Identification of bovine sperm specific polypeptides reactive with antisperm antibodies

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Bovine infertility has been associated with presence of antisperm antibodies which impair the physiological processes of reproduction. The relative importance of antisperm immunity as a cause of unexplained infertility in bovines is largely unknown. The high circulatory levels of antisperm antibodies have been positively correlated with unexplained infertility in artificially inseminated infertile cows. Bovine sperm antigens are capable of eliciting the production of antisperm antibodies. Identification and characterization of these antigens are important for (a) understanding the mechanism involved in antibody mediated impairment of reproduction; (b) to develop reliable diagnostics; and (c) to prevent and treat the cases of unexplained immuno-infertility. Extensive studies have been performed in human sperm specific antigens and associated consequences. Such studies are rather sketchy in farm animals.

Auto and isoantibodies directed against the surface of spermatozoa may cause infertility through immobilization by agglutination or complement fixation or inhibition of cervical mucus penetration. These antibodies have been conventionally detected by agglutination and precipitation techniques which are comparatively less sensitive and show considerable degree of cross reactivity. The use of ELISA has increased the sensitivity and reproducibility of the diagnosis. Antigens specific to spermatozoa were reported to show genetic polymorphism. Some of them were identical between the species while in some species, two genetically distinct population of spermatozoa having different antigenic characteristics have been reported. This polymorphism is related to genetic configuration and may be utilized for prevention of immuno-infertility by changing antigenically related bulls. Accurate knowledge of these factors may explore the better understanding of immuno-infertility mechanism and could greatly aid in developing preventive procedures.

Molecular characterization through intervention of electrophoretic separation of sperm polypeptides by SDS-PAGE and detection of their immunoreactivity to hyperimmune sera as well as sera from immuno-infertile individual may give more deeper insights. Similar approaches have been made in human and in buffalo.

In the present study presence of circulatory antisperm IgG antibodies as a cause of unexplained infertility has been assessed in artificially inseminated repeat breeder cows. Bovine spermatozoid polypeptides are also characterized by SDS-PAGE and immunoblotting with homologus/heterologus hyperimmune sera and with sera from immuno-infertile cows.

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Materials and Methods

Collection of serum samples—Serum samples were collected from cross-bred (Holstein Friesian Sahiwal) cattle maintained in organised dairy herds under standard conditions of husbandry in Bareilly, U.P., India. Repeat breeder cows (75), 3-8 years old, who failed to become pregnant in 3 or more subsequent inseminations, were examined per rectally and those which appeared normal, were included in this study. Samples were also collected from pregnant (2-4 months) cows (15) and heifers (5) of 18-24 months of age.

Preparation of spermatozoa antigen—Semen from 4 fertile cross-bred (H.F. x Sahiwal) bulls was collected by means of artificial vagina. Individual ejaculate containing more than $1 \times 10^8$ sperm/ml and > 70% motility was centrifuged at 500 g for 20 min. at 20°C to separate seminal plasma. Spermatozoal pellets were washed four times with phosphate buffer saline (PBS, pH 7.4) by resuspending and centrifuging at 500 g for 20 min. Finally prepared spermatozoa were suspended in PBS (containing 1 mM of PMSF and 1:10000 of thiomersal) and concentration was adjusted to $1 \times 10^9$ sperm/ml and stored at -80°C till further use.

Immunization of rabbits and calf—Female rabbits (4) of Newzeland white strain, 4 months old were immunized by inoculating emulsion containing Freund’s complete adjuvants (FCA; Sigma) and $1 \times 10^8$ spermatozoa. One rabbit was kept as control. Three subsequent boosters were given in same manner at 15 days interval with Freund’s incomplete adjuvant (FIA) and $1 \times 10^8$ sperm. Blood was collected from all rabbits after one week of last inoculation and separated sera were stored at -80°C.

Sperm antisera was also raised in calf against pooled spermatozoa from 4 bulls. The procedure was same as in rabbits except number of spermatozoa which in this case was $1 \times 10^9$ spermatozoa.

Standardization of cellular ELISA for detection of antisperm antibodies—Different serum dilutions (1:25, 1:50, 1:100, 1:200) from known positive (hyper immune calf antibovine sperm sera) and known negative (pooled heifer sera) were analyzed. The best differential optical density (O.D.) was obtained at 1:50 dilution. To decide seropositivity in test sera, the cut off value of O.D. was decided as per Cuzppon\textsuperscript{15} i.e. positive if O.D.is＞mean O.D. + 2×SD of fertile group.

Detection of antisperm antibodies by cellular ELISA (cELISA)—Detection of IgG class of antibodies was done in serum samples from repeat breeder, pregnant control, heifers, hyperimmunized rabbits and calf as per Lander et al.\textsuperscript{1} Washed spermatozoa antigen was diluted in PBS (pH 7.4) to make the dilution of $1 \times 10^6$ cells/ml. Hundred µl of this was coated in wells of microtitre ELISA plates (Tarson, India). Plates were kept at 20°C overnight in front of air blower. Once the wells were completely dried, remaining sites were completely blocked by filling whole wells with 5% skimmed milk in PBS for overnight at 4°C. Assay plates were washed thrice with PBS (pH7.4) containing 0.05% (v/v) Tween-20 (PBS-T) between all incubation steps. After washing with PBS-T, 100 µl test sera (1.50 dilute in PBS-T) in triplicate was added in all wells and then plates were incubated at 37°C for 2 hr in humified chamber. Following washing and filling the wells with 100 µl of 1:10,000 diluted rabbit anti bovine IgG-HRPO conjugate (10K; NII, India), plates were again incubated for 1 hr. After washing 100 µl of substrate solution (0.5 mg orthophenylene diamine/ml of citrate buffer) was added to each well. The enzyme action was stopped by adding 100 µl of 5 N H₂SO₄ after 20 min. Absorbance of each well was measured at 450 nm using an anths Labtec ELISA reader.

Detection of sperm specific polypeptides by SDS-PAGE—One dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed as per Hansen and Bjerrum\textsuperscript{10} with slight modification. Briefly, stacking gels composed of 4% acrylamide and resolving gels containing 10% acrylamide were run in a vertical slab gel unit (Atto, Japan). Spermatozoa protein was obtained by suspending washed spermatozoa in Tris-HCK (6.25 mM) buffer (pH 6.8) containing 2% SDS 10% glycerol, 5% β-mercaptoethanol and 0.1% bromophenol blue and immersing for 5 min in water bath at 95°C. Gels were loaded with 100 µl well of sperm protein and run for 8 hr at a constant current of 12 mA. Molecular weight standard at the range of 14.3 to 66 kDa (Sigma) were run in parallel. Gel was stained with coomassie brilliant Blue R-250 (Sigma).

Detection of humoral responsive polypeptides by immunoblotting—Humoral responsive polypeptides were detected as per Kanchev et al.\textsuperscript{14} with slight modification. Proteins from gel were electro-
phoretically transferred to nitrocellulose paper (NCP) by semi-dried system (Atto, Japan) for 90 min at a constant current of 2 mA/cm². The transfer buffer was used as per instruction manual of the apparatus. Remaining sites in NCP were blocked by placing it in 5% skimmed milk in Tris-NaCl buffer (50 mM tris-HCl, 150 mM NaCl, pH 10.2 and 0.1% Tween-20) for 2 hr at 37°C. Strips of NCP with transferred spermatozoal proteins were allowed to react with 1:50 dilute hyper immune sera of rabbit/calf or sera of immuno-infertile repeat breeder for 18 hr at 4°C followed by three washings for 4 min in Tris-NaCl buffer. The strips were then incubated in 1:500 diluted goat antirabbit IgG-HRPO/rabbit antibovine IgG-HRPO conjugate (10K, NII, India) for 2 hr at 37°C. Strips were again washed 4 times and subjected to substrate solution (Di-amino benzidine solution) for 5 min at room temperature. The reaction was terminated by washing the NCP strips with distilled water.

Results

Testing of hyperimmune sera for antisperm IgG antibodies—The hyperimmune sera raised in rabbits and calf were tested for presence of antisperm IgG antibodies by cellular-ELISA. A titre of 1:1600 was obtained in rabbit sera but it was only 1:400 in calf serum.

Detection of antisperm IgG antibodies in test samples—Detection of IgG antisperm antibodies by cELISA was carried out in repeat breeder cattle (75) pregnant control (15) and heifers (5) using sperm antigens from 4 bulls. The results pertaining to percentage and frequency of seropositivity of antisperm antibodies are given in Figs 1 and 2.

Detection of sperm specific polypeptides—Sperm antigens from 4 bulls were subjected to SDS-PAGE to detect various polypeptides. After staining of gel (Fig. 3) 16 polypeptides were detectable in all 4 bulls. The number of polypeptides were same but comparative difference was there which has been depicted by difference in colour intensity of different peptides among bulls.

Immunoblotting to detect humoral responsiveness of sperm specific antigens—Rabbit antibovine sperm sera were analysed against spermatozoal proteins by immunoblotting (Fig. 4). Seven spermatozoal polypeptides were found immuno-reactive with IgG antibodies in rabbit antibovine sperm sera (Table I). These reactions were present in immunoblotting by the sera from all rabbits and sperm antigen from all bulls. Serum from control rabbit did not react with any polypeptide. Calf antibovine sperm sera reacted with 3 polypeptides of spermatozoa. These were of 158.5, 69.2 and 51.3 kD (Fig. 5). Sera from 4 repeat breeder cows which were found seropositive for anti

![Fig. 1—Seropositivity for antisperm antibodies in repeat breeder and control cattle](P:*<0.05; **<0.01).
sperm antibodies for all 4 bulls, were also subjected for immuno-reactivity. Two peptides of 69.2 and 51.3 kDa molecular weight reacted invariably with these sera (Fig. 6).

**Discussion**

Spermatozoa contains proteins foreign to female reproductive tract, therefore, there are chances of initiations of immune response against them. The chances are further increased when inseminations are frequently repeated in repeat breeder cattle. Variable occurrence of anti-sperm antibodies in repeat breeder have already been reported. The analysis of sera from repeat breeders by cELISA revealed the variable occurrence of antisperm antibodies (14.67-26.67%). Wang\textsuperscript{16} studied the relationship between sperm antibody reaction of ELISA and infertility in Chinese black and white dairy cows. He found that 34.5% of infertile cows had sperm antibodies in blood serum as compared to 6.7% in non-pregnant cows with a history of normal fertility. The present findings in repeat breeder group is slightly lower in occurrence which may be due to variation in individual susceptibility and number of inseminations received by animals. However, the present findings are in agreement with Wang\textsuperscript{16} in pregnant control group except for one bull. This may be due to antigenic similarity between the bull tested and the bull from

![Graph showing seropositivity for antisperm antibodies](image)

**Fig. 2**—Frequency of seropositivity for antisperm antibodies [− = negative for all 4 bulls; positive for 1(1+), 2(++) and 3(+++) and 4(++++) bulls. $P^{*}<0.05; P^{**}<0.01$].
which animal has been served earlier or it may be due to presence of cross-reactive antibodies in serum of particular animal. All heifers tested were negative for antisperm antibodies. These findings are in agreement with findings of Farahani et al. who reported that agglutination reaction was not seen in virgin heifers because there is no chance of exposure of heifers to sperm antigen. Variation in occurrence of antisperm antibodies for different bulls may be because of the fact that repeat breeders included in the present study were belonging to different farms where they received inseminations from semen of different bulls. Variable agglutination titre has been reported in repeat breeders when testing was carried out using the sperm antigen of same bull from which they have been served as compared to other, non specific bull.

Frequency of sero positivity of antisperm antibodies—Most of repeat breeders in present study were positive for one bull, followed by some of the animals for two bulls. Very less number of repeat breeders were positive for three or four bulls. Investigators have commonly used pooled spermatozoa antigen from different bulls for testing purpose. Antigenic variation in spermatozoa from different bulls may have contributed to these findings. These results indicated the possibility of solving the problem of immuni-infertility by changing bulls.

![Table 1](image)

**Table 1—Detection and identification of humoral responsive polypeptides of sperm**

<table>
<thead>
<tr>
<th>Polypeptides (kDa)</th>
<th>Rabbits</th>
<th>Calf</th>
<th>Repeat breeder</th>
</tr>
</thead>
<tbody>
<tr>
<td>158.5</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>81.3</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>69.2</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>51.3</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>45.7</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>40.7</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>30.5</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>27.8</td>
<td>+</td>
<td>-</td>
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</tr>
<tr>
<td>25.4</td>
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<td>-</td>
</tr>
<tr>
<td>22.6</td>
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</tr>
<tr>
<td>21.4</td>
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</tr>
<tr>
<td>19.0</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>17.0</td>
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</tr>
<tr>
<td>14.1</td>
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<td>-</td>
</tr>
<tr>
<td>11.7</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

+= immunoreactivity; -= non-reactivity
Humoral responsiveness of sperm specific polypeptides to calf antiovine sperm sera. [Lane PB = Seroreactive polypeptide of pooled sperm from 4 bulls].

Identification of sperm specific polypeptides by SDS-PAGE—The present finding in cattle regarding similar number of polypeptides in different bulls is in agreement for same in buffalo. Variation in intensity of bands among spermatozoa from different bulls may be due to differences at the level of expression for different proteins. Variable numbers of polypeptides have been reported in different species.

Detection of humoral responsiveness of polypeptides—Reactivity of 7 polypeptides in bovine spermatozoa have been reported by double immunodiffusion technique with antisera raised in rabbit. When human sperm specific polypeptides were tested for immunoreactivity with rabbit antihuman sperm sera by SDS-PAGE and immunoblotting, 12 polypeptides were found immunoreactive, whereas, immunoreactivity of only 3 human spermatozoal polypeptides could be antigenically correlated to agglutinating activity from immuno-infertile sera. Their observations are also supportive of species variation regarding antigenicity of human spermatozoa.

Identification and molecular characterization of sperm specific isoantigens in human beings revealed that 2 polypeptides of 44 and 72 kDa reacted significantly more frequently with serum antibodies from immunoinfertile female. Three isoantigens were also identified and rabbits; as compared to 7 antigenic polypeptides of rabbit spermatozoa were found immuno-reactive with rabbit antimouse sperm sera by SDS-PAGE and immunoblotting. These findings are also supportive of species variation in antigenicity of rabbit sperm polypeptides.

Immuno-infertility was diagnosed as a cause of unexplained infertility in cows but seropositivity was variable for sperm antigen from different bulls. Antigenicity of sperm polypeptides was also variable in different species and even in between the 2 sexes.

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