INTERLABORATORY INTERPROTOCOL
COMPARISON OF INDIRECT IMMUNOBEAD ASSAY
FOR SPERM-ASSOCIATED ANTIBODIES IN SERUM

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P. Prakash thanks Sundar Melo-Abreu of the Andrology Laboratory at the Hospital of the University of Pennsyl-
vania, Department of Obstetrics and Gynecology, Philadelphia, Pennsylvania, USA, for the gift of positive
control serum for the immunobead assay. The work at Massachusetts General Hospital was supported in part by the
Vincent Research Fund.

Presented in part as a poster at the Annual Meeting of the American Society of Andrology, Bethesda, Maryland,

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This investigation was designed to study the effect of two different protocols on the indirect sperm-associated antibody test on serum performed using Bio-Rad immunobead (IBT) at two andrology laboratories. Aliquots of 31 serum samples from infertile couples were analyzed by both protocols. The IBT was negative by both protocols (100% concordance) for 18 serum samples. Seven of the 13 remaining samples were positive by both protocols (≥10% bead attachment by protocol A and ≥20% bead attachment by protocol B), for a concordance of 54%. The remaining six samples were positive by one of the two protocols. The overall concordance for positive and negative results was 81% (25 of 31 samples). Protocol A detected a higher percentage of bead attachments for IgG and IgA, while protocol B detected a higher percentage of bead attachments for IgM. The discordance in the results of IgA attachment obtained by the two protocols was statistically significant (p < .05). A standardized, uniform protocol for the indirect IBT is needed.

Keywords antisperm antibodies, comparison, human spermatozoa, immunobead test, serum

The advantages of immunobead test (IBT) include the ability to identify class- and region-specific sperm-associated antibodies and to assess the proportion of motile sperm that are coated with antibody. Its disadvantages include the requirement for semen from a negative control donor tested to be negative for sperm-associated antibodies [14], the subjective nature of the interpretation of results [13], and the technical differences in the protocols used by various authors [9]. Different procedures have been used to obtain indirect IBT for sperm-reactive antibodies. Bronson et al. [3] used donor semen filtered through Pyrex glass wool before washing it and subsequently used a modified swim-up protocol for the negative control donor semen [4]. Adeghe et al. [1] used motile sperm from donor semen collected by the layering and swim-up technique of Hellem and Rumke [12]. Other authors have used only washed sperm [13] and not the motile fraction after swim-up. In their first description of the IBT, Clarke et al. [8] did not dilute the unknown serum before passive antibody transfer. However, in a recent text, Clarke [7] has argued that 1:10 should be the minimum serum dilution tested and that 1:100 may be more appropriate to ensure clinical significance of the results. Bronson et al. [2, 4] have been consistent about the dilution of the unknown serum, i.e., 0.4 mL diluted to a final volume of 1.6 mL. These authors have cited 30–90 min as the incubation period for the passive transfer of antibodies from the test serum to the donor sperm [5], while others have used 60 min [9, 10, 15]. Likewise, there are differences among protocols with regard to the final reading. Most authors incubate the immunobead and sperm mixture for 10–15 min before reading [13], while Bronson et al. [4] believe that binding occurs immediately. Clarke et al. [8] initially scored ≥5% of sperm bound with immunobeads as positive; in subsequent reports they had considered the binding of 20% of sperm [9, 10] with immunobeads as a positive result for sperm-reactive antibody. Other authors have also considered the latter criterion to be the cutoff point for a positive immunoreaction [2, 4]. Clarke [7] recently proposed ≥50% as the cutoff for clinically significant results.

Bronson et al. [2] compared the classic techniques used for the detection of sperm-reactive antibodies in different laboratories. Mettler et al. [16] compared the results of various enzyme-linked immunosorbent assays performed in different laboratories on the WHO collection of reference sera. However, no interlaboratory comparison of the indirect IBT for serum has been published in the English language literature. Thus, we designed the present study to compare the effect of two protocols undertaken at two andrology laboratories in the Boston area on the results of the indirect IBT.
MATERIALS AND METHODS

A total of 31 serum samples from infertile couples were used. Each was divided into two aliquots for testing by the participating laboratories at Brigham and Women's Hospital (laboratory A) and Massachusetts General Hospital (laboratory B). Protocol A (used in laboratory A) was developed from G.N. Clark, BIO-RAD Bulletin 1170 [6], and protocol B (used in laboratory B) was modified from Bronson et al. [2, 4]. Serum samples were stored at −70°C (laboratory A) or −20°C (laboratory B) before testing. Semen specimens from donors found in pretests to be negative for sperm-reactive antibodies and to have a good sperm count and good forward progressive motility were obtained on the day of use by each laboratory. Negative and positive controls were tested in each assay. Immunobeads (Bio-Rad Laboratories, Richmond, CA, USA) specific for binding to human IgA, IgG, and IgM were used by both laboratories. Each serum sample was tested for sperm-associated antibody by region-specific immunobead attachment on the antibody-negative sperm. The differences between protocol A and protocol B are summarized in Table 1 and are detailed below.

Protocol A. Liquefied semen from healthy donors was washed twice with Tyrode's solution with 0.4% bovine serum albumin (T-BSA 0.4%) at 600g for 10 min. The final pellet was resuspended in Tyrode's solution with 4% bovine serum albumin (T-BSA 4.0%), and the sperm concentration was adjusted to 30 × 10^6/mL. Test and control sera were heat inactivated in a 56°C water bath for 30 min and centrifuged at 8000 rpm for 5 min in an Eppendorf microcentrifuge (Fisher Scientific, USA) for removal of the precipitate. To 100 μL of test serum, negative control serum, and positive control serum, 100 μL of straight Tyrode's solution was added. A 0.2 mL of the washed sperm suspension (30 × 10^6/mL) was added to 0.2 mL of the above serum solution. The sperm/serum mixture was incubated for 1 h at 37°C and then centrifuged for 10 min at 600g. The pellet was washed twice in T-BSA 0.4% and finally resuspended in 0.2 ml of T-BSA 4.0%.

For preparation of the immunobeads, 0.5 mL of immunobead stock (50 mg/10 mL) was washed three times in T-BSA 0.4% for 10 min at 600g. The pellet was resuspended in 1.0 mL of BSA-4.0%.

One drop (8 μL) of IgA-, IgG-, or IgM-specific immunobeads and one drop of serum-incubated sperm were mixed on a microscope slide, covered with a coverslip, and incubated at room temperature for 10–15 min. The slides were scored for immunobead attachment as visualized by 400× phase-contrast microscopy. A total of 200 motile sperm were counted. The number of motile sperm with more than two

<p>| TABLE 1 Differences Between Protocol A and Protocol B Used for the Indirect IBT |
|---------------------------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Criterion</th>
<th>Protocol A</th>
<th>Protocol B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>Tyrode's</td>
<td>PBS</td>
</tr>
<tr>
<td>Bovine serum albumin concentration</td>
<td>4%</td>
<td>5%</td>
</tr>
<tr>
<td>Negative control donor semen</td>
<td>Wash × 2, no swim-up</td>
<td>Layer 1:2 PBS, swim-up</td>
</tr>
<tr>
<td>Test and control sera</td>
<td>Dilute 1:1 in medium</td>
<td>Dilute 1:3 in medium</td>
</tr>
<tr>
<td>Incubation</td>
<td>1 h, wash × 2</td>
<td>1 h, wash × 2–3</td>
</tr>
<tr>
<td>Centrifuge</td>
<td>600g × 10 min</td>
<td>600g × 8 min 15,000 rpm in microcentrifuge 8–10 s × 2</td>
</tr>
<tr>
<td>Reading protocol</td>
<td>Follows 10–15 min of incubation in moist chamber</td>
<td>Add horse serum and read immediately</td>
</tr>
<tr>
<td>Cutoff for positivity</td>
<td>≥10% with &gt;2 immunobeads</td>
<td>≥20% with ≥1 immunobead</td>
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</tbody>
</table>
beads attached was divided by the total number of motile sperm (bound and unbound) to determine the percentage attachment. Samples with immunobead binding at a level of ≥10% were considered positive.

Protocol B. Liquefied semen from healthy known sperm-antibody-negative donors was layered under PBS-BSA 0.5% medium (phosphate-buffered saline with 0.5 g of bovine serum albumin/100 mL) 1:2 volume to volume in a conical test tube, and sperm were allowed to swim-up for 1 h at 37°C. After swim-up, the supernatant was removed and centrifuged for 8 min at 600g. The pellet was resuspended in PBS-BSA 0.5% and the sperm concentration was adjusted to 30 × 10^6/mL. The test serum and the positive control serum were heat-inactivated in a 56°C water bath for 30 min. A 0.4 mL volume of test serum or positive control serum was combined with 1.1 mL of straight PBS solution in separate conical test tubes. A 0.3-mL volume of the washed sperm suspension (30 × 10^6/M/mL) was added to the above serum solutions. The serum/sperm mixtures were incubated for 1 h at 37°C and then centrifuged for 8 min at 600g. The sperm pellet was resuspended in 0.35 mL of PBS-BSA 0.5% and centrifuged twice more in a microcentrifuge (Abbott Laboratories, Irving, TX, USA) at 15,000 rpm for 8–10 s. The final suspension was in 0.35 mL of PBS-BSA 0.5% medium. To the above suspension a 20-μL volume of heat-inactivated horse serum (Sigma Chemical Co., St. Louis, MO, USA) was added to maintain adequate motility of sperm for the final reading.

For preparation of the immunobeads, 0.35 mL of a working solution of immunobeads (10 mg/mL) was first washed in 10 mL of PBS-BSA 0.5% medium at 600g for 8 min, and then washed twice with PBS-BSA 0.5% in a microcentrifuge at 15,000 rpm for 8–10 s each time. The final pellet was resuspended in 0.35 mL of PBS-BSA 0.5% solution. A 50-μL volume of sperm suspension and a 40-μL volume of IgA-, IgG-, or IgM-specific immunobeads were mixed in an Eppendorf tube. One drop (5–10 μL) of this mixture was placed on a microscopy slide, and a coverslip was applied. The slides were read immediately and scored for immunobead attachment as visualized by 400× phase contrast microscopy. In general, 100 motile sperm were counted; in doubtful or borderline cases, 200 sperm were counted. The number of motile sperm with any bead attachment was divided by the total number of motile sperm (bound and unbound) to determine the percentage attachment. If required, bead attachment was confirmed or ruled out by periodic tapping on the coverslip. Samples with immunobead binding at a level ≥20% were considered positive.

Statistical Methods. The results obtained by the two protocols for immunobead binding of all three isotopes (IgA, IgG, and IgM) were compared by calculation of p values in the nonparametric Wilcoxon signed rank test.

RESULTS

All 31 serum samples were analyzed by both protocols. The IBT was negative by both protocols for 18 serum samples (58%), i.e., no sperm-associated antibody (IgA, IgG, or IgM) was detected, or bead attachment was below the cutoff point for the given protocol. Thirteen samples (42%) were found to be positive: seven by both protocols (54% positive correlation for any isotope) and six by one protocol. There was an overall concordance of 76.9% (10/13) for IgG and IgM attachment, between the two protocols. The concordance for IgA attachment between the two protocols was 38.5% (5 of 13) (Table 2).

For both positive and negative sera, the interlaboratory difference in the percentage attachment of IgG and IgM was not statistically significant (p > .05). For IgA, there was no statistical difference between the two laboratories among the sera testing negative. However, there was a statistically significant difference between laboratories among the sera testing positive for IgA
TABLE 2 Immunoglobulin-Specific Analysis of Results of the Indirect IBT for 13 Serum Samples
Found to be Positive by One or Both Protocols

<table>
<thead>
<tr>
<th>Immunoglobulin</th>
<th>Positive and Concordant</th>
<th>Negative and Concordant</th>
<th>Discordant</th>
<th>Overall Concordance</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgA</td>
<td>2</td>
<td>3</td>
<td>8</td>
<td>5/13 (38.5%)</td>
</tr>
<tr>
<td>IgG</td>
<td>5</td>
<td>5</td>
<td>3</td>
<td>10/13 (76.9%)</td>
</tr>
<tr>
<td>IgM</td>
<td>1</td>
<td>9</td>
<td>3</td>
<td>10/13 (76.9%)</td>
</tr>
</tbody>
</table>

\( p < .05 \). The discrepancy resulted in an overall statistically significant difference between laboratories for the attachment of IgA \( p < .05 \) (Table 3).

In summary, aliquots of 31 sera were analyzed in two laboratories using two technically different protocols (Table 1). Eighteen samples (58%) tested negative by both protocols; thus there was 100% concordance of negative results. Thirteen samples (42%) tested positive by one or both protocols (54% concordance). The overall concordance of results (positive and negative) between the two laboratories was 81\% (25 of 31 samples). Transposing the cutoff for positive score of laboratory A to the data from laboratory B (and vice versa) did not change this figure significantly. However, if a cutoff \( \geq 50\% \) for positive score [7] was applied, the correlation dropped to 67.7\%.

DISCUSSION

The following technical factors could play a role in the variation of results obtained with different protocols:

1. **Swim-up fraction vs. only washed sperm as negative controls.** According to Hellema and Rumke [12], making use of only motile (washed) sperm reduces nonspecific clumping and minimizes specific tail-to-tail agglutination. However, in the highly capacitated swim-up fraction, the sperm may actually be moving too fast to mechanically bind to immunobeads [11]. This situation may have led to the higher number of positive scores for IgA detected by protocol A.

2. **Dilution of unknown and control sera.** A high concentration of immunoglobulins and proteins in general and the competition between antibodies of different avidities in an insuffi-

TABLE 3 Comparison of Percentage of Sperm Binding (%) of IgA-, IgG-, and IgM-Specific Immunobead Binding Among the Same Serum Samples Tested in Two Laboratories

<table>
<thead>
<tr>
<th>Types of Sample (n)</th>
<th>IgA (%)</th>
<th></th>
<th>IgG (%)</th>
<th></th>
<th>IgM (%)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lab A</td>
<td>Lab B</td>
<td>Lab A</td>
<td>Lab B</td>
<td>Lab A</td>
<td>Lab B</td>
</tr>
<tr>
<td>Positive (13)</td>
<td>26.5 ± 8.5*</td>
<td>19.7 ± 8.9*</td>
<td>44.2 ± 11.7</td>
<td>27.7 ± 10.1</td>
<td>6.6 ± 2.5</td>
<td>9.8 ± 7.6</td>
</tr>
<tr>
<td>Negative (18)</td>
<td>1.3 ± 0.6</td>
<td>0.9 ± 0.5</td>
<td>1.4 ± 0.5</td>
<td>0.9 ± 0.5</td>
<td>0.3 ± 0.2</td>
<td>1.0 ± 0.5</td>
</tr>
<tr>
<td>Total (31)</td>
<td>11.8 ± 4.1*</td>
<td>8.8 ± 4.0*</td>
<td>19.4 ± 6.2</td>
<td>12.1 ± 4.8</td>
<td>2.9 ± 1.2</td>
<td>4.7 ± 3.2</td>
</tr>
</tbody>
</table>

*Note. Values are means ± standard error.
*p < .05.
ciently diluted serum in the sensitization mixture used in protocol A may have resulted in the exclusion of some IgM class antibodies [9]. The use of undiluted or insufficiently diluted serum can lead to nonspecific binding.

(3) Differences in the final reading protocols. In protocol B (but not in protocol A), the addition of horse serum probably renders the already-motile sperm too active to mechanically bind to immunobeads. This effect may explain the lower level of overall immunobead binding detected by protocol B. Even though protocol A used a higher minimum number of bound immunobeads (more than two), as recommended by Clarke et al. [8], but a lower cutoff level (≥10%) and protocol B used a lower minimum number of bound immunobeads (at least one) and a higher cutoff level (≥20%), as recommended by Bronson et al. [2] and Clarke et al. [9, 10], this factor did not appear to account for the difference between the protocols.

In conclusion, 100% concordance was seen in all serum samples testing negative for sperm-associated antibody of any isotype. Therefore, a negative result in the indirect IBT for serum appears to be reliable. Protocol A detected more antibody class-specific interactions and a higher percentage of bead attachments for IgG and IgA, while protocol B detected a higher percentage of bead attachments for IgM. The frequency of exact concordance was 54% for samples with positive results. There was a statistically significant discordance between the two protocols for IgA attachment. Therefore, we recommend that all borderline and positive results be confirmed. To reduce variability due to technical differences, uniform, standardized protocols with uniform cutoff points should be used for the interpretation of investigative procedures in andrology, including the indirect IBT.

REFERENCES


