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Relationship of Naturally Occurring Antisperm Antibodies in Blood Serum and Seminal Plasma of Cattle Bulls with Sperm Function and Fertility Tests

V. Zodinsanga[1], Ranjna S. Cheema[1*], P. S. Mavi[2]

[1]Department of Veterinary Gynaecology and Obstetrics, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, India
[2]Department of Teaching Veterinary Clinical Complex, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, India
Email: *Ranjna.cheema@gmail.com

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Abstract

The study was planned with an objective to assess the level of antisperm antibodies (ASA) in the blood serum and seminal plasma of breeding cow bulls and their relationship with sperm function and fertility tests. ASA was analyzed in blood serum and seminal plasma by SpermMar test, Immuno peroxidase assay (IPA) and Enzyme linked immunoabsorbant assay (ELISA). In SpermMar test, about 54% bulls were with >40% IgG in blood serum against sperm surface antigens, whereas none of the bulls were with >10% IgG in seminal plasma. More than 20% and >10% IgA against sperm surface antigens were detected in the blood serum and seminal plasma of 65.8% and 37% bulls, respectively. Out of 26 bulls, seminal plasma of 21 bulls reacted with spermatozoa both in IPA and IgA latex particles and that of only 12 bulls reacted with IgG. In IPA, about 50% of the bulls had >40% ASA against head surface antigens, whereas, there were 23% bulls with >10% ASA in seminal plasma. Also ELISA indicated a higher antibody titre in blood serum (3200 - 6400) and seminal plasma (40 - 80) of 50% and 42% bulls, respectively. There were 11 bulls with low values of HOST/*in vitro* acrosome reaction/cervical mucus penetration assay and higher level of either serum or seminal plasma ASA. Our study revealed that a significant level of ASA in serum or seminal plasma may have effect on the fertility of bulls by affecting the sperm function.

Keywords

ASA, Cattle Bulls, Sperm-Function, Fertility-Tests, Relationship

*Corresponding author.

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1. Introduction

Sperm has long been known to be antigenic and antisperm antibodies (ASA) are immune-reactive proteins produced by the body in response to the sperm antigens. The blood-testis barrier (BTB), a tight junction among sertoli cells, separates the spermatozoa and immune system. It prevents testicular cells, which express foreign antigens from coming into contact with lymphoid tissue and immunocompetant cells. However, BTB is commonly disrupted by physiological leakage of normally sequestered sperm antigens. Generally, ASA formation can be induced primarily during infectious and non-infectious inflammations, or by obstruction of testicular efferent duct [1]. Sperm antibodies may be both transudates from the blood and secreted locally by plasma cells within the reproductive tract [2]. Sperm-reactive antibodies can also be present in serum yet undetectable in semen or within female reproductive tract secretions [3]. ASA has harmful effects on sperm function and may prevent motility or lead to sperm death. Negative effect of ASA on sperm function during *in vitro* fertilization (IVF) was demonstrated by Kim *et al.* and Lombardo *et al.* [4] [5]. An elaborative study done by Zraly *et al.* [1] indicated that ASA was significantly higher in active donors of semen compared with the candidate breeders that had not been used as regular donors (56.4% vs. 39.2%; $P < 0.01$) and fertility in those bulls was insignificantly lower. The study was done with an objective to evaluate the relationship of ASA with sperm function and fertility tests in cattle bulls.

2. Material and Methods

2.1. Procurement of Samples

Frozen semen of 26 breeding cattle bulls (Pure HF and HF crosses) were procured from Semen Freezing Laboratory, GADVASU, Ludhiana, Semen Bank Bhattian, Khanna and Government Semen Bank, Ropar, Punjab, India during 2013-2014. Freshly ejaculated semen and blood of these bulls were also collected to harvest seminal plasma and serum, respectively from the respective farms. All the tests were performed in duplicate.

2.2. Semen Function and Fertility Tests

2.2.1. Sperm Concentration

Sperm concentration was taken from the records of respective semen freezing laboratories.

2.2.2. Individual Motility

A drop of frozen-thawed semen was placed on micro slide, covered with cover slip and progressively motile spermatozoa were observed under bright field microscope (400×) at 37˚C. A total of 200 spermatozoa were counted in different fields and percentage motility was calculated.

2.2.3. Sperm Viability and Abnormalities [6]

Frozen thawed semen was evaluated for viability and abnormalities through Eosin Nigrosin staining method. A total of 150 sperms were counted in different fields under oil immersion at 1000 X and per cent live sperm was calculated. Spermatozoa with various abnormalities of head, tail and mid piece were also observed in eosin-nigrosin stained slides.

2.2.4. Hypo Osmotic Swelling Test [7]

A total of 150 spermatoazoa were counted at 400X in different fields and total number of coiled tailed sperms was calculated. The number of coiled tailed spermatozoa in phosphate buffered saline (PBS) was deducted from the number in hypo osmotic solution and the resultant figure was taken as the HOS reactive spermatozoa.

2.2.5. Cervical Mucus Penetration Test [8]

Cervical mucus was collected from a normal cycling cow in estrus and was filled in a capillary by capillary action. Capillary was sealed from one side with PVA powder and pre heated at 37˚C for 10 min. Approximately 100 µl of frozen semen was placed at the bottom of an eppendorf tube and a capillary tube was placed with its open end in the semen. After 30 min of incubation at 37˚C, the capillary tube was fixed on scaled glass slide and viewed under microscope at 400×. The length of the tube was then scanned to establish the distance furthest from the semen reservoir attained by spermatozoa. The maximum distance of migration of spermatozoa after 30

min of incubation was defined as the migration distance. Number of migrated spermatozoa was counted in the peak 0.5 cm.

2.2.6. Acrosomal Integrity [9]

Sperm smears stained with giemesa were examined microscopically under oil immersion at 1000 X. About 200 spermatozoa with intact acrosomes (stained dark purple) and damaged acrosome (without stain/stained light purple) was counted in different fields and percentage of spermatozoa with intact acrosomes was calculated.

2.2.7. *In Vitro* Capacitation and Acrosome Reaction [10]

About 200 × 10^6 spermatozoa/ml were incubated in Tyrode albumin lactate pyruvate (TALP) medium (100 mM NaCL, 3.1 mM KCl, 25 mM $NaHCO_3$, 0.3 mM Na_2HPO_4, 21.6 mM Na lactate, 2 mM $CaCl_2$, 0.4 mM $MgCl_2$ $4H_2O$, 10 mM Hepes, 1 mM Na pyruvate, 0.6% bovine serum albumin (BSA), 5 mM glucose and heparin 10 µg/ml) at 37˚C in an incubator for 4 - 6 hrs. Motility was checked every hour and sperm smears were prepared at 0, 4 and 6 hrs of incubation. Sperm smears were stained with giemsa to observe different stages of acrosome reaction. Giemsa stained slides were observed under bright field microscope at 1000X and about 200 spermatozoa with swollen heads, vesiculated and shedded acrosomes were counted.

2.2.8. Calculation of Percentage of Spermatozoa

Percentage of motile, viable, HOS reactive and acrosome reacted spermatozoa was calculated by the following formula:

$$\frac{\text{Number of motile/viable/HOS reactive/acrosome reacted spermatozoa}}{\text{Total counted spermatozoa}} \times 100$$

2.3. Detection of ASA in Serum and Seminal Plasma

2.3.1. Preparation of Blood Serum and Seminal Plasma

Blood serum and seminal plasma were obtained by centrifugation of clotted blood and fresh semen at 3000 rpm for 10 min, respectively. Blood serum and seminal plasma were heated at 56˚C for 30 min to inactivate complements.

2.3.2. Immunoperoxidase Assay (IPA) [11]

Sperm smears on clean slides were incubated with 1% bovine sperm albumin for 2 hours at 4˚C. Slides were washed thrice with PBS, pH 7.4, incubated with 1:200 diluted serum/1:10 diluted seminal plasma for 1 hour at 37˚C, again washed thrice with PBS. Smears were incubated with rabbit anti-bovine IgG (Sigma) for 45 minutes at 37˚C and washed thrice with PBS. Colour was developed with 3, 3'-Diaminobenzindine tetrahydrochloride in Tris buffer (0.05M, pH 7.6 at 25˚C) and 27 µl of 3% hydrogen peroxide for 5 minutes at room temperature. Washed with distilled water, slides were mounted in 10% glycerol in PBS, covered with coverslip and examined under the microscope at 10 × 100 X for dark brownish colouration of the sperm. About 200 sperms with browning on acrosome, post acrosomal cap or whole head were counted in different fields and percentage of IPA positive sperms were calculated (**Figure 1**).

Figure 1. Showing browning of sperm membrane in immunoperoxidase assay.

2.3.3. Indirect Sperm Mar Test with Sperm Mar Kit [12]

Diluted inactivated serum/cervical mucus 1/4 with TALP medium, pH, 7.4 and incubated at 37˚C for 30 min. Collected the motile sperms by centrifugation through Histopaque, suspended the sperm pellet in TALP and adjusted the sperm conc to 20×10^6. Incubated 100 µl of the sperm suspension of motile spermatozoa with 100 µl of inactivated ¼ diluted serum or seminal plasma, incubated for 1 hour at 37˚C. Added 2 ml of TALP, mixed well and centrifuged for 10 minutes at 400 g. Resuspended the pellet with 50 µl of TALP. On a slide, mixed 10 µl of sperm suspension and 5 µl of sperm Mar latex particles bound to IgG/IgA, covered with cover slip, kept in humid chamber for 5 min and observed under microscope at 400 X. Attachment of latex particles to the head/tail or whole sperm was observed (**Figure 2**). About 200 sperms in different fields were counted and percentage was calculated.

2.3.4. Enzyme Linked Immunoabsorbant Assay (ELISA) [13]

Sperm antigen/extracts were prepared by suspending washed spermatozoa in 62.5 mM Tris-HCl, pH 6.8 containing 2% SDS, 1 mM PMSF, 25 mM benzidine, 10 mM aprotinin, 10 mM pepstatin and 5 mM soyabean trypsin inhibitor, sonicated (3 bursts of 20 sec each) and centrifuged at 15,000 rpm for 30 minutes. ELISA plates were coated with 5 µg protein (sperm antigen) per well by incubating at 37˚C for three hrs. After washing thrice with PBS, antigen coating was blocked by incubating with 300 µl of 2% BSA per well for overnight at 4˚C. Again washed thrice with PBS pH 7.4 and added serial dilutions of serum/cervical mucus into the wells and incubated at 37˚C for three hours. Washed again with PBS and incubated with 100 µl/well of HRP conjugated anti bovine IgG for three hours at 37˚C. Washed the plate twice with PBS and incubated with 100 µl of o-phenyldiamine + 0.06% H_2O_2 as a substrate for 20 min at room temperature. Stopped the reaction with 5 N H_2SO_4 and measured the absorbance at 492 nm using ELISA reader.

2.3.5. Statistical Analysis

The data obtained was analyzed statistically according to Independent Sample T-Test and One-Way ANOVA using difference between means of two groups and means of different group application at 5 per cent level of significance (SPSS, Version 16.0).

3. Results

3.1. Immunoperoxidase Assay

Browning of acrosome, equatorial segment and whole head surface in the presence of blood serum or seminal

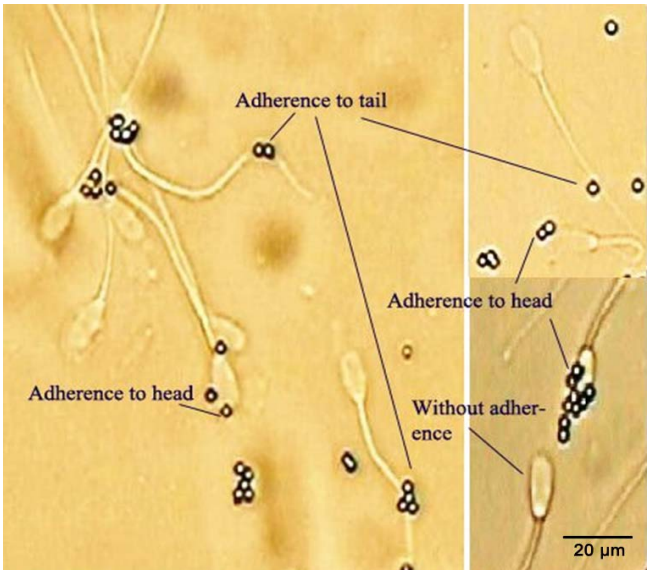

Figure 2. Showing adherence of latex particles of IgG/IgA class to various parts of cattle bull spermatozoa.

plasma indicated the development of antibodies against surface proteins of acrosome, equatorial segment and whole head in bulls (**Figure 1**). There was 39.5% ± 2.1% and 8.2% ± 1% browning of acrosome, equatorial segment and whole head surface in the presence of blood serum and seminal plasma of bulls respectively. However, values ranged from 14.4% - 57.8% and 0% - 19.3% in blood serum and seminal plasma, respectively. It indicated the presence of 39.5% ± 2.1% and 8.2% ± 1% ASA in blood serum and seminal plasma against sperm head surface antigens, respectively (**Table 1**).

3.2. Indirect Sperm Mar Test

Binding of IgA/IgG latex particles to sperm head, tail and head + tail indicated the development of antibodies of IgA/IgG type against sperm surface antigens in the blood serum or seminal plasma (**Figure 2**). Mean values for attachment of IgG type latex particles to motile spermatozoa were 41.2% ± 2% and 2.4% ± 0.4%, ranging from 21.5% - 62.5% and 0% - 5.4% in the presence of blood serum and seminal plasma, respectively. Whereas, IgA latex particles attached to a mean of 26.4% ± 1.7% and 10.7% ± 1.5% motile spermatozoa, ranging from 7.3% - 39.8% and 0% - 29.2% in the presence of blood serum and seminal plasma, respectively (**Table 1**).

3.3. Enzyme-Linked Immunosorbent Assay

Mean ELISA titre for ASA were 2361.5 ± 258.2 and 35.9 ± 5.1 and ranged from 2000 - 6400 and 0 - 80 in blood serum and seminal plasma of tested bulls (**Table 1**). Mean 28.5 ± 1.0 per cent positivity in the range of 18.5 - 34.7 was detected in the blood serum of tested bulls, which was significantly higher ($P < 0.05$) than that of negative control. It again indicated the presence of ASA in blood serum of breeding bulls.

Percentage of bulls with higher level of ASA, detected by IPA, Sperm Mar test and ELISA in serum and seminal plasma is given in **Figure 3**.

3.4. Relationship between ASA in Blood Serum and Seminal Plasma of Bulls and Sperm Function/Fertility Tests

3.4.1. Immunoperoxidase Assay

Sperm parameters of bulls with >40 vs. <40% ASA in blood serum and >20 vs. <20% ASA in seminal plasma were compared (**Table 2**). A positive correlation was found between post-thawed motile (+0.18), viabile (+0.17), HOST (+0.18), acrosome reacted (+0.13) and cervical mucus penetrated spermatozoa in peak 0.5 cm (+0.19) and ASA in blood serum. HOS-positive and capacitated/acrosome reacted spermatozoa showed a significant

Table 1. Percent ASA (IPA), tested by IPA, SpermMar test and ELISA in serum and seminal plasma of cattle bulls.

	IPA (%)		SpermMar test				ELISA (titre)	
			IgG (%)		IgA (%)			
	Serum	SP	Serum	SP	Serum	SP	Serum	SP
Mean ± SE	39.5 ± 2.1	8.2 ± 1.0	41.2 ± 2.0	2.4 ± 0.4	26.4 ± 1.7	10.7 ± 1.5	2361.5 ± 258.2	35.9 ± 5.1
Range	14.4 - 57.8	0 - 19.3	21.5 - 62.5	0 - 5.4	7.3 - 39.8	0 - 29.2	200 - 6400	0 - 80

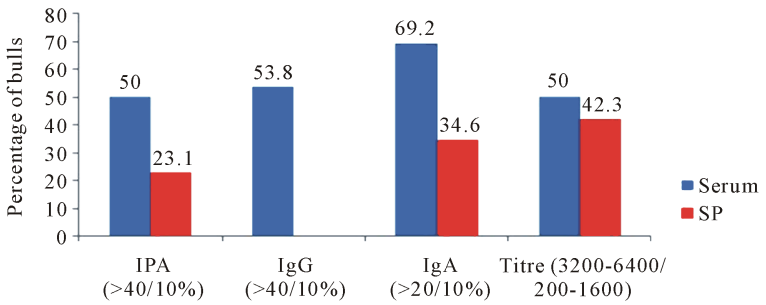

Figure 3. Occurance of ASA in total tested bulls.

difference (45.4% ± 3.5%/36.5% ± 2.5% vs. 39.6% ± 3.3%/31.1% ± 2.9%, P < 0.05) between the two groups (Table 2). Sperm count (million/ml) and percent HOS-positive spermatozoa were significantly higher (P < 0.05; 991.3 ± 79.3 vs. 879.5 ± 37.3, 43.1 ± 2.9 vs. 39.8 ± 4.0) in bulls with <20% seminal plasma ASA. Significant correlation (P < 0.05) between seminal plasma ASA and post-thaw motile, distance covered by spermatozoa in cervical mucus/30 min and number of spermatozoa penetrated in peak 0.5 cm was high (+0.62), moderate (+0.32) and weak (+0.19), respectively.

3.4.2. SpermMar Test (IgG)

There was no difference in post-thaw motile, viable, acrosome intact/cervical mucus penetrated spermatozoa in the bulls with >40% and <40% blood serum IgG. Mean HOS-positive and acrosome reacted spermatozoa were higher (47% ± 3.4% Vs 37.1% ± 2.8% and 35.5% ± 2.1% Vs 30.4% ± 2.9%) in bulls with >40% IgG than with <40% IgG (Table 3) and there was also significant correlation (P < 0.05; r = +0.22, +0.25) between the two. However, none of the tested bulls was with >10% IgG in seminal plasma.

3.4.3. Sperm Mar test (IgA)

Abnormal/*in vitro* acrosome reacted and HOS-positive/acrosome intact spermatozoa were significantly (P < 0.05)

Table 2. Relationship between ASA (IPA) in serum and seminal plasma of bulls and sperm function/fertility tests.

Percent ASA (% of bulls)	Post thaw motility (%)	Viability (%)	Abnormalities (%)	HOST (%)	Acrosome reaction (%)	CMPT		Acrosome integrity (%)	Sperm count (ml/million)
						Distance (mm)	Sperm count		
Blood serum									
>40% (50)	41.2 ± 0.8[a] (35 - 45)	65.4 ± 2.3[a] (55.2 - 84.8)	16.9 ± 1.2[a] (9.7 - 23.2)	45.4 ± 3.5[a] (31.1 - 66)	36.5 ± 2.5[a] (26.5 - 41.4)	22.9 ± 2.0[a] (12 - 28)	363.7 ± 29.7[a] (231 - 578)	88 ± 3.6[a] (61.3 - 100)	898.9 ± 46.2[a] (655 - 1178)
<40 % (50)	40.4 ± 1.1[a] (30 - 50)	66.7 ± 2.2[a] (58 - 80.2)	17.7 ± 1.2[a] (11.1 - 24.3)	39.6 ± 3.3[b] (24.6 - 58.7)	31.1 ± 2.9[b] (13.1 - 50)	23.0 ± 2.5[a] (9.0 - 37)	375.5 ± 30.3[a] (219 - 529)	87.6 ± 3.6[a] (66.2 - 98.3)	882.2 ± 48.4[a] (573 - 1301)
Seminal plasma									
>20% (23)	44.2 ± 1.6[a] (40 - 50)	71.6 ± 4.2[a] (60 - 84.8)	13.5 ± 1.3[a] (11.1 - 1.7)	39.8 ± 4.0[a] (29.5 - 53)	33.7 ± 1.9[a] (26.5 - 38.3)	26.2 ± 3.0[a] (18 - 37)	423.8 ± 40.6[a] (297 - 529)	92.8 ± 4.3[a] (71.2 - 100)	991.3 ± 79.3[a] (744 - 1301)
<20% (77)	39.7 ± 0.7[b] (30 - 45)	64.3 ± 1.5[b] (51.9 - 75.9)	18.1 ± 0.9[b] (9.7 - 24.3)	43.1 ± 2.9[b] (24.6 - 66)	32.9 ± 2.2[a] (13.1 - 55)	21.7 ± 1.6[b] (9.0 - 35)	371.2 ± 24.9[b] (219 - 578)	87 ± 2.7[a] (61.3 - 97.3)	879.5 ± 37.3[b] (573 - 1178)

[a]Figures in parentheses represent range; [b]Values with different superscripts are significant (P < 0.5).

Table 3. Relationship between ASA (IgG type) in blood serum and seminal plasma of bulls and sperm function/fertility tests.

Percent ASA (% of bulls)	Post thaw motility (%)	Viability (%)	Abnormalities (%)	HOST (%)	Acrosome reaction (%)	CMPT		Acrosome integrity (%)	Sperm count (million/ml)
						Distance (mm)	Sperm count		
Blood seum									
>40% (53.7)	40 ± 1.0[a] (30 - 45)	66.1 ± 2.4[a] (51.9 - 84.8)	16.2 ± 1.1[a] (9.7 - 23.2)	47 ± 3.4[a] (28.7 - 58.7)	35.5 ± 2.1[a] (26.5 - 43.1)	23.1 ± 1.7[a] (1.2 - 3.3)	385.3 ± 29.5[a] (231 - 578)	88.6 ± 3.5[a] (61.3 - 100)	900.8 ± 46.9[a] (573 - 1178)
<40% (46.3)	41.6 ± 0.9[a] (40 - 50)	65.9 ± 2.3[a] (57.3 - 80.2)	18.2 ± 1.2[a] (11.3 - 23.2)	37.1 ± 2.8[b] (24.6 - 58.6)	30.4 ± 2.9[a] (13.1 - 50)	22.3 ± 2.6[a] (9.0 - 37)	381.2 ± 32.3[a] (219 - 529)	88 ± 3.4[a] (66.2 - 98.3)	910.5 ± 50.5[a] (655 - 1301)
Seminal plasma									
>10% (0)									
<10% (100)	40.7 ± 0.5[a] (30 - 50)	66 ± 1.7[a] (51.9 - 84.8)	17 ± 0.8[a] (9.7 - 24.3)	42.4 ± 2.4[a] (24.6 - 66)	33.1 ± 1.7[a] (13.1 - 54.9)	23.1 ± 0.2[a] (9.0 - 37)	383.3 ± 21.3[a] (219 - 578)	87.1 ± 2.6[a] (61.3 - 100)	870.6 ± 46.6[a] (573 - 1301)

[a]Figures in parentheses represent range; [b]Values with different superscripts are significant (P < 0.05).

and non-significantly (P > 0.05) higher in bulls with >20% than <20% serum IgA, respectively (**Table 4**). HOS-positive, *in vitro* acrosome reacted and acrosome intact spermatozoa were non-significantly (P > 0.05) higher in bulls with <10% than with >10% seminal plasma IgA. Whereas, number of spermatozoa in peak 0.5 cm in cervical mucus was significantly (P < 0.05) higher in bulls with <10% seminal plasma IgA than >10% and there was also a significant correlation (r = +0.36) between seminal plasma IgA and CMPT. Sperm count (million/ml) in ejaculated semen was also significantly (P < 0.05) different among the two groups.

3.4.4. Enzyme-Linked Immunosorbent Assay

There was not much difference in motile, viable, abnormal morphology, HOS-positive, acrosome intact, *in vitro* arosome reacted and cervical mucus penetrated spermatozoa between the bulls with 3200 - 6400 and <3200 serum antibody titre (**Table 5**). But a very weak correlation (+0.004 to +0.17) was found between serum ELISA titre and abnormal, viable, motile, HOS-positive, *in vitro* arosome reacted and cervical mucus penetrated spermatozoa. Acrosome intact, sperm count (million/ml) and numbers of spermatozoa penetrated in peak 0.5 cm of cervical mucus were higher in bulls with seminal plasma antibody titre of 0 - 20 than that of 40 - 80, but difference was significant only in later two parameters. Seminal plasma antibody titre and motile/cervical mucus penetrated spermatozoa also showed a moderate positive correlation (+0.49 and +0.28/+0.37).

Table 4. Relationship between ASA (IgA type) in blood serum and seminal plasma of bulls and sperm function/fertility tests.

Percent ASA (% of bulls)	Post thaw motility (%)	Viability (%)	Abnormalities (%)	HOST (%)	Acrosome reaction (%)	CMPT		Acrosome integrity (%)	Sperm count (million/ml)
						Distance (mm)	Sperm count		
Blood serum									
>20% (69.2)	40.5 ± 0.9[a] (30 - 45)	66.9 ± 2.2[a] (51.9 - 84.8)	15.7 ± 0.9[a] (9.7 - 23.2)	45 ± 2.9[a] (28.7 - 54.2)	34.8 ± 1.7[a] (26.2 - 54.9)	24 ± 1.7[a] (9.0 - 37)	383.7 ± 24.9[a] (231 - 578)	90.1 ± 3.0[a] (61.3 - 100)	908.9 ± 39.6[a] (573 - 1178)
<20% (30.8)	41.2 ± 1.2[a] (40 - 50)	64.1 ± 2.0[a] (58 - 74.4)	20.1 ± 1.3[b] (14.5 - 23.2)	36.6 ± 3.8[a] (24.6 - 58.6)	20.1 ± 1.3[b] (13.15 - 50)	22.1 ± 3.0[a] (11 - 35)	382.5 ± 43.3[a] (219 - 528)	86.7 ± 4.1[a] (66.2 - 96.6)	897.1 ± 72.4[a] (655 - 1301)
Seminal plasma									
>10% (34.6)	42.8 ± 1.2[a] (40 - 50)	68.9 ± 3.5[a] (56.6 - 84.8)	14.6 ± 1.1[a] (11.1 - 20.6)	37.9 ± 2.9[a] (29.5 - 53)	31.6 ± 1.7[a] (23.3 - 38.3)	24 ± 3.2[a] (9.0 - 37)	427 ± 33.6[a] (297 - 578)	86.9 ± 4.6[a] (66.2 - 100)	971.5 ± 60.5[a] (744 - 1301)
<10% (65.4)	39.8 ± 0.7[a] (30 - 45)	64.6 ± 1.5[a] (51.9 - 71.8)	18.3 ± 1.0[a] (9.7 - 24.3)	44.8 ± 3.4[a] (24.6 - 66)	34 ± 2.5[a] (13.15 - 54.9)	22.1 ± 1.5[a] (11 - 31)	460 ± 26.4[b] (219 - 528)	89.2 ± 2.8[a] (61.3 - 7.2)	870.2 ± 39.5[b] (573 - 1178)

[a]Figures in parentheses represent range; [b]Values with different superscripts are significant (P < 0.05).

Table 5. Relationship between ASA (ELISA) in blood serum and seminal plasma of bulls and sperm function/fertility tests.

Titre (% of bulls)	Post thaw motility (%)	Viability (%)	Abnormalities (%)	HOST (%)	Acrosome reaction (%)	CMPT		Acrosome integrity (%)	Sperm count (million/ml)
						Distance (mm)	Sperm count		
Blood serum									
200 - 1600 (50)	40.8 ± 1.3[a] (35 - 45)	67.1 ± 2.2[a] (51.9 - 84.8)	17.7 ± 1.3[a] (9.7 - 23.2)	40.5 ± 3.6[a] (28.7 - 66)	32.9 ± 2.5[a] (13.1 - 54.9)	2.3 ± 2.3[a] (9.0 - 33)	380 ± 31.9[a] (231 - 578)	86.9 ± 3.5[a] (61.3 - 100)	917 ± 48.9[a] (655 - 1301)
3200 - 6400 (50)	40.7 ± 0.7[a] (30 - 50)	65.1 ± 24[a] (57.3 - 80.2)	16.6 ± 1.1[a] (11.1 - 24.3)	44.1 ± 3.4[a] (24.6 - 58.7)	33.2 ± 2.5[a] (16.1 - 50)	2.2 ± 2.0[a] (11 - 37)	385 ± 29.7[a] (219 - 529)	89.8 ± 3.0[a] (66.3 - 98.3)	894.5 ± 47.8[a] (573 - 1178)
Seminal plasma									
0 - 20 (57.7)	41.8 ± 1.0[a] (40 - 50)	66.8 ± 2.77[a] (56.6 - 84.8)	15.7 ± 1.3[a] (11.5 - 24.3)	39.7 ± 3.7[a] (28.7 - 66)	32.6 ± 1.8[a] (23.3 - 43.1)	22.9 ± 0.8[a] (9.0 - 37)	447 ± 27.4[a] (297 - 578)	84.8 ± 4.3[a] (61.3 - 100)	863.1 ± 42.1[a] (573 - 1178)

[a]Figures in parentheses represent range; [b]Values with different superscripts are significant (P < 0.05).

4. Discussion

4.1. Anti Sperm Antibodies

SpermMar test indicated the presence of very high percentage of IgG in blood serum (41.2% ± 2%) as compared with seminal plasma (2.4% ± 0.4%), but IgA (10.7% ± 1.5%) class ASA was in higher percentage than IgG (2.4% ± 0.4%) in seminal plasma. Higher percentage of IgA in seminal plasma may be due to the reason that these are secreted by the accessory sex glands [14]. IgA class antibodies are present in seminal plasma and also attach to the sperm surface, but are usually absent in serum. Milovanovic *et al.* [15] confirmed the hypothesis that immune mechanisms might be involved in reproductive disturbances due to high levels of ASA of IgA class. ASA of the IgA class, which mainly has agglutinating properties [16], rarely occurs without antibodies of the IgG class. Therefore, both classes are important for male infertility.

IPA, SpermMar test and ELISA confirmed the occurrence of ASA in blood serum and seminal plasma of 26 and 21 tested bulls, respectively. It has been suggested that molecular mimicry between bacteria and the sperm can be a major factor inducing antisperm immunological reactions [17]. In SpermMar test, 40% reaction between motile spermatozoa and coated latex particles of IgG class is considered as the lower limit of significant activity. In general, the proportion of motile spermatozoa reacting in the SpermMar-IgA test is smaller than that reacting in the SpermMar-IgG test, but the contrary may occasionally occur [18]. In rare cases, there is a positive reaction in the SpermMar-IgA test in the absence of any reaction in the SpermMar-IgG test, indicating the presence of antibodies of the IgA class without antibodies of the IgG class. Therefore, bulls with >40% IPA/IgG, >20% IgA, 3200 - 6400 titre and > 0% IPA/IgG/IgA, 40 - 80 ELISA titre in blood serum and seminal plasma, respectively were considered for significant presence of ASA. In SpermMar test, about 54% bulls were with >40% IgG in blood serum against sperm surface antigens, but none of the bulls were with >10% IgG in seminal plasma. Higher percentage of IgA against sperm surface antigens was detected in the blood serum (>20%) and seminal plasma (>10%) of 65.8% and 37% bulls, respectively. In IPA, about 50% of the bulls also had >40% ASA against head surface antigens, whereas, there were only 23% bulls with >10% ASA in seminal plasma. ELISA indicated a higher antibody titre in blood serum (3200 - 6400) and seminal plasma (40 - 80) of 50% and 42% bulls, respectively. Out of 26 tested bulls, seminal plasma of 21 bulls reacted with spermatozoa both in IPA and IgA latex particles and that of 12 bulls reacted only with IgG. This indicated the presence of IgA class antibodies without IgG in seminal plasma of 57.1% tested bulls. Occurrence of mixed agglutination reaction of 40% or more in semen indicates a positive reaction to the SpermMar-IgA test. Although mixed reaction of IgG and IgA was not >40% in seminal plasma of all tested bulls, IgG class antibody was >40% in serum of 54% of tested bulls. Therefore, a combination of tests revealed higher percentage of ASA in blood serum of about 50% of the tested bulls. Higher level of ASA in serum than seminal plasma indicated that ASA in blood serum of bulls was of circulatory type. IgG/IgA class antibodies in the blood can cross testis/epididymis and may affect spermatogenesis and sperm maturation; therefore, elevated level of ASA in blood serum of 50% tested bulls may have effects on the process of sperm maturation in these bulls.

4.2. Relationship between ASA in Blood Serum and Seminal Plasma of Bulls and Sperm Function/Fertility Tests

Higher mean values of motile, viable, intact acrosomes, capacitated/acrosome reacted spermatozoa in bulls with >20% serum-ASA (IPA) as compared to <20% ASA observed during the present study may be due to individual variation. Romano *et al.* [19] also found that the proportion of acrosome reacted spermatozoa was higher in ASA-coated spermatozoa. The immunological defense is activated and production of ASA initiated, when there is both acute and chronic infection and/or inflammation, especially of the epididymis [20] [21]. First, IgM antibodies will be produced, but these are not secreted into the genital tract because their size is too large to pass the epithelial barrier. Shortly afterwards, antibodies of the IgG class appear, and these can enter the genital tract. The ASA of the IgG class come into contact and attach with the spermatozoa [22]. During the present study, inspite of higher percentage of (>40%) IgG in serum of 53.7% bulls, sperm parametes were not affected. It is possible that IgG against spermatozoa were present in serum of these bulls, but did not enter the genital tract.

Sperm parameters, *i.e.* HOST, acrosome integrity, *in vitro* acrosome reaction and CMPT were higher in bulls with <10% IgA as compared to those with >10% IgA in seminal plasma. In some cases and more commonly indeed during infection, secretory IgA-ASA are produced locally in the genital tract, probably the epididymis [23].

Antibodies of the IgA class can then be detected on the ejaculated spermatozoa, but not in serum, and this is associated with an additional reduction of their fertilizing capacity [14]. In the present study, IgA class antibodies of significance (>10%) were produced in genital tract of only 57.1% bulls, which are attached to the spermatozoa and had effect on penetration of spermatozoa through cervical mucus and *in vitro* acrosome reacton. Milovanovic *et al*. [15] also gave the hypothesis that immune mechanism may be involved in reproductive disturbances due to high level of ASA of IgA class. Recently, a study done by Jarora *et al*. [24] indicated that higher percentage of IgG-ASA and IgA-ASA in cervical mucus of cross bred cows reduced *in vitro* penetration of spermatozoa through cervical mucus in 44% of the tested animals.

It can be concluded that higher level of ASA, especially IgA class antibodies in seminal plasma reduced post-thaw motility, *in vitro* capacitation/acrosome reaction and cervical mucus penetration of spermatozoa. Presence of ASA can inhibit passage of spermatozoa through cervical mucus, prevent membrane fluidity changes needed for capacitation, reduce the ability of spermatozoa to undergo the acrosome reaction, and interfere with binding to the zona pellucida and fertilization [25]. Experimentally induced ASA were shown to affect the ability of bull spermatozoa to fertilize oocytes *in vitro* [4], while naturally occurring ASA were associated with reduced spermatozoal motility and infertility in two bulls [26]. Various parts of the sperm are surrounded by a common plasma membrane. ASA have been shown to react with plasma membrane with variable biological effects [27].

There were only 5 bulls among those with >40% blood serum ASA (IPA/IgG) and 3200 - 6400 antibody titre, who also had low values for HOST, *in vitro* acrosome reaction and CMPT. Among the bulls with >10% seminal plasma IPA/IgA and 40 - 80 antibody titre, only 3 bulls had low values for HOS-positive, *in vitro* acrosome reacted and cervical mucus penetrated spermatozoa. There were also one and two bulls with higher level of seminal plasma ASA and low values of only CMPT/HOST and *in vitro* acrosome reaction, respectively. Therefore, there were 11 bulls with low values of HOST/*in vitro* acrosome reaction/CMPT and higher significant level of either serum—or seminal plasma—ASA. Hence, it can be interpreted that significanct level of serum/seminal plasma ASA may have effect on the fertility of bulls by affecting any of the sperm function, *i.e.* membrane integrity/capacitation/acrosome reaction/CMPT. Moreover, in the absence of any standard and universally accepted assay for the detection of ASA, hence, as per our findings, it is suggested that combination of tests gives higher accuracy in comparison to any particular single test.

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