EFFECT OF AGE AND BREED ON ANTISPERM ANTIBODIES (ASA) IN BLOOD SERUM AND SEMINAL PLASMA OF BULLS: IMMUNO-REACTIVITY OF SPERM SURFACE PROTEINS WITH ASA

V.ZODINSANGA, RANJNA.S, CHEEMA AND P.S.MAVI*

Department of Veterinary Gynaecology and Obstetrics, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana-141004, Punjab, India

**Department of Teaching Veterinary Clinical Complex*

Received : 27.01.15 Accepted : 10.07.15

ABSTRACT

Occurrence of ASA in blood serum / seminal plasma of bulls with respect to age / breed and immunoreactivity of sperm surface proteins with serum of bulls were determined during the present study. There was an increase of 15.3% IPA, 12.4% IgG and 9.5% IgA in serum of tested bulls with increase in age. Higher significant level of IgG antibodies and ELISA titre was detected in the serum of bulls in the age group of > 6 years, whereas, significant level of IPA / IgA (> 10%) and higher ELISA titre in seminal plasma was detected in higher percentage of bulls in the age group of 3-6 years. Occurrence of ASA (IPA / IgG / IgA) in serum was higher in cross-bred bulls as compared to pure-bred bulls and reverse was the case in seminal plasma. Serum of all bulls showed a reaction with sperm extracted proteins on immunoblots and a total of 19 antigens (16 ¬ 130 kDa) were detected, but only 4-10 antigens were recognized by serum in Sperm Extracts (SE) of respective bulls. Immunofluorescence of sperm smears with serum of bulls predominantly stained post-acrosomal region, thus indicated the presence of ASA mainly against surface proteins of this region. A similarity in molecular weights of sperm proteins involved in various aspects of fertilization and that detected for immunoseropositivity with serum of presently tested bulls was found, therefore, it can be predicted that such ASA may affect the fertility of bulls. In future, the functional effects of these antibodies on bull«s fertility can be explored.

Key words: ASA, Cattle bulls, Age, Breed, Immunorectivity, Sperm surface proteins.

INTRODUCTION

A wide range of antigenic structures is expressed in the testis during spermatogenesis and protection against autoimmunity is provided by the hemotesticular barrier composed predominantly of sertoli cells, which isolates the tubular contents from the vasculature, and limited lymphatic drainage of the testis. Several other immunoregulatory mechanisms e.g. immunosuppressive factors of seminal plasma and both systemic nonspecific and specific factors (immunoregulation cells, cytokines etc.) also prevent formation of antisperm antibodies (ASA). Generally, ASA formation can be induced primarily during infectious and noninfectious inflammations, or by obstruction of testicular efferent duct. The incidence of ASA was also induced by an accident (Zhang *et al.*, 1990), very low temperature (Fayemi *et al.*, 1992), cryptorchism (Pinart *et al.*, 1999), vasectomy (Jessop and Ladds 1995), and by excessive male exploitation (Wicher *et al.*, 1987). Recently, Thaper *et al.* (2014) suggested that cross-reactivity between certain epitopes on the bacterial surface and spermatozoa, particularly involving carbohydrate determinants might be one potential triggering mechanism for induction of antisperm antibodies in males and females. The prevalence of ASA was reported in sera of boars and sows (Fayemi *et al.*, 1992), bulls and boars (Zraly *et al.*, 2002). Proteomics represents an extraordinary tool for evaluating the molecular mechanisms

regulating sperm function for understanding the male fertility / infertility. Several antibody-binding proteins have been characterized in isolated sperm surface membranes and several proteins have been described whose ASA could be associated with agglutination (Koide *et al.,* 2000), motility (Munuce *et al.,* 2000), cervical mucus penetration (Bronson 1999), acrosome reaction (Bohring *et al.,* 2001), zona pellucida binding, penetration and oolemma binding (Focarelli *et al.,* 1998). This study was performed with an objective to perceive the effect of age and breed on occurrence of ASA in blood serum and seminal plasma of cow bulls. Immunoreactivity of sperm surface proteins with blood serum of bulls was also detected.

MATERIAL AND METHODS

Frozen semen of 14 cross-bred (Holstein Friesian X Red Dane X Sahiwal) and 12 pure-bred (Holstein Friesian) bulls was procured from Semen Freezing Laboratory, GADVASU, Ludhiana, Semen Bank Bhattian, Khanna and Government Semen Bank, Ropar, Punjab, India. Freshly ejaculated semen and blood of these bulls were also collected to harvest seminal plasma and serum, respectively from the respective farms.

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

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±l 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 x 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.

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 concentration to 20 X 10^6. Incubated 100 ±l of the sperm suspension of motile spermatozoa with 100 ±l of inactivated 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 ±l of TALP. On a slide, mixed 10 ±l of sperm suspension and 5 ±l of sperm Mart latex particles IgG or IgA, mixed, 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. About 200 sperms in different fields were counted and percentage was calculated.

Sperm extracts (SDS-SE) were prepared by suspending washed spermatozoa in 62.5 mM Tris-HCl, pH 6.8 containing 2% SDS, 1mM PMSF, 25 mM benzidine, 10mM aprotinin, 10mM pepstatin and 5mM 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 ±g 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 ±l 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 ±l / well of HRP conjugated anti bovine IgG for three hours at 37≥C. Washed the plate twice with PBS and incubated with 100 ±l 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.

SDS-SE of 15 cattle bulls were reacted with serum of respective bulls on immunoblots. Proteins separated by SDS-PAGE under reducing conditions were transferred to nitrocellulose membrane using wet electrophoresis transfer apparatus at 100 V for 2.30 hrs. Transfer quality was checked by 0.2 % ponceau dye and proteins were blocked in 2% BSA as blocking solution for overnight at 4°C. After washing the membrane with PBS+0.05% Tween-20, it was incubated in 1:200 diluted serum for 2.5 hrs. Again washed thrice with PBS+0.05% Tween-20 and incubated with 1:10000 diluted HRP conjugated anti bovine IgG as secondary antibody for 45 min. Washed thrice with PBS + Tween-20 and incubated with substrate (0.05% Diaminobenzidine + 0.06% Hydrogen Peroxide) for 10 min. Gel images were captured on Syngene gel doc using GeneSnap image acquisition software and analyzed by using GeneTools gel analysis software (Syngene).

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).

RESULTS AND DISCUSSION

Pure and cross-bred bulls were grouped as < 3 years (G-I), 3-6 years (G-II) and > 6 years (G-III). ASA were detected in all the bulls irrespective of age. Therefore, bulls with > 40% IPA / IgG, > 20% IgA, 3200-6400 titre and > 10% IPA / IgG / IgA, 40-80 ELISA titre in serum and seminal plasma, respectively were considered for significant presence of ASA. Zraly *et al.* (2002) observed a first incidence of antibodies at the age of 5 to 6 months (13.8%), with a significant increase (58.1%; $p<0.01$) at 9 to 10 months of age. ASA, detected by IPA, SpermMar test in serum of bulls with respect to age are presented in. There was an increase of 15.3% ASA, 12.4% IgG and 9.5% IgA and ELISA titre in serum of tested bulls with increase in age. Fayemi *et al.* (1992) also recorded a continuous increase of antibodies in boars. Increase in percentage of ASA (IPA) was significant ($P < 0.05$) in G-II and G-III as compared to G-1, whereas, increase in IgG / IgA / ELISA titre was significant in G-III as compared to G-I and G-II. Percentage of bulls with > 40% IPA and > 20% IgA increased with an increase in age. But > 40% IgG and higher ELISA titre were in serum of higher percentage of bulls in G-II as compared to G-I / G-III. It indicated higher significant level of IgG antibodies and ELISA titre in the age group of > 6 years. Jarora *et al.* (2014) were also of the view that higher percentage of IgG-ASA and IgA-ASA reduced *in vitro* penetration of spermatozoa through cervical mucus in 44% of the tested cross-bred cows

In seminal plasma, per cent ASA, IgG, IgA and ELISA titre were non - significantly ($P > 0.05$) higher in G-II as compared to G-I and G-III. Significant level of IPA / IgA (> 10%) and higher ELISA titre in seminal plasma was also detected in higher percentage of bulls in the age group of 3-6 years. Comparison of ASA incidence in mature breeding bulls with regard to their sexual activity revealed significantly higher incidence ($p<0.01$) in active semen donors than in the candidate breeders (Zraly *et al.*, 2002).

Mean values for total ASA, IgG and IgA were higher in the blood serum of cross-bred as compared to pure-bred bulls (Table 1). However, mean ELISA titre remained similar in the two breeds (Table 1). Reverse was the case with seminal plasma. Differences were significant ($P < 0.05$) only for ASA (IPA) in serum and ELISA titre in seminal plasma (Table 1). It indicated the presence of more antibodies against sperm surface antigens in blood serum of cross-bred as compared to pure-bred bulls. Occurrence of ASA (IPA / IgG / IgA) in blood serum was higher in cross-bred (64.3-78.6%) as compared to pure-bred bulls (40-50%). Percentage of bulls with higher ELISA titre was same in two breeds. In seminal plasma, occurance of ASA (IPA / IgG / IgA / ELISA) was higher in pure-bred (0-60%) as compared to cross-bred bulls (0-21.4%). Zraly *et al.* (2002) also

compared ASA in two breeds of bulls and detected higher frequency of antibodies in bulls of the Black Pied Holstein cattle and their cross-breeds compared with the Czech Red Pied cattle and their cross-breeds (48.4% Vs 35.2%; $p< 0.01$).

Serum of all bulls showed a reaction with sperm extracted proteins on immunoblots and a total of 19 antigens 130, 80, 70, 65, 60, 55, 50, 48, 45, 40, 37, 35, 33, 30, 28, 24, 20, 18 and 16 kDa were detected (Fig. 1-2). A study, done by Tripathi *et al.* (1999) revealed the reaction of only two sperm polypeptides out of 16 with serum of infertile cows. In other species, ASA have recognized antigens of 40 / 44 kDa, 62 kDa and 50, 55, 57, 62, 72 kDa in mice, horses [Teuscher *et al.*, 1994] and infertile humans [Paradisi *et al.*, 1996], respectively. Although, 19 antigens were not detected in SE of all tested bulls, but 4-10 antigens were recognized by serum in SE of respective bulls. Antigens of 24, 35, 30 / 33 / 16, 55 / 45, 48, 65, 18, 28 / 70, 80 / 130 kDa were detected in the SE of 12, 10, 9, 8, 7, 6, 5, 4, 3 and 2 bulls, respectively. Proteins with molecular weight of 60 / 50, 40 and 37 kDA were detected only in SE of bull no. 1, 9 and 14, respectively. Post-immunization IgG1 and IgG2 recognized a 45-kDa sperm antigen and incubation of spermatozoa with post-immunization serum reduced *in vitro* fertilization rates [$p < 0.01$, Kim *et al*, 1999]. Surface antigens of 35, 40, 47 and 65 kDa also reacted with circulating ASA in blood serum of infertile patients [Feng *et al.*, 2008]. Proteins of 55, 31, 26, 24 kDa were characterized in seminal plasma and spermatozoa of bulls and related to their fertility (McCauley *et al.*, 2001). FA-1 antigen composing of a monomer of 23 kDa and / or a dimer of 47-50 kDa was shown to completely block binding and penetration of zona-free hamster ova by human sperm / significantly inhibit sperm penetration in IVF trials in mice [Naz *et al.*, 1986], Cheema *et al.* [2015] were also of the view that 60, 45 and 16 kDA-FA-1like proteins may possibly serve as indicators of higher rate of *in vitro* acrosome reaction vis a vis fertility of cattle bulls. Since a similarity in molecular weights of sperm proteins involved in various aspects of fertilization and that detected for immunoseropositivity with serum of presently tested bulls was found, therefore, it can be predicted that such ASA may affect the fertility of bulls. In future, the functional effects of these antibodies on bull«s fertility can be explored.

Sperm smears of two cattle bulls were reacted with their respective serum to detect immunolocalization of antigens, responsible for generation of ASA (Fig.3). It indicated localization of sperm antigens mainly on the post-acrosomal region. A mild signal was also obtained on acrosome surface. There was also a weak signal on the tail in some sperms. Intensity of signal on post-acrosomal cap, acrosome surface varied from sperm to sperm. Therefore, it indicated that ASA, generated in serum of bulls were mainly against proteins of post-acrosomal cap and acrosome surface.

Table 1: Presence of ASA (evaluated by IPA, SpermMar test and ELISA, Mean ± SE) in serum and seminal plasma of bulls of different age and breeds.

Serum/ Seminal Plasma	Age (Years)			Breed	
	<3 (8)*	3-6 (13)*	>6 (5)*	Cross-bred (13)*	Pure HF (14)*
Serum					
IPA (%)	34.2 Ø 3.5[a] (14.4-43.8)	38.9 Ø 3.2[b] (22.4-57.8)	49.5 Ø 0.6[c] (47.5-51.1)	41.5 Ø 2.2[a] (22.4-51.2)	36.4 Ø 4.4[b] (14.4-57.8)
IgG (%)	37.3 Ø 3.4[a] (21.5-47.2)	40.2 Ø 3.0[a] (24.8-62.5)	49.7 Ø 1.8[b] (44.8-53.7)	42.7 Ø 2.2[a] (24.8-54.2)	38.9 Ø 4.3[a] (21.5-62.5)
IgA (%)	37.3 Ø 3.4[a] (21.5-47.2)	40.2 Ø 3.0[a] (24.8-62.5)	37.3 Ø 3.4[a] (21.5-47.2)	28.2 Ø 2.2[a] (12.1-38.4)	23.8 Ø 3.0[a] (7.3-39.8)
ELISA titre	2125 Ø 435.7[a] (200-3200)	2308 Ø 430[a] (400-6400)	3200 Ø 0[b] (3200)	2428 Ø 260[a] (400-3200)	2420 Ø 575[a] (200-6400)
Seminal Plasma					
IPA (%)	4.8 Ø 2.0[a] (0-15.9)	7.6 Ø1.6[a] (0-19.3)	7.2 Ø 1.0[a] (5.2-9.8)	5.0 Ø 1.5[a] (0-19.3)	7.8 Ø 1.4[a] (3.7-16)
IgG (%)	1.2 Ø 0.5[a] (0-3.4)	1.3 Ø 0.5[a] (0-5.4)	0.4 Ø 0.2[a] (0-1.3)	0.5 Ø 0.3[a] (0-5.4)	1.4 Ø 0.4[a] (0-3.4)
IgA (%)	6.7 Ø 2.7[a] (0-21.8)	10.4 Ø 2.4[a] (0-29.2)	6.9 Ø 0.6[a] (5.6-9.3)	5.3 Ø 1.4[a] (0-17.8)	19.3 Ø 5.8[a] (0-80)
ELISA titre	23.7 Ø 9.9[a] (0-80)	35.3 Ø 7.9[a] (0-80)	29.8 Ø 4.4[a] (20-40)	11.6 Ø 2.7[a] (0-29)	40 Ø 7.4[b] (20-80)

✧ Figures in () represent range and ()* number of bulls

✧ Values with different superscripts are significant ($p<0.05$)

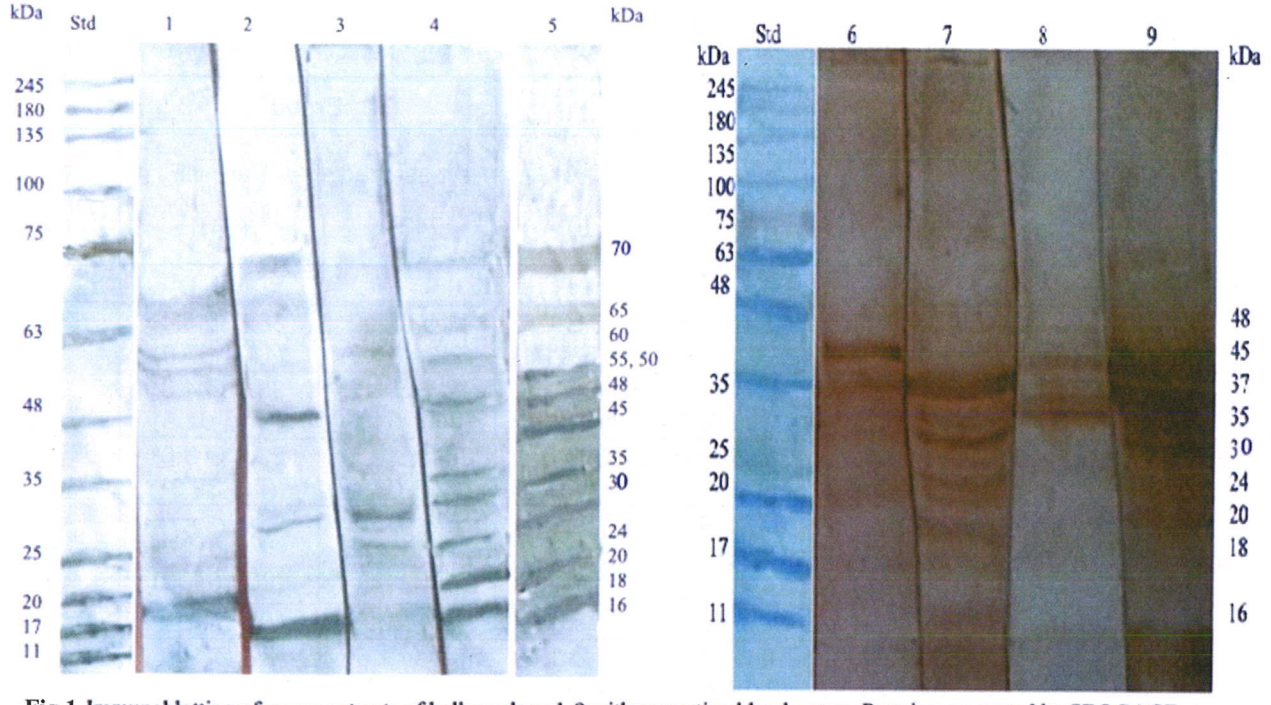

Fig.1. Immunoblotting of sperm extracts of bull numbers 1-9 with respective blood serum. Proteins, separated by SDS-PAGE were transferred to nitrocellulose membrane and reacted with blood serum.

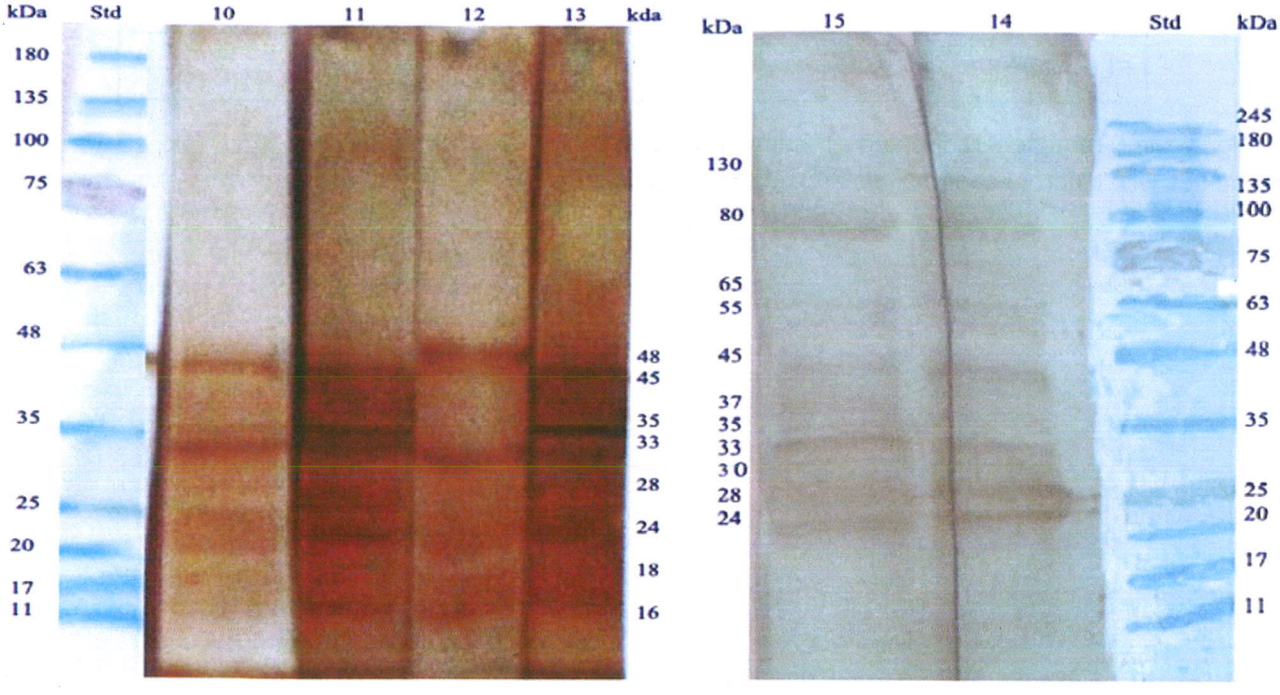

Fig.2. Immunoblotting of sperm extracts of bull numbers 10- 15 with respective blood serum. Proteins, sepa rated by SDS-PAGE were transferred to nitrocellulose membrane and reacted with blood serum.

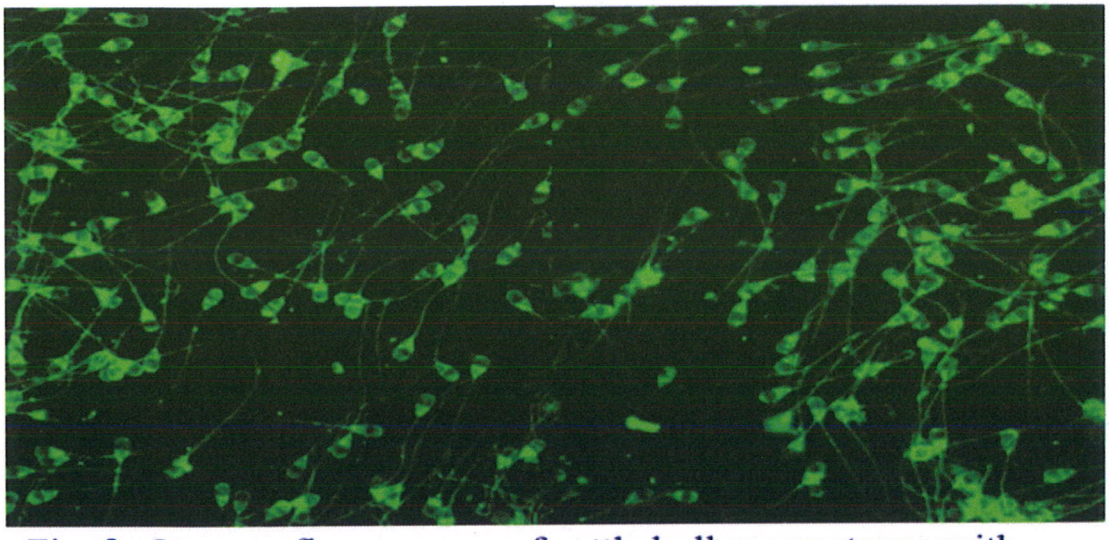

Fig. 3. Immunofluorescence of cattle bull spermatozoa with blood serum.

REFERENCES

Bohring, C., Krause W., Habermann B. and Krause E. (2001). Isolation and identification of sperm membrane antigens recognized by antisperm antibodies, and their possible role in immunological infertility disease. *Mol Hum Reprod.,* **7**: 113-118.

Bronson, R. (1999). Antisperm antibodies: A critical evaluation and clinical guidelines. *J Reprod Immunol.,* **45**: 159-183.

Cheema Ranjna, S., Bansal, A.k. and Zodinsanga, V. (2015) Characterization and immunolocalization of HBP, FA-1 and TIMP-2 like proteins in cattle bull semen: HBP modification in in vitro capacitated spermatozoa. *Theriogenol Insight.*, **5**, 53-67.

Crowther, J. R. (1995). Methods in molecular biology. *ELISA, Theory and Practice, Volume 43 Humana Press New Jersey, Product Code,* **236**: 415-420.

Fayemi, O. (2005). Sperm antibodies and reproductive efficiency in the Zebu cattle in South Western Nigeria. *Pak Vet J.*, **25**: 111-114.

Fayemi, O.E., Morrison, R.B. and Joo, H.S. (1992) Seroprevalence of sperm antibody in selected Minnesota Swine Breeding Herds. *Ani Reprod Sci.,* **27**: 341-345.

Feng, H.L., Han, Y.B., Sparks, A.E.T. and Sandlow, J. I. (2008). Characterization of human sperm antigens reacting with antisperm antibodies from an infertile female patient«s serum. *J Androl.,* **29**: 440-448.

FertiPro, N.V. (2011). A qualitative latex test for detection of sperm antibodies. *Bijsluiter SpermMar Indd.,* **12**: 31-32.

Focarelli, R., Giuffrida, A., Capparelli, S., Scibona, M., Menchini. F.F, Francavilla, F., Fancavilla, S., Giovampaola, C.D. and Rosati, F. (1998). Specific localization in the equatorial region of gp20, a 20 kDa sialyl-glycoprotein of the capacitated human spermatozoon acquired during epididymal transit which is necessary to penetrate zona-free hamster eggs. *Mol Hum Reprod.,* **4**: 119-125.

Jessop, T.S. and Ladds, P.W. (1995). The immunopathology of unilateral vasectomy in the ram. *Vet Immunol Immunopathol.,* **47**: 123.

Koide, S.S., Wang, L. and Kamada, M. (2000). Antisperm antibodies associated with infertility: Properties and encoding genes of target antigens. *Proc Soc Exp Biol Med.,* **224**: 123-132.

Munuce, M.J., Berta, C.L., Pauluzzi, F. and Caille, A.M. (2000). Relationship between antisperm antibodies, sperm movement, and semen quality. *Urol Int.,* **65**: 200-203.

Naz, R.K., Phillips, T.M. and Rosenblum, B.B. (1986). Characterization of the fertilization antigen- 1 for the development of a contraceptive vaccine. *Proc Natl Acad Sci USA.*, **83**, 573-577.

Paradisi, R., Bellavia, E., Pession, A., Venturoli, S. and Flamigni, C. (1996). Characterization of human sperm antigens reacting with sperm antibodies from autologous serum and seminal plasma in an infertile population. *Biol Reprod.,* **55**: 54-61.

Pinart, E., Sancho, S., Briz, M.D, Bonet, S., Garcia, N. and Badia, E. (2000). Ultrastructural study of the boar seminiferous epithelium: changes in cryptorchidism. *J Morphol.,* **244**: 190-202.

Teuscher, C., Kenney, R.M., Cummings, M.R., Catten, M. (1994) Identification of stallion sperm-specific proteins and their autoantibody response. *Equine Vet J.*, **26**:148¬151.

Thaper, D., Vander, H. and Prabha, V. (2014) Association of antisperm antibodies with bacterial infection: An Insight to Infertility. Androl Gynecol: *Curr Res.*, 2:1-6.

Towbin, H., Staehelint, T. and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc Nat Acad Sci USA.,* **76**: 4350-4354.

Tripathi, D., Sharma, N.C., Singh, S.K. and Gupta, L.K. (1999). Identification of bovine sperm specific polypeptides reactive with antisperm antibodies. *Ind J Exp Biol.,* **37**: 655-661.

Wicher, V., Wicher, K. and Gruhn, R.F. (1987). Increased production of antibodies to spermatozoa and seminal fluid in rabbits used as semen donors. *Int Arch Allergy Appl Immunol* .,**83**: 104-108.

Zhang, J., Ricketts, S.W., Tanner, S.J. (1990). Antisperm antibodies in the semen of a stallion following testicular trauma. *Equine Vet J.,* **22**: 138-141.

Zraly, Z., Bendova, J., Sisak, M., Diblikova, I., Svecova, D., Zajicova, A. and Veznik, Z. (2002). Occurrence of antibodies to the sperms in blood sera of bulls and boars. *Vet Med Czechslovakia.,* **43**: 137-144.