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Molecular Characterization of Sperm Antigens with *In Vivo* Developed Antisperm Antibodies in Variably Inseminated Cross Bred-Heifers/Cows

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Abstract Molecular characterization of sperm proteins and their immunoreactions to sera from subfertile/infertile animals may give deeper insight to the immunological reason of infertility. The purpose of this study was to characterize antigenic sperm proteins by reacting with blood serum and cervical mucus of variably inseminated heifers/cows using immunoblotting and immunofluorescence techniques. Immunoblot analysis of sperm proteins with blood serum/CM of variably inseminated cows/heifers revealed around 23 polypeptides in cattle sperm, but all proteins did not react with blood serum and cervical mucus of all tested animals. Number of sperm polypeptides that reacted with blood serum of non-inseminated, 1-3, 4-6 and >6 times inseminated heifers/cows were in the range of 11-14, 8-13, 3-14 and 5-13 respectively. Correspondingly cervical mucus of 1-3, 4-6 and >6 times inseminated heifers/cows also recognized about 2-7, 2-13 and 2-12 sperm polypeptides respectively. Immunofluorescence of sperm smears with blood serum and cervical mucus of variably inseminated heifer/cows indicated the presence of antisperm antibodies (ASA) mainly against acrosome surface, post -acrosome and principal piece surface proteins. There was not any difference in range of number of polypeptides detected with blood serum in regard to number of insemination. But range of number of polypeptides detected was higher with cervical mucus of >3 times inseminated animals, which indicated increase in ASA against antigenic sperm proteins in cervical mucus with increase in number of inseminations. It was concluded that ASA are produced in blood serum and cervical mucus of cows/heifers against sperm proteins, irrespective of number of inseminations. In future further characterization of sperm proteins and their homogeneity with bacterial proteins is needed to understand the mechanism of ASA mediated fertility impairment and to develop treatment protocol on the basis of purified antigens.

Keywords Sperm; Antigens; ASA; Heifers; Cows

1. Introduction

Infertility or sub fertility is a cause of concern in dairy industry. Infertility in the female animals may involve a number of factors, including problems of ovulation, obstruction of oviducts, presence of pathological lesions in the uterus and poor quality of cervical mucus [1]. Bovine infertility has also been associated with the presence of antisperm antibodies, which impairs the physiological process of reproduction. Antibodies against sperm prevent their motility through female reproductive tract and hamper the process of fertilization [2]. Antibodies to sperm or egg yolk have been suggested to be possible cause of sub fertility in cows [3, 4]. The association of sperm antibodies with infertility/sub fertility in zebu cattle [5, 6, 7], goat [8] and mare [9] has been studied using ELISA, immunoperoxidase assay or immunobead assay. During cryopreservation of semen, antigenic structure of sperm cell is changed due to addition of extender, freezing/thawing procedures and seminal plasma is also significantly reduced during semen preparation for AI. The varying degree to which the antibodies impair fertilization suggests that identification of relevant sperm surface antigens and their role in the fertilization process would lead to formulation of an effective therapeutic plan to treat infertility [10]. It was also demonstrated by Jawad [11] that elevated level of ASA can be treated with zinc sulfate. However, molecular characterization through intervention of electrophoretic separation of sperm polypeptides by SDS-PAGE and detection of their immunoreactions to sera from immunological infertile animals may give deeper insight to the immunological reason of infertility. Therefore, in the present study, sperm antigens were characterized by immunoblotting and indirect immunofluorescence with blood serum and cervical mucus of variably inseminated cows and correlated with the number of estrus / AI's.

2. Materials and Methods

2.1. Procurement of Semen

Frozen semen straws were procured from GADVASU, dairy farm.

2.2. Collection of Samples

Blood and cervical mucus (CM) were collected from 43 (3 non-inseminated heifers; 4 inseminated heifers and 36 inseminated cows) cross-bred cows (HF X Red dane X Sahiwal) from dairy farm, Guru Angad Dev Veterinary and Animal Sciences University and private dairy farms around Ludhiana, Punjab, India. Blood was collected in sterilized vials from jugular vein without anticoagulant, centrifuged at 3000 rpm for 5 min to separate serum. At the time of estrus, CM was collected with the help of sterilized AI pipette, sonicated at 20 watts, 3 X 20 seconds. Serum and CM were inactivated at 56° C for 30 min and stored in aliquots at -20°C till further use. Number of estrus and AI's of the cows were also obtained from the farms. Out of 43 samples, collected, there were about 4, 8, 22 and 9 cows exhibiting about 0, 1-3, 4-6 and 6-14 times estrus viz a viz number of AI's, respectively.

2.3. Preparation of Sperm Extracts

Frozen semen straws were thawed, centrifuged and washed three times with PBS, pH 7.4. About 500 x10⁶ spermatozoa were suspended in 1.0 ml of 2% SDS in 62.5 mM Tris-HCl (pH 6.8), containing 10 µl of cocktail protease inhibitors (SERVA), sonicated at 4°C (20 W, thrice for 20 seconds each) and centrifuged at 16,000 g for 30 minutes to prepare sodium dodecyl sulphate sperm extract (SDS -SE).

2.5. SDS-PAGE [12] and Immunoblotting [13]

Blood serum and CM of variably inseminated heifers and cows were reacted with SDS-SE. 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 blood serum (1:200) and CM (1:50) of variably inseminated heifers and cows 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).

2.6. Immunolocalization of Antigenic Proteins using FITC Labeling [14]

Smears of frozen-thawed-washed cattle bull spermatozoa were prepared on glass slides, air-dried, and fixed in ethanol for 30 minutes. Slides were then covered with PBS containing 1% BSA for 45 minutes to block nonspecific antibody binding. They were then incubated at 37°C in a humidified chamber for 2 hours with 1:200 blood serum, 1:50 CM of variably inseminated heifers and cows. Slides were then washed and incubated for 1 hour with 1: 100 diluted rabbit anti-bovine IgG for one hour. Slides were again washed and incubated with 1: 100 diluted goat anti rabbit-FITC-conjugated antibody (Sigma). After 3 washings, slides were mounted with PBS-glycerol (1:1 v/v) containing 1, 4 diazabicyclo (2.2.2) octane as anti-fade and observed under fluorescent microscope (olympus), blue filter and images were captured on digital camera.

3. Results and Discussion

3.1. Characterization of Sperm Surface Antigens with Blood Serum and Cervical Mucus of Variably Inseminated Cows/Heifers

The blood serum and CM of all cows irrespective of number of heats or artificial inseminations reacted with SDS-SE on immunoblots and indicated variation in immunodominant sperm proteins for development of antibodies in variably inseminated cows. Immunoblot analysis of SDS-SE with blood serum/CM of variably inseminated cows/heifers revealed around 23 polypeptides of 300, 270, 245, 230, 200, 170, 150, 130, 115, 100, 90, 80, 75, 70, 65, 48, 45, 42, 35, 24, 18, 16, 11 kDa in cattle sperm (Figure 1 and 2). However, all proteins did not react with blood serum and cervical mucus of all tested animals. Number of polypeptides of SDS-SE that reacted with blood serum of non-inseminated, 1-3, 4-6 and >6 times inseminated heifers/cows were in the range of 11-14, 8-13, 3-14 and 5-13 respectively. There was also difference in intensity of bands reacting with blood serum/ cervical mucus of tested animals. Only two polypeptides out of 16 sperm polypeptides reacted with blood serum of infertile cows [15]. Circulating anti-sperm antibodies in blood serum of infertile patients also reacted with 4 surface antigens of 35, 40, 47 and 65 kDa [10].

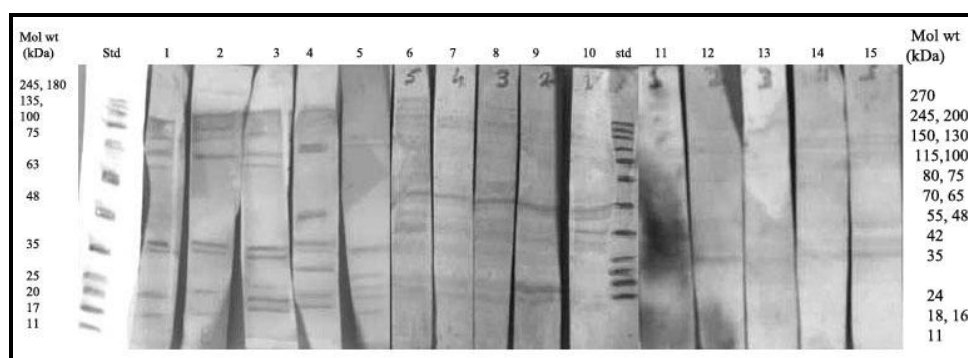


Figure 1: Showing Reaction of SDS-SE Proteins with Blood Serum of Some of Variably Inseminated Cows/Heifers on Immunoblot. SDS-SE were Separated on 10% Acrylamide Gel, Transferred to Nitrocellulose Membrane and Blot was developed with Blood Serum of Variably Inseminated Heifers/Cows

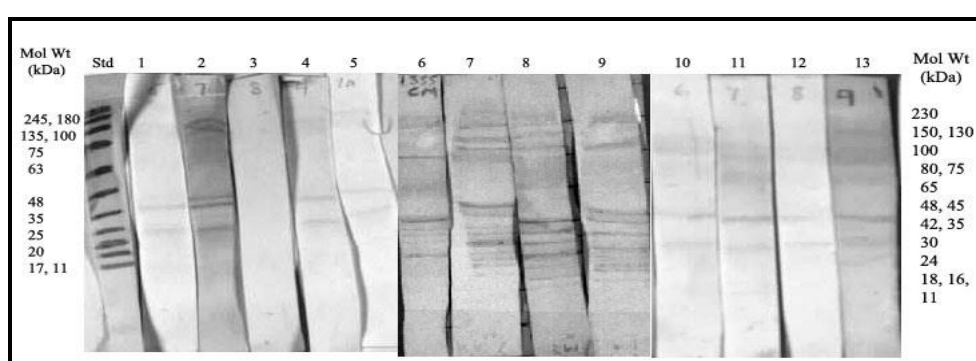


Figure 2: Showing Reaction of SDS-SE Proteins with Cervical Mucus of Some of Variably Inseminated Cows/Heifers on Immunoblot. SDS-SE were Separated on 10% Acrylamide Gel, Transferred to Nitrocellulose Membrane and Blot was developed with Cervical Mucus of Variably Inseminated Heifers/Cows

Correspondingly cervical mucus of 1-3, 4-6 and >6 times inseminated heifers/ cows also recognized about 2-7, 2-13 and 2-12 polypeptides in SDS-SE respectively. Bohring et al. [16] identified a total of 18 antigens by 2-D western blotting using ASA from seminal plasma samples of infertile patients. Six of the recognized proteins were identified as heat shock proteins HSP70 and HSP70-2, the disulphide isomerase ER60, the inactive form of caspase-3 and two subunits of the proteasome. There were 11.6-13.9% and 8.3-19.4% cows irrespective of number of estrus/ AI, which had ASA against 5-9 and 2-7 sperm proteins in blood serum and cervical mucus respectively (Table 1). Percentage of cows having ASA against higher number of sperm proteins i.e. 10-14 and 8-14 was only 0-9.3% and 2.7-5.5% in blood serum and CM respectively. Cervical mucus of only 2.7% tested cows could not recognize any polypeptide in SDS-SE.

Table 1: Number of Sperm Polypeptides Detected with Blood Serum/ Cervical Mucus and Percentage of Cows

Number of Proteins		0	2	3	4	5	6	7	8	9	10	11	12	13	14
% cows	Serum	-----	-----	4.6	---	11.6	11.6	13.9	13.9	13.9	4.6	6.9	4.6	9.3	4.6
	CM	2.7	11.1	16.6	8.3	11.1	19.4	16.6	2.7	2.7	----	----	2.7	5.5	---

3.5. Immunolocalization of Sperm Antigens with Blood Serum and Cervical Mucus of Variably Inseminated Cows/Heifers

Immunofluorescence of sperm smears with blood serum/cervical mucus of variably inseminated animals gave variable signals. A very strong signal was observed on acrosome surface, post-acrosome region and principal piece of spermatozoa (Figure 3). It indicated the presence of ASA

against mainly acrosome surface, post –acrosome and principal piece surface proteins in the blood serum/ cervical mucus of variably inseminated animals. Milovanovic et al. [17] also observed fluorescence on the head, tail or neck of the sperm cell with blood serum and cervical mucus of artificially inseminated Holstein cows. They confirmed the hypothesis that immune mechanisms may be involved in reproductive disturbances due to high levels of ASA of Ig A class and found high levels of ASA in animals with longer open day's period. The mean age at first calving and the mean intercalving interval were significantly higher in the group positive for sperm antibodies compared to the negative animals [5].

Therefore, immunofluorescence as well as immunoblotting revealed the presence of antisperm antibodies in the blood serum and cervical mucus of all tested animals irrespective of number of inseminations. However, there was not any difference in range of number of polypeptides detected with blood serum in regard to number of insemination. But range of number of polypeptides detected was higher with cervical mucus of >3 times inseminated animals. It indicated increase in ASA in cervical mucus with increase in number of inseminations. The cows having more unsuccessful inseminations also showed higher ASA with high titre in serum and mucus [7]. Farahani et al. [18] found agglutinating and immunofluorescent antibodies in serum from repeat breeder, fertile cows and virgin heifers with no sperm-immobilizing antibodies. The antibodies were assumed to be produced naturally with no need of female exposure to sperm antigens as all virgin heifers also demonstrated agglutinating and immunofluorescence antibodies in their serum. Similarly, in our study 11-14 polypeptides were detected even with blood serum of virgin heifers (<6 months), which were reduced to 8-13, 3-14 and 5-13 in 1-3 and >3 times inseminated animals. Reaction of 11-14 sperm polypeptides with blood serum of virgin heifers indicated the presence of cross reacting antibodies in their blood serum. Paolichhi et al. [19] also postulated that cross reactive antibodies may develop in animals and impair fertility. Many species of bacteria and anaerobic microorganisms inhabit the vagina, uterus, cervix of cows and many bacteria were isolated from the uteri of cows with a history of repeat breeding, retained placenta and metritis [20]. Recently Thapar et al. [21] were of the opinions that cross reactivity between certain epitopes on the bacterial surface & spermatozoa particularly involving carbohydrate determining domains might be potential triggering mechanism for induction of ASA in male and female. An amazing analogy between the chlamydial heat shock proteins and human proteins; the relationships between *Ureaplasmaurealyticum* infection, antisperm antibodies, and infertility, homology between Ure-G and Nuclear sperm autoantigenic protein was noticed in human in different studies [22]. Therefore, reaction of sperm proteins with blood serum/cervical mucus of virgin heifers and cows may also be due to the presence of cross reacting antibodies against microorganisms.

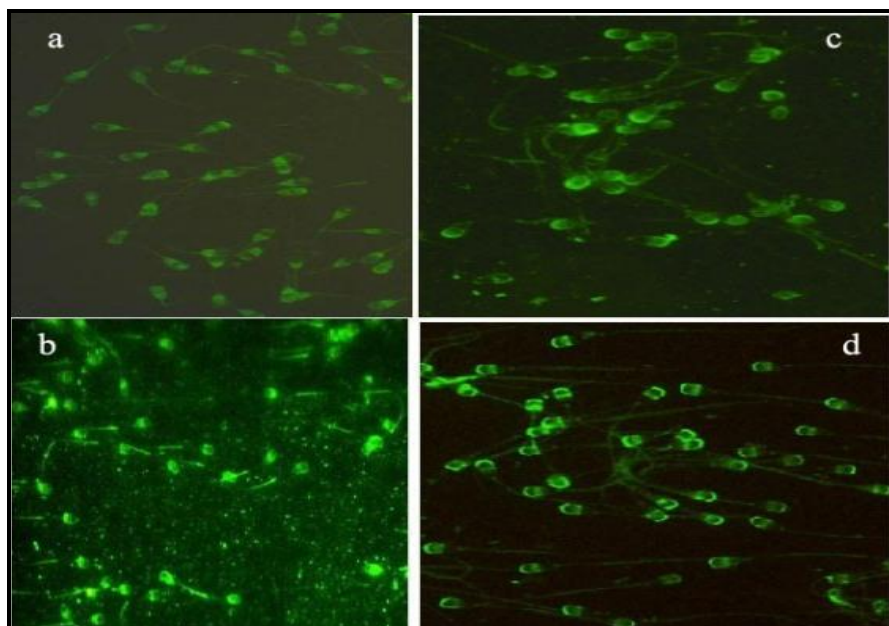


Figure 3: Showing Localization of Sperm Antigens upon Reaction of Sperm Smears with Blood Serum (A, B) and Cervical Mucus (C, D) of Variably Inseminated Cows. Slides Fixed in Ethanol were Reacted with Blood Serum / Cervical Mucus and Fluorescence was Developed with Goat Anti Rabbit-FITC-Conjugated Antibody and Observed at 400 X

4. Conclusions

It was concluded that ASA are produced in blood serum and cervical mucus of cows/heifers against sperm proteins, ranging from 11-300 kDa, irrespective of number of inseminations. In future further characterization of sperm proteins and their homogeneity with bacterial proteins is needed to understand the mechanism of ASA mediated fertility impairment and to develop treatment protocol on the basis of purified antigens.

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