NaOH, 0.2 mM EGTA [ethyleneglycol tetraacetic acid] to 1 liter distilled H_2O , pH 7.1) in a 15-ml plastic conical centrifuge tube. The cells were then treated as described for target spermatozoa including fixation with 2% paraformaldehyde and subsequent adjustment to a concentration of 2×10^7 spermatozoa per ml.

Absorbed Seminal Fluid and Removal of Nonspecifically Bound Ig from Target Spermatozoa

Absorbed seminal fluid was used in the ELISA as a blank in the indirect seminal plasma assay. Seminal fluid determined to be negative for antisperm antibody was absorbed 2:1 with hard packed, washed target spermatozoa for 3 hours at 20°C with constant rotation, followed by overnight incubation at 4°C . The absorbed seminal fluid was centrifuged for 10 minutes at $18,000 \times g$ and the supernatant was removed, aliquoted, and stored frozen at -70°C .

To provide a blank for the direct assay, nonspecifically bound immunoglobulin was removed from target spermatozoa by thorough washing of normal spermatozoa prior to fixation. Unfixed spermatozoa from three normal fertile men were washed with PBS-EGTA six times, which effectively removed nonspecifically bound Ig on these normal cells. The final supernatant was discarded and the cells were resuspended in PBS-EGTA at pH 7.0. The cells were then fixed with 2% paraformaldehyde and washed as for the target or test spermatozoa, adjusting the final concentration to 2×10^7 spermatozoa per ml.

ELISA for Sperm-Bound or Bindable Immunoglobulin

The indirect seminal fluid ELISA was performed as previously described (Howe and Lynch, 1986) and the assay is summarized as follows. Target spermatozoa were adsorbed to the surface of microtiter plate wells using bicarbonate buffer (1.59 g Na_2CO_3 , 2.93 g NaHCO_3 , 0.2 g NaN_3 , in 1 liter H_2O , pH 9.6) to enhance binding to the plastic. After blocking reactive sites with PBS in which 0.2% gelatin was dissolved, seminal fluid at a 1:3 dilution was added and the plate incubated for 1 hour at 37°C . Absorbed seminal fluid at a 1:3 dilution was used in all indirect assays using either pooled target spermatozoa or the patients' own spermatozoa. The direct assay was performed identically to the indirect assay except for the elimination of the seminal fluid incubation step. The wells of the microtiter plate were washed free of excess unbound immunoglobulin and then the indicator antibody was added in the form of peroxidase-conjugated F(ab) $'_2$ fragment of goat antihuman immunoglobulins (Cappel Laboratories, Malvern, PA) at a dilution of 1:200 and incubated for 1 hour at 37°C . After the wells were washed free of the indicator antibody, orthophenylenediamine was added to the wells and allowed to react. The intensity of the colored end product was proportional to the amount of immunoglobulin attached to the spermatozoa. The test specimens and controls were tested in triplicate. A standard reference curve was prepared consisting of purified human secretory IgA (Cappel Laboratories, Malvern, PA). The binding of spermatozoa and immunoglobulin were determined as previously described (Howe and Lynch, 1986; Lynch et al, 1986) using the iodination method of Markwell (1982). Under the assay conditions specified, when 2×10^6 spermatozoa were added to the wells, $48 \pm 2\%$ bound to the plastic. In the direct test, the level of immunoglobulin bound to spermatozoa *in vivo* or *in vitro* did not significantly influence the percent binding to the microtiter plate wells.

At the conclusion of the assay, the nanograms of immunoglobulin derived from the standard curve after subtraction of the appropriate blanks were divided by the number of spermatozoa per well (approximately 10^6 cells) to obtain the number of femtograms of Ig per spermatozoon.

Statistical Analysis

Statistical analysis, including descriptive statistics, *t*-test for difference between two independent sample means, simple linear regression analysis, and the Yates corrected chi-square test, were performed using the Stats Plus General Statistics Package of Human Systems Dynamics (Northridge, CA).

Results

Indirect Seminal Fluid and Direct Sperm-Bindable Immunoglobulin

The normal ranges for the indirect seminal plasma and direct assays are presented in Table 1. The nor-

mal direct and indirect seminal fluid values, determined by the mean and 3 standard deviations, were both ≤ 1.5 fg Ig per spermatozoon.

Sixty-six infertile men were evaluated for the presence of elevated levels of sperm-bindable immunoglobulin in seminal fluid and that bound directly to their own spermatozoa. The results of these assays are presented in Table 1. The indirect seminal plasma results from the 66 infertile men ranged from 0.1 to 16 fg of Ig per spermatozoon. Fourteen subjects (21%) had values outside the normal range. The direct assay of sperm-associated immunoglobulin in the 66 infertile males resulted in a mean and standard deviation of 1.75 ± 3.6 fg Ig per spermatozoon with a range of 0.1 to 21. Seventeen (26%) of the infertile men had elevated levels of Ig attached directly to their spermatozoa. A simple linear regression analysis between the direct and indirect seminal fluid values of normal and infertile men is illustrated in Fig. 1. The correlation coefficient was $r = 0.907$ and the equation for the regression analysis was direct = 1.190 , indirect = 0.27 . The student's *t*-test was applied to the numerical data to determine if the sample populations were equivalent based on the variance of the individual sample means. The analysis indicated no statistically significant difference between the two samples.

When designated as positive (elevated) or negative (normal), the indirect seminal fluid and direct assay provided equivalent results in 62 of 66 infertile patients, for a 94% correlation between the two sets of assay results. Four patients had elevated direct and normal indirect assays while the reverse occurred in one patient. Chi-square analysis comparing the positive and negative results of the direct and indirect assays resulted in a chi-square value of 38.75, indicating that the indirect and direct results were significantly related ($P < 0.001$). Table 2 compares the seminal fluid analyses of males who were negative for indirect seminal fluid antisperm antibody and those who were positive. Using the *t*-test to detect differences between populations, the only parameter that was significantly different ($P < 0.01$) was the percent motility.

Indirect Seminal Fluid ELISA Using the Patient's Spermatozoa as Target Antigen

Spermatozoa from a subgroup of 14 of the infertile men were prepared as target antigen. Each individual's seminal plasma was then tested against his own spermatozoa to detect possible autospecificity. Data

TABLE 1. Statistical Evaluation of the Results of the Indirect Seminal Fluid and Direct ELISA Procedures for Quantitating Antisperm Antibody Concentrations in Normal and Infertile Men

Assay	Normal Fertile Males		Infertile Males		
	No.	Mean \pm SD	No.	Mean \pm SD	Range
Seminal fluid	52	0.53* \pm 0.32	66	1.54 \pm 2.5	< 0.1-16
Direct sperm	12	0.36 \pm 0.36	66	1.75 \pm 3.6	< 0.1-21

*Quantitation in femtograms of Ig per spermatozoon.

from this experiment are presented in Table 3. Statistical analyses (mean, standard deviation, the student's *t*-test for independent sample means, and simple linear regression analysis) indicated that there was no significant difference in the amount of antibody bound to pooled target spermatozoa or to the patient's own spermatozoa in the indirect seminal fluid assays ($P = 0.631$). Similarly, there was no significant difference between the direct assay and the indirect seminal fluid assay using either the pooled target spermatozoa ($P = 0.684$) or the patient's spermatozoa ($P = 0.615$). Simple linear regression analy-

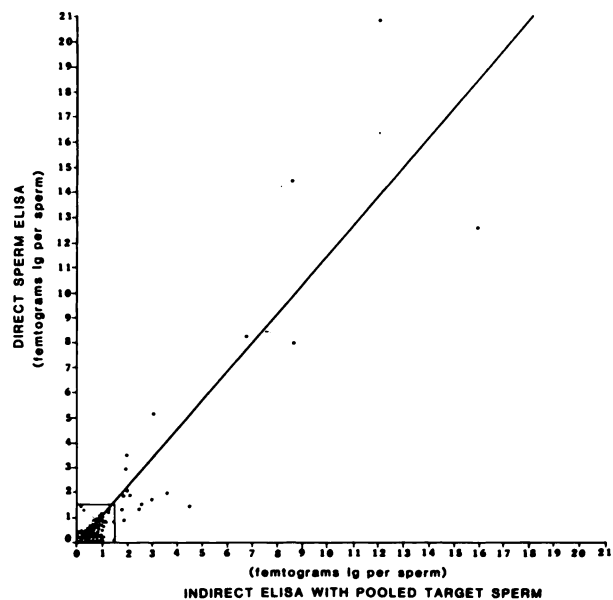


Fig. 1. Simple linear regression analysis of the indirect seminal plasma ELISA with pooled target spermatozoa compared with the direct sperm ELISA. The units of measurement are expressed in femtograms of Ig per spermatozoon. The closed circles (●), $N = 66$, are infertile patients. The box at the lower left indicates the normal range, as determined by the normal fertile males, open circles (○), $N = 12$. The coefficient of correlation was $r = 0.907$.

TABLE 2. Seminal Fluid Analysis of the Infertile Males Categorized as Having a Negative or Elevated Indirect Seminal Fluid ELISA

	Negative ELISA (N = 48)	Positive ELISA (N = 10)
Volume (ml)	2.5 ± 1.2*	2.3 ± 1.0
Sperm count (10 ⁶)	122 ± 94	55 ± 88
Percent motile spermatozoa	55 ± 21	36 ± 19
Percent abnormal forms	25 ± 16	30 ± 15

*Standard deviation.

sis of paired data showed a positive correlation with no significant difference in antibody concentration for the pooled target or the patient's own spermatozoa. In one patient (#14), however, it is important to note that indirect seminal fluid and direct values did not correlate except when the patient's own spermatozoa were used as substrate. To determine if spermatozoa from this patient bound excessive immunoglobulin from normal semen, his spermatozoa were evaluated with four normal seminal fluids and one semen sample with elevated sperm antibodies. The four normal semen samples remained normal and the elevated semen showed no significant difference (2.0 fg per spermatozoon using the pooled target spermatozoa and 1.9 fg per spermatozoon using the spermatozoa from patient 14). This suggests there may indeed be antisperm antibody specificity in some patients. In this small group, the incidence was one person, or 7%.

Discussion

The indirect approach to the determination of antisperm antibodies in semen has encompassed a variety of assays and interpretations. Previously, we presented an indirect seminal fluid antisperm antibody ELISA method with quantitative capability (Howe and Lynch, 1986). The results of this test method compared favorably with results obtained by the sperm agglutination and sperm immobilization tests. The present study describes a direct ELISA assay for antisperm antibody and compares it with the indirect seminal plasma ELISA. We questioned whether the two tests would provide equivalent quantitative results. For the assays to be comparable, major assumptions must be valid for both assays. First, for the indirect assay, there must be unbound antisperm antibodies in the seminal plasma in equilibrium with that bound *in vivo* to spermatozoa. Second, the target spermatozoa utilized must pro-

TABLE 3. Results of a Subgroup of the Infertile Patients in which the Direct Test is Compared With the Results of the Indirect Seminal Fluid Assays Using Pooled Target Spermatozoa and the Patient's own Spermatozoa as Target Cells

Patient	Indirect Seminal Fluid		Direct Patient Spermatozoa
	Pooled Target	Patient Target	
1	8.8	2.6	8.5
2	1.2	3.2	1.2
3	0.6	0.1	0.5
4	1.8	0.9	2.2
5	0.6	0.2	< 0.1
6	2.1	1.8	2.2
7	1.2	0.1	3.0
8	0.7	0.1	0.5
9	1.1	0.1	1.6
10	0.3	0.1	0.1
11	0.8	0.9	0.4
12	0.7	1.3	0.1
13	8.5	18	15
14	1.4	3.5	4.5
Mean	2.13	2.35	2.58
SD	2.70	4.49	4.05

vide the specific antigenic sites recognized by the patient's antibodies. The direct assay assumes that the antibody binding measured is sperm specific: that antibody present is not directed toward cellular or protein contaminants present in many of these abnormal specimens or due to immunoglobulin binding to the Fc receptor on certain cells or through other nonspecific interactions.

To evaluate the two assays, we tested the seminal fluid of 66 men using an indirect and direct ELISA measurement of antisperm antibody. A point-for-point comparison of the indirect seminal fluid and the direct measurements of sperm-associated antibody for each patient (Fig. 1) provided a correlation coefficient of 0.907 with a slope of 1.19. The upper limits of normal for the two assays were similar, with the direct assay at 1.5 fg of Ig per spermatozoon and 1.5 fg Ig per spermatozoon for the indirect assay. The *t*-test for independent sample means indicated that the two sample populations were not statistically different, and the chi-square analysis indicated that the two sets of data were highly correlated at $P < 0.001$. The overall correlation between positive and negative results in these patients was 94%. These data suggest that antisperm antibody in seminal fluid generally is not completely absorbed from the fluid onto the patient's spermatozoa. It appears that the antibody is

in excess of the antigenic sites *in vivo*, i.e. the equilibrium constant between free and bound antibody prevailing *in vivo* or in the seminal fluid does not result in total binding of specific immunoglobulin.

The second assumption inherent in indirect assays is that relevant antigens are available on target spermatozoa. Target spermatozoa, whether from one donor or a pooled collection of donors, must provide sufficient specific antigenic epitopes for a quantitative measurement of antibodies. In this study, we used the patient's own spermatozoa as target cells and compared these results to parallel results generated with pooled target spermatozoa. The results of these assays presented in Table 3 represent statistically similar groups. However, it is noteworthy that patient 14 appeared to demonstrate specificity to his own spermatozoa. This patient represents less than 10% of the group and does not account for a statistically significant variance when evaluated within the group. He may, however, represent a category of patients who respond to low incidence antigen present on the patient's own sperm membrane but which is not adequately represented in the pooled target spermatozoa in the indirect assay.

There are other theoretical sources of noncorrelation using an indirect measurement of antisperm antibodies in the seminal fluid. Variations in antibody concentration in the seminal plasma may be inversely proportional to the number of spermatozoa in the specimen available to bind antibody. The stage of the individual's immune response and the contribution of suppressor T cells may affect the concentration, avidity, and affinity of antibody produced and may alter the equilibrium constant either *in vivo* or in the assay. Antibody may be binding to the spermatozoa in the upper testicular compartment and not account for a measurable amount of free antibody in the seminal plasma. The indirect assay may produce false positives as a result of cross-reacting antibodies to epitopes on the target spermatozoa not present on the patients' own spermatozoa. And yet, for a statistically significant number of patients, the indirect test for antisperm antibody appears adequate for clinical interpretation.

Direct assays also have inherent problems that make the indirect measurement of seminal fluid for antisperm antibody advantageous in spite of occasional noncorrelators. Spermatozoa may not be available to perform a direct assay in azoospermic and vasectomy patients and low sperm counts in oligozoospermic men may render the direct assay impractical. Spermatozoa may be aggregated, adversely

affecting the washing and preparation steps often employed in direct assays, or the spermatozoa may be poorly motile and invalidate direct assays that require motility, such as the Immunobead® Test. Cells other than spermatozoa or protein contaminants in the washed sperm preparation may allow false positive results due to binding of the primary or secondary antibody either through a specific antigen-antibody complex or nonspecifically to the cells.

In conclusion, the indirect assay correlates well with the direct measurement of antisperm antibody (94%). The potential sources of error in this indirect assay are common to all assays that utilize target sperm indicators. The ELISA methods presented are specific for immunoglobulin, in contrast to the traditional indirect functional agglutination or immobilization assays of Kibrick or Isojima. Furthermore, the indirect or direct ELISA procedures provide quantitative results that may improve quality control and accuracy of assay and patient monitoring. The indirect assay is easily performed in contrast to the direct assay where washing of individual patient sperm specimens greatly complicates the procedure. The use of pooled target spermatozoa controlled for antigenic specificity may minimize the incidence of false negative results. The direct assay of antisperm antibody may function well as a complementary method to the indirect assay when there is suspicion of elevated antisperm antibody and a normal indirect assay.

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References

- Ackerman SB, Wortham JWE, Swanson RJ. An indirect enzyme-linked immunosorbent assay (ELISA) for the detection and quantitation of antisperm antibodies. *Am J Reprod Immunol* 1981; 1:199-205.
- Alexander NJ, Bearwood D. An immunosorption assay for antibodies to spermatozoa: comparison with agglutination and immobilization tests. *Fertil Steril* 1984; 41:270-276.
- Bronson R, Cooper G, Rosenfeld D. Sperm antibodies their role in infertility. *Fertil Steril* 1984; 42:171-183.
- Clarke GN, Elliott PJ, Smaila C. Detection of sperm antibodies in semen using the immunobead test: A survey of 813 consecutive patients. *Am J Reprod Immunol Microbiol* 1985; 7:118-123.
- Czuppon AB, Mettler L. Estimation of anti-spermatozoa antibody concentration by a [¹²⁵I]-Protein-A binding assay in sera of infertile patients. *J Clin Chem Clin Biochem* 1983; 24:357-362.
- Haas GG, Cines DB, Schreiber AD. Immunologic infertility: identification of patients with antisperm antibody. *N Engl*

- J Med 1980; 303:722-727.
- Hendry WF, Stedronska J, Lake RA. Mixed erythrocyte-spermatozoa antiglobulin reaction (MAR test) for IgA antisperm antibodies in subfertile males. *Fertil Steril* 1982; 37:108-112.
- Howe SE, Lynch DM. Quantitation of sperm bindable IgA and IgG in seminal fluid. *Am J Reprod Immunol* 1986; 11:17-23.
- Isojima S, Li TS, Ashitaka Y. Immunologic analysis of sperm-immobilizing factor found in sera of women with unexplained sterility. *Am J Obstet Gynecol* 1968; 101:677-683.
- Jager S, Kremer J, van Slochteren-Draaisma T. A simple method of screening for antisperm antibodies in the human male: detection of spermatozoal surface IgG with the direct mixed antiglobulin reaction carried out on untreated fresh human semen. *Int J Fertil* 1978; 23:12-21.
- Kibrick S, Belding DL, Merrill B. Methods for detection of antibodies against mammalian spermatozoa. II. A gelatin agglutination test. *Fertil Steril* 1952; 3:430-438.
- Lynch DM, Leali BA, Howe SE. A comparison of sperm agglutination and immobilization assays with a quantitative ELISA for anti-sperm antibody in sera. *Fertil Steril* 1986; 46:285-292.
- Markwell MAK. A new solid-state reagent to iodinate proteins 1. Conditions for the efficient labeling of antiserum. *Anal Biochem* 1982; 125:427-432.
- Mather S, Williamson HO, Derrick FC, Madyastha PR, Melchers JT, Holtz GL, Baker ER, Smith CL, Fudenberg HH. A new microassay for spermocytotoxic antibody: comparison with passive hemagglutination assay for antisperm antibodies in couples with unexplained infertility. *J Immunol* 1981; 126:905-909.
- Meinertz H, Hjort T. Detection of autoimmunity to sperm: mixed antiglobulin reaction (MAR) test or sperm agglutination? A study of 537 men from infertile couples. *Fertil Steril* 1986; 46:86-91.
- Witkin SS, Zelikovsky G, Good RA, Day NK. Demonstration of 11S IgA antibody to spermatozoa in human seminal fluid. *Clin Exp Immunol* 1981; 44:368-374.

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