Chapter 12

Isolation and Purification of Sperm Immobilizing/Agglutinating Factors from Bacteria and Their Corresponding Receptors from Human Spermatozoa

Vijay Prabha and Siftjit Kaur

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/48594

1. Introduction

For successful fertilization, motility is the most obvious and most essential sperm function and has been repeatedly shown to be predictive of fertilization in vitro [1]. Several studies have shown that the motility characteristics of spermatozoa are of the utmost importance for the men's fertility [2]. Spermatozoa dysfunction is the single most important cause of infertility. A decrease in spermatozoa motility with time is universal phenomenon. This reduction differs from species to species and also among individuals of the same species, as in the human male. Most investigators agree that the majority of spermatozoa cease to move within the first 24 hours. The survival of spermatozoa after ejaculation is dependent on the environmental conditions under which they are kept. In the female genital tract they may remain active for several days [3], but their activity is of much shorter duration if they remain in the seminal fluid outside the body.

Male genital infections are relevant cause in the etiology of infertility due to abnormalities in sperm quality [4,5], affecting spermatozoal motility. The comparison of semen characteristics between infected and non-infected men show that motile spermatozoa are lower when the microorganisms are present in the semen [6]. It appears that bacteria have a direct effect on sperm motility with negative consequences in fertility. Among bacterial species that interact with spermatozoa are well-known causative pathogens of genitourinary infections such as *Escherichia coli, Ureaplasma urealyticum, Mycoplasma hominis, Chlamydia trachomatis* [7]. Of the various bacteria, *E. coli* is the most extensively studied microorganism in relation to infertility as a result of interaction with spermatozoa [8]. It is also the primary bacteria associated with prostatitis and epididymitis [9]. Several authors describe sperm

agglutination and immobilization by *E. coli* [10,11]. Paulson & Polakoski [12] investigated the mechanism of how *E. coli* immobilizes spermatozoa and they reported a factor, apparently excreted by the bacteria which immobilizes spermatozoa without agglutinating it. However, Diemer et al. [13] reported that *E. coli* inhibits sperm motility by directly adhering to and agglutinating spermatozoa. Rapidity and extent of sperm–*E. coli* agglutination indicated strong adhesive forces. Bartoov et al. [8] proposed that mannose plays a critical role in adherence of *E. coli* to sperm. Although, a number of studies have evaluated the ability of *E. coli* to affect sperm motility by adherence, agglutination and dialyzable factors, however, none have identified the exact mechanism of interaction between spermatozoa and bacteria.

In addition to *E. coli*, *Staphylococcus aureus,* the predominant flora in infertile men, has also been reported to cause a significant decrease in sperm motility [14]. Emokpae et al. [15] while studying the contribution of seminal tract infection to sperm density, asthenozoospermia and teratozoospermia, observed *S. aureus* as the causative organism accounting for 68.2% of seminal infections. Most practitioners dismiss this infection as mere contamination, which is assumed to have no significance. Semen that passes through the genital tract is routinely contaminated with staphylococci. However, since the prevalence of abnormal sperm indexes is high, it was suggested that *S. aureus* infection should be treated and no longer ignored when managing male factor infertility [15]. *S. aureus* is known to produce various toxins and enzymes that may be exerting damaging effect on human sperm, but its mechanism of action also needs further investigation. Therefore, the present work was undertaken to study the mechanism of immobilization/agglutination of spermatozoa by *S. aureus* and *E. coli*.

2. Microorganisms

The bacterial isolates *Staphylococcus aureus* either showing immobilization or agglutination of human spermatozoa, were isolated from the cervices of women suffering from unexplained infertility, attending the Department of Obstetrics and Gynecology, Government Multi Speciality Hospital, Sector-16, Chandigarh, India. The isolates of *Escherichia coli* were isolated from semen samples of infertile males attending the infertility clinic at the Department of Urology, Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh.

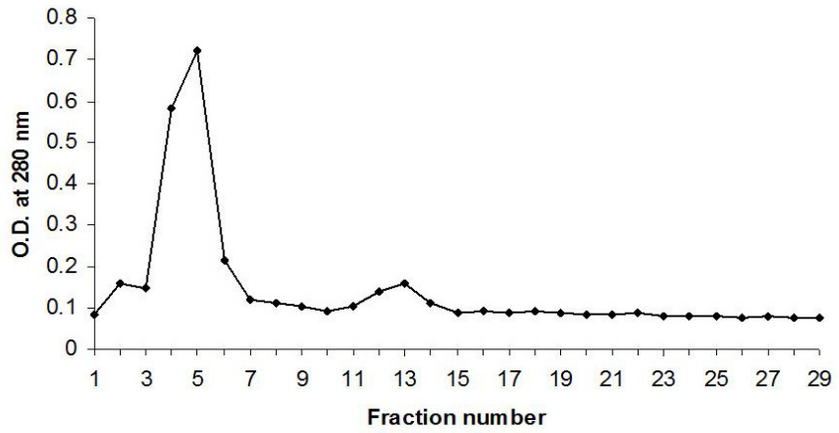
3. Extraction and purification of sperm agglutinating factor (SAF) from *S. aureus*

Sperm agglutinating factor was extracted and purified from 72 h old cell culture of *S. aureus* by the method standardized in the laboratory [16]. The cell culture was centrifuged at 10,000 g for 20 minutes and the clear supernatant and the cells obtained were washed twice with sterile phosphate buffered saline (PBS) (50 mM, pH 7.2) and resuspended in the same buffer. When washed cells and cell free supernatant were studied for their sperm agglutinating property, the results showed that only washed cells were able to agglutinate the spermatozoa whereas culture

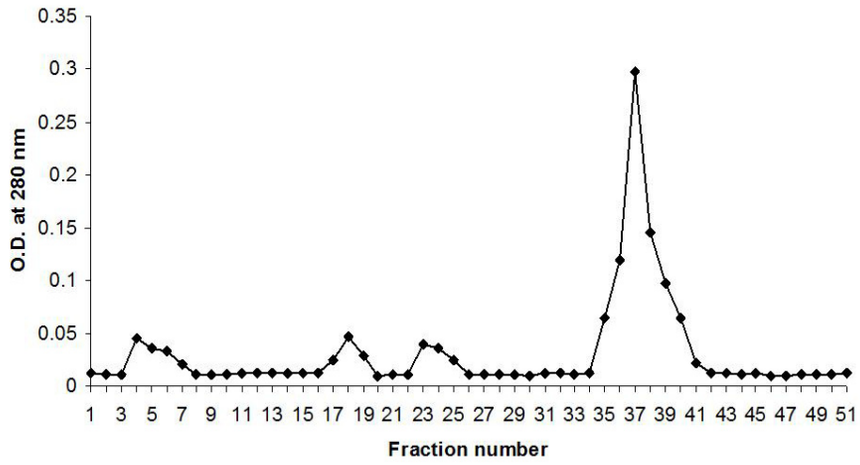
supernatant failed to do so indicating that the agglutination of sperm might be associated with bacterial cells and not their metabolites. Pretreatment of *S. aureus* by sonication produced bacterial fragments that were unable to agglutinate sperm. Centrifugation of *S. aureus* fragments at 10,000 g for 5 min did not eliminate sperm agglutinating elements from the solution, indicating the sperm agglutinating factor to be present in sonicated supernatant. Based on its sperm agglutinating activity, the bioactive molecule from the sonicated supernatant was purified by ammonium sulphate precipitation, gel permeation chromatography and ion exchange chromatography. The sonicated supernatant was fractionated with ammonium sulphate so as to get 20, 40, 60 80 and 100% saturation. The flasks were kept at 4°C overnight and next day the precipitates were collected by centrifugation at 10,000 g for 15 min at 4°C and were redissolved in minimum amount of PBS. SAF was precipitated by ammonium sulphate at 40% saturation. The protein was dialyzed in preactivated dialysis bags against PBS under cold conditions and concentrated against polyethylene glycol (PEG) 6000 at 4°C. Further purification was done using Sephadex G-200 and DEAE cellulose column chromatography. The fractions containing approximately 1mg protein were applied on Sephadex G-200 (Pharmacia Fine Chemicals, Uppsala), column (2 cm X 31 cm) equilibrated and eluted with PBS. The aspirator bottle with PBS was joined with a fine capillary tube to maintain constant head pressure and allowed to run for 24 h. Fractions of 3 ml each were collected and each fraction was read at 280 nm on U.V. spectrophotometer. The fractions showing spermagglutinating activity i.e. fraction (4-7) with a peak value in fraction 5 were pooled and concentrated against PEG 6000 (Figure 1a) and applied to DEAE cellulose (Hi Media Laboratories Ltd., Mumbai, India) column. A column of 2 cm x 15 cm was made with activated slurry of DEAE cellulose, an anion exchanger with a pressure of about 1 cm water/cm of height of gel bed. The column was washed with PBS until the ion exchanger reached ionic equilibrium with the starting buffer. The aspirator bottle with PBS was joined with a fine capillary tube to maintain a constant head pressure and allowed to run. Final elution was done with PBS containing 0.05, 0.1, 0.2, 0.4 and 0.6 M NaCl. Fractions of 4 ml each were collected and read at 280 nm on U.V. spectrophotometer (Hitachi U-2900). All fractions were again checked for agglutination of spermatozoa. The fractions showing agglutination of sperm (35-39) were pooled and concentrated (Figure 1b). The purified pooled fractions were dialyzed, lyophilized and then subjected to protein estimation. The purification status of SAF was checked by gel electrophoresis (PAGE). Molecular weight estimation was done by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using a 12% resolving gel containing SDS [17]. 10µl of the pooled and concentrated sample obtained from DEAE cellulose column was mixed with loading dye (Bromophenol blue: glycerol) in the ratio of 1:50. The sample was applied to the gel and subjected to a current of 15 mA and run initially at 50V and thereafter at 100V. The gel was visualized by staining with Coomassie Brilliant Blue R-250. The molecular weight of SAF was estimated to be approximately, 65 kDa (Figure 1c).

4. Extraction and purification of sperm immobilization factor (SIF) from *S. aureus*

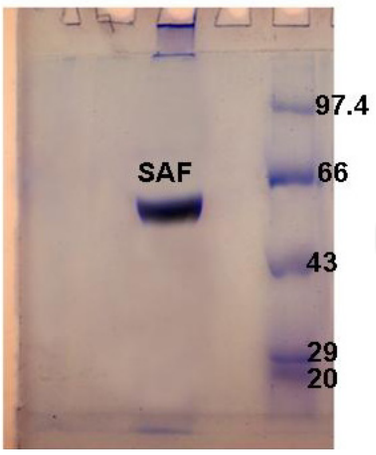
For extraction of SIF, *S. aureus* was grown in Brain Heart Infusion (BHI) broth for 72 h at 37°C under shaking conditions (150 rpm). The cell culture was subjected to centrifugation at



1a



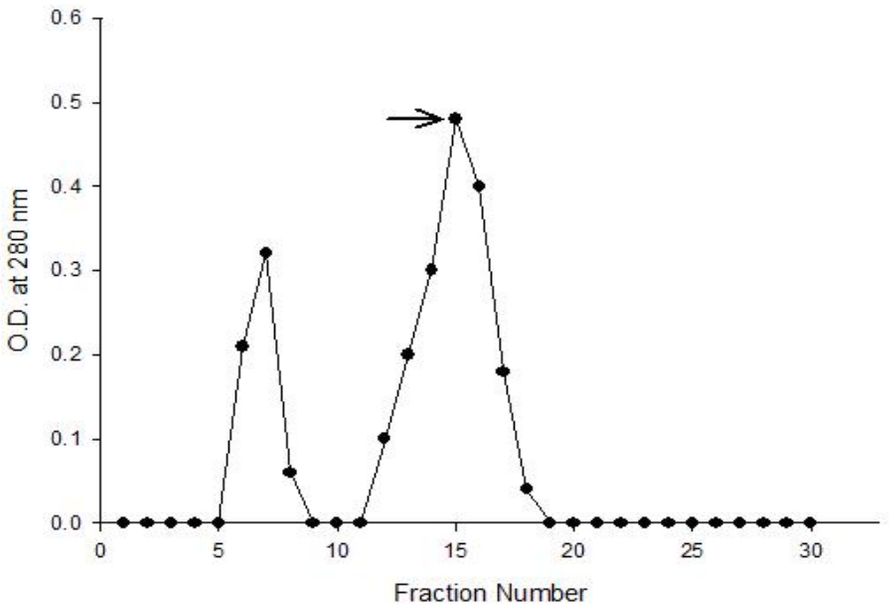
1b



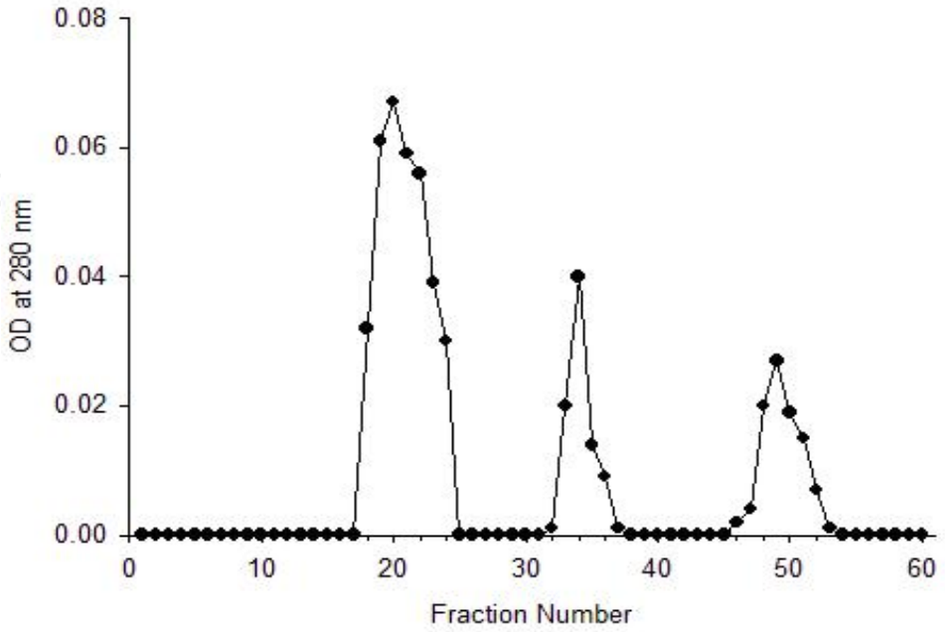
1c

Figure 1. **(a)** Elution pattern of SAF from *S. aureus* after gel filtration through Sephadex G-200 column showing the presence of SAF in fractions 4-7 with a peak value in fraction 5. **(b)** Elution pattern of SAF obtained after DEAE cellulose column showing sperm agglutination activity in fractions 35-39 with peak value in fraction 37. **(c)** SDS-PAGE of purified SAF, with **Lane1** containing DEAE cellulose purified and concentrated fraction. Molecular weight approximately, 65 kDa and **Lane2** containing Standard protein markers.

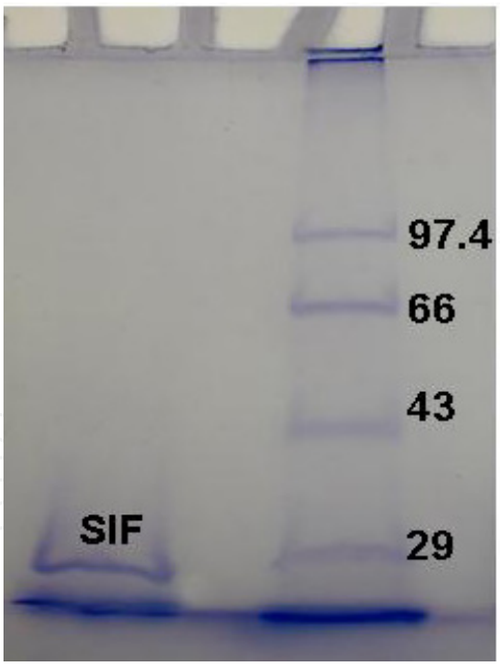
10,000 g for 30 min at 4°C. Both the resultant cell pellet and culture supernatant were checked for sperm immobilization activity. As the activity resided with cell supernatant it was subjected to ammonium sulphate precipitation so as to get 20, 40, 60, 80 and 100% saturation. SIF could be precipitated at 60-80% saturation. These precipitated proteins containing the bioactive molecule were dialyzed extensively against PBS and applied to Sephadex G-100 column (2 cm x 31 cm) equilibrated and eluted with PBS. The head pressure maintained to achieve a flow rate of 10 ml/h. Fractions of 3 ml each were collected, and the absorbance of each fraction was read at 280 nm. Fractions (12–18) showing the immobilization activity were pooled and concentrated against PEG 6000 at 4°C (Figure 2a). These fractions were then applied onto DEAE cellulose column. The column was equilibrated with PBS and head pressure was maintained to achieve a flow rate of 60 ml/h. Final elution was done with 0.05, 0.1, 0.2, 0.4 and 0.6 M NaCl dissolved in PBS. Fractions of 4 ml each were collected, and the absorbance was read at 280 nm. The fractions (47–52) that caused immobilization of spermatozoa were pooled and concentrated (Figure 2b). The fractions concentrated after DEAE cellulose chromatography were dialyzed, lyophilized, and subjected to PAGE analysis. The presence of a single band suggested apparent homogeneity of the protein. This purified fraction was then analyzed by SDS-PAGE against standard molecular weight markers (Figure 2c). The molecular weight of SIF was estimated to be ~20 kDa [18].



2a



2b



2c

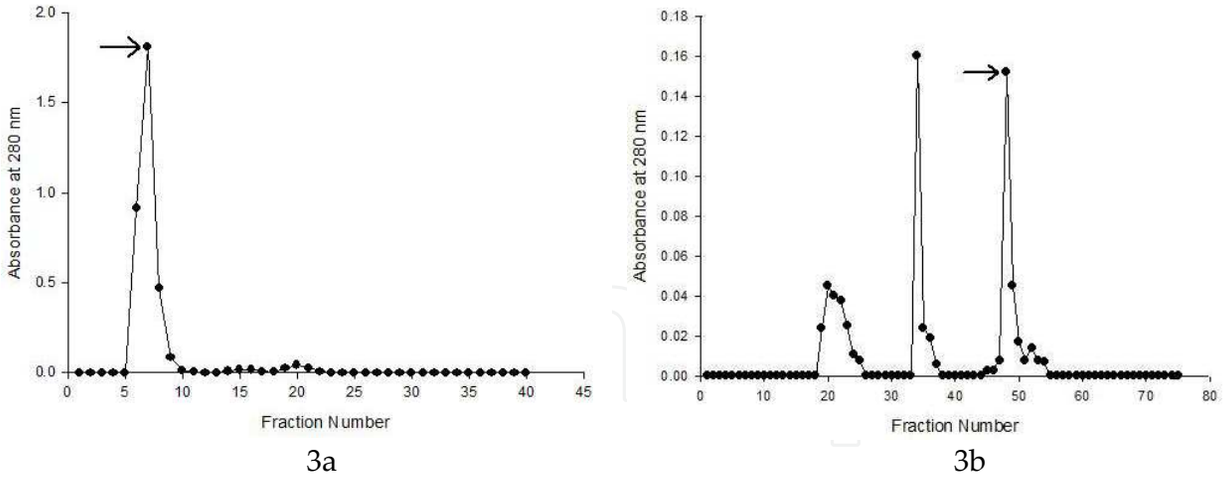
Figure 2. **(a)** Elution pattern of SIF from *S. aureus* after gel filtration through Sephadex G-100 column showing the presence of SIF in fractions 12-18 with a peak value in fraction 15. **(b)** Elution pattern of SIF from *S. aureus* obtained after DEAE cellulose column showing sperm immobilization activity in fractions 47-52 with peak value in fraction **49**. **(c)** SDS-PAGE of purified SIF, with **Lane1** containing DEAE cellulose purified and concentrated fraction. Molecular weight approximately, 20 kDa and **Lane 2** containing standard protein markers.

5. Extraction and purification of SAF from *E. coli*

48 h old culture of *E. coli* grown in Luria broth was centrifuged at 10,000 g for 10 min at 4ºC. Cell free supernatant was prepared by passing the supernatant through a 0.22 µm Millipore filter. The cell pellet was washed twice with sterile PBS. Both the cell free supernatant and the washed cells were checked for spermagglutinating activity by incubating with semen samples. As the washed cells showed spermagglutinating activity, therefore further studies were carried out with washed cells. Extraction of sperm ligand from washed cells was done by salt treatment. The washed cells of *E. coli* (1000 ml, 48 h old cell culture) were incubated with 1, 2, 3, 4 and 5 M solution of NaCl under shake conditions (150 rpm) at 37ºC for different time intervals 2, 4, 8, 12 and 24 h, separately. The cells were centrifuged at 10,000 g for 30 min. The resulting cell pellet and supernatant (which was dialyzed against double distilled water overnight at 4ºC and passed through the UM05 Amicon filter) were analyzed for sperm agglutinating activity. As pellet did not show sperm agglutinating activity, further work was carried out with the supernatant. SAF could be efficiently extracted by treatment of cell pellet with 3 M NaCl for 12 h under shaking conditions (150 rpm) at 37ºC. Purification of crude sperm ligand consisted of filtration of dialyzed and concentrated fraction through a Sephadex G-200 column, equilibrated and eluted with PBS. Fractions of 3 ml each were collected and read at 280 nm on U.V. spectrophotometer. Fractions showing sperm agglutinating activity i.e. 6-9, with a peak in fraction 7 (Figure 3a) were pooled and concentrated using polyethylene glycol (PEG) 6000 at 4ºC. These fractions were applied to DEAE cellulose column. Final elution was done with 0.05, 0.1, 0.2, 0.4 and 0.6 M NaCl dissolved in PBS. Fractions of 4 ml each were collected and read at 280 nm on U.V. spectrophotometer. Most of the SAF could be eluted with PBS containing 0.4 M NaCl (fractions 46-49, Figure 3b). Fractions showing agglutinating activity were further pooled, dialyzed and concentrated. The protein content at each step was assayed by the method of Lowry et al. [19] against a standard curve calibrated with bovine serum albumin. PAGE of purified sperm ligand was carried out to check the purification status. Molecular weight estimation by SDS-PAGE, using the standard molecular weight markers showed the presence of ~71 kDa protein band (Figure 3c) [20].

6. Extraction and purification of SIF from *E. coli*

The isolate of *E. coli* showing immobilization of spermatozoa was grown in BHI broth under shaking conditions (150 rpm) at 37ºC for 72 h. The culture was centrifuged at 10,000 g for 15 min at 4ºC and cell-free supernatant was prepared by passing the supernatant through a 0.22 µm Millipore filter. The supernatant was then subjected to ammonium sulphate precipitation so as to get 20, 40, 60, 80, and 100% saturation. The precipitates so obtained were dissolved in a minimum amount of PBS. The precipitated protein was dialyzed against PBS under cold conditions and checked for sperm immobilization. SIF could be saturated at 60-80% of ammonium sulphate. Further purification of the factor was done by gel filtration through a Sephadex G-100 column (2 cm x 31 cm) equilibrated, and eluted with PBS.



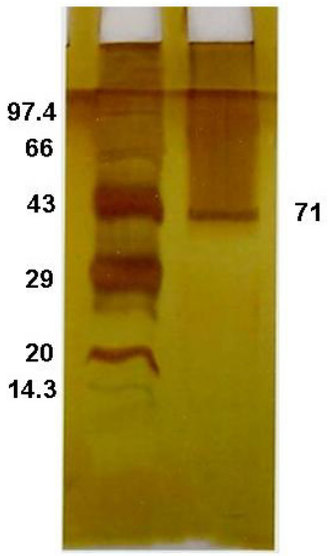
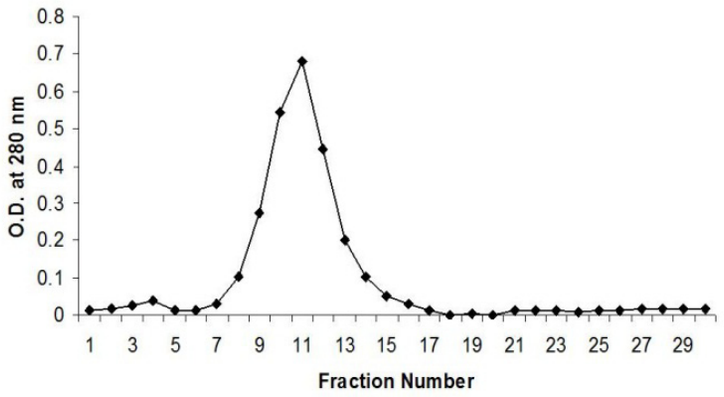


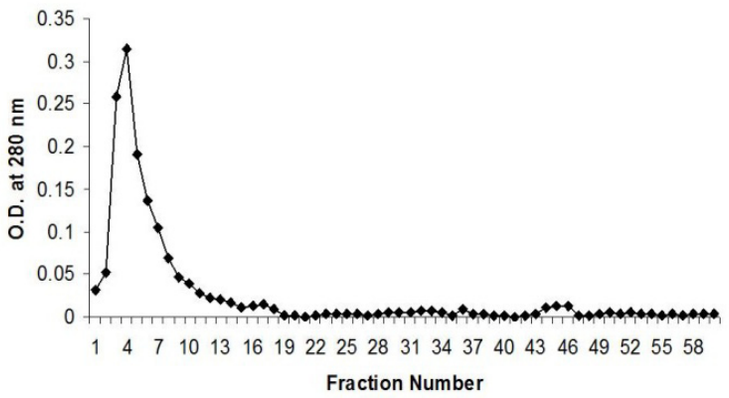
Figure 3. **(a)** Elution pattern of SAF obtained after Sephadex G-200 gel filtration of dialyzed and filtered supernatant containing sperm ligand on *E. coli*, showing the presence of ligand in fractions 6-9 with peak value in fraction 7 (arrow refers to sperm ligand on *E. coli*). **(b)** DEAE cellulose chromatography of G-200 pooled and PEG concentrated fractions revealed the presence of three peaks but the agglutinating activity was present only in the fractions 46-49 with peak value in fraction 48 (arrow refers to sperm ligand on *E. coli*). **(c)** SDS-PAGE of purified sperm ligand, with **Lane1** containing Standard protein marker and **Lane2** containing DEAE cellulose purified and concentrated fraction. Molecular weight approximately, 71 kDa.

Fractions of 3 ml each were collected and each fraction was read at 280 nm on U.V. spectrophotometer. Fractions 8-14, showing the immobilization of spermatozoa were pooled and concentrated using PEG 6000 under cold conditions (Figure 4a). These fractions were applied on to a DEAE cellulose column. First of all, 80 ml of elution buffer, PBS (50 mM pH 7.2) was allowed to run down the column. Final elution was done with 0.05, 0.1, 0.2, 0.4, and 0.6 M NaCl dissolved in PBS (50 mM, pH 7.2). Fractions of 4 ml each were collected and read at 280 nm on U.V. spectrophotometer. The fractions showing immobilization of spermatozoa i.e. 3-7 were again pooled and concentrated (Figure 4b).

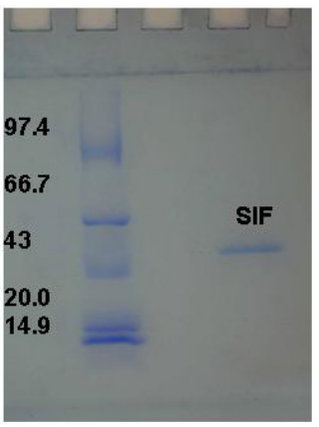
To verify the purification status of all the preparations, PAGE was carried out (10% resolving and 5% stacking gel). Estimation of molecular weight by SDS-PAGE, showed that SIF had a molecular weight of ~56 kDa (Figure 4c), compared to standard protein markers [21].



4a



4b



4c

Figure 4. (**a**) Elution pattern of SIF from *E. coli* on Sephadex G-100 column. **(b)** Elution pattern of SIF from *E. coli* on DEAE cellulose column. **(c)** SDS-PAGE of purified SIF, with **Lane1** containing Standard protein marker and **Lane2** containing DEAE cellulose purified and concentrated fraction. Molecular weight approximately, 56 kDa.

7. Isolation and purification of receptor from human spermatozoa for SAF isolated from *S. aureus*

Spermatozoa were washed twice with PBS and resuspended in the same buffer. Washed spermatozoa were treated with 1, 2, 3 and 4 M NaCl for different intervals to optimize the best concentration and time combination for receptor extraction. The treated mixture was centrifuged at 10,000 g for 20 min. The pellet and supernatant were examined for blockage of agglutination activity. Receptor was efficiently extracted by 3 M NaCl when incubated for 12 h at 37°C while shaking at 150 rpm. Crude receptor was further dialyzed extensively against PBS under cold conditions and then concentrated using PEG 6000. Receptor was purified by filtration through a 2 x 31 cm Sephadex G-200 column, equilibrated and eluted with PBS. Head pressure was maintained to achieve a flow rate of 10 ml/h. Fractions (3 ml) were collected and each was read at 280 nm using a U.V. spectrophotometer. Fractions were analyzed for the presence of receptor by blocking the agglutination of spermatozoa by SAF. Fractions (12-17) representing receptor were pooled, concentrated against PEG 6000 at 4°C (Figure 5a). One-dimensional SDS-PAGE was done using a vertical slab gel apparatus with stacking gel containing 5% polyacrylamide and resolving gel containing 12% polyacrylamide. The protein sample was diluted 1:1 with reducing sample buffer and heated for 5 min at 100°C. The sample was loaded and subjected to electrophoresis at 50 V for 15 min, followed by 150 V for 45 min. The gel was stained with Coomassie Brilliant Blue R-250 and calibrated using molecular weight markers. The molecular weight of the receptor was estimated to be approximately, 57kDa (Figure 5b) [22].

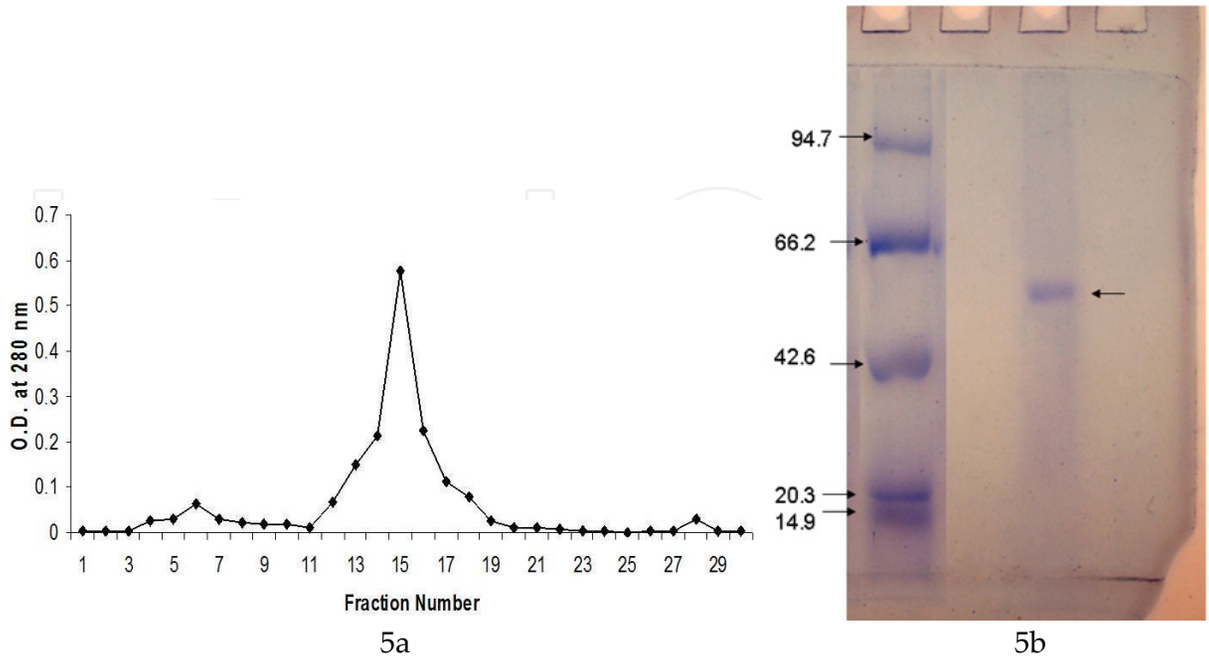
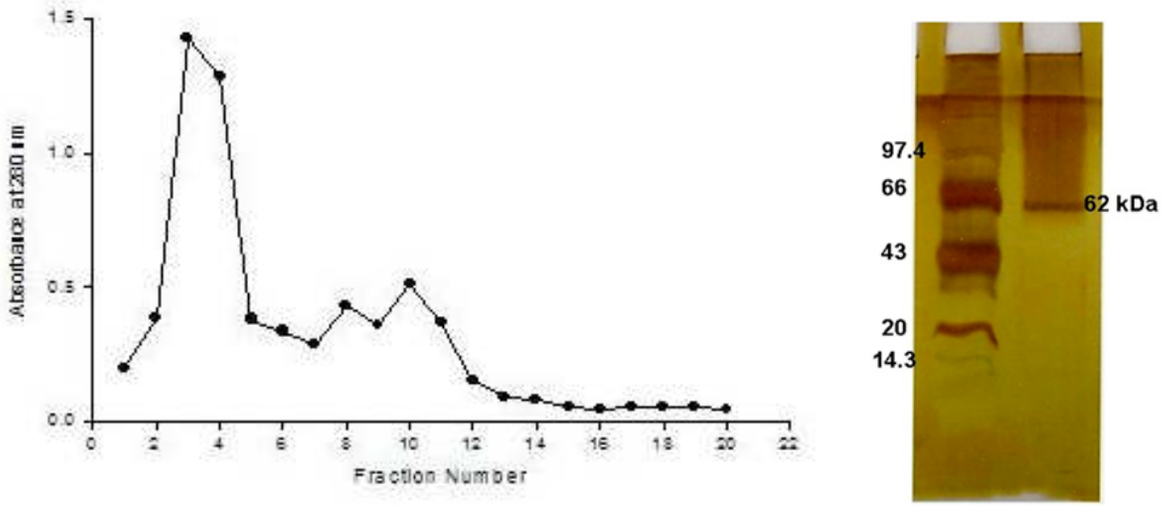


Figure 5. **(a)** Elution pattern of receptor from human sperm on Sephadex G-200 column. **(b)** SDS-PAGE (molecular weight determination) **Lane 1**: Standard proteins markers, **Lane 2**: Purified receptor protein.

8. Isolation and purification of receptor for SIF isolated from *S. aureus*

Salt extraction of the receptor from human spermatozoa was done by treating the washed sperm sample with 1, 2, and 3 M NaCl for different time intervals i.e. 2, 4, 8, 12, 24 and 48 h. Purification of the receptor was further done by gel filtration through a Sephadex G-100 column (2 cm × 31 cm) equilibrated and eluted with PBS (50 mM, pH 7.2). Fractions of 3 ml each were collected and absorbance read at 280 nm to determine the SIF receptor concentration. Fractions showing the blockage of immobilization of spermatozoa (2-5, with a peak in fraction 3) were pooled and concentrated using PEG 6000 under cold conditions (Figure 6a). To check the purification status and biological activity of pooled and concentrated fractions, PAGE was carried out. Molecular weight of the purified receptor as estimated using SDS-PAGE was found to be approximately, 62kDa (Figure 6b) [23].



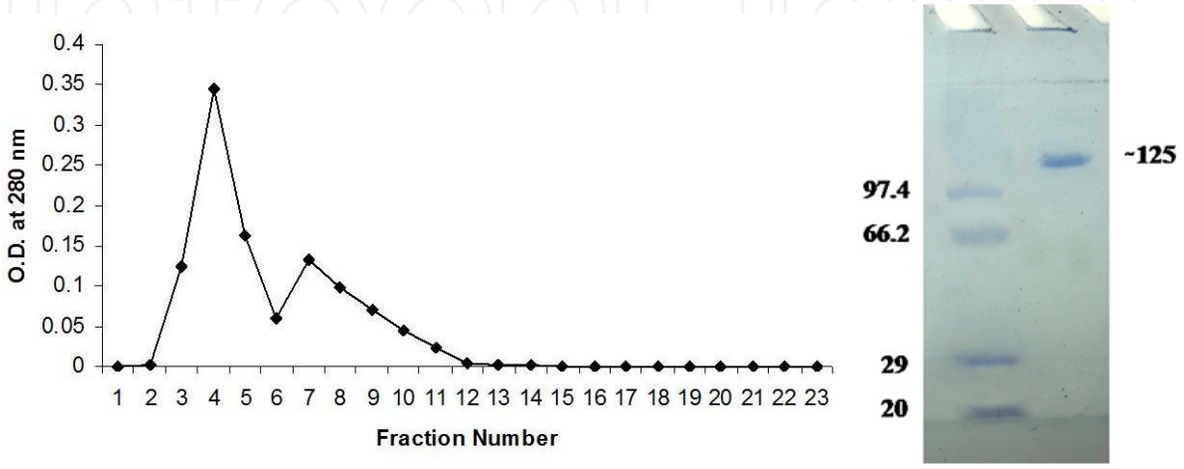
6a 6b

Figure 6. **(a)** Elution pattern of receptor from human sperm on Sephadex G-100 column. **(b)** SDS-PAGE (molecular weight determination) **Lane 1**: Standard proteins markers, **Lane 2**: Purified receptor protein

9. Isolation and purification of receptor for SAF isolated from *E. coli*

The corresponding receptor for SAF was isolated from human spermatozoa using the ligand as a tool. The procedure involved the use of salt solution for the extraction of molecules bound to the cell surface. The spermatozoa were washed twice with PBS and then resuspended in same buffer. They were then treated with different concentrations of NaCl i.e. 1, 2, 3 and 4 M for different time intervals and incubated at 37°C under shaking conditions (150 rpm). The salt treated sample was centrifuged at 1500 g for 15 min. Cell debris was suspended in minimum amount of PBS. Both cell debris and supernatant were dialyzed against PBS at 4°C overnight, concentrated against PEG 6000 and checked for blocking of agglutination induced by SAF. Results showed that receptor for SAF from *E. coli* could be efficiently extracted by 2 M NaCl when incubated for 18 h. Purification of the receptor was further carried out by filtration through a Sephadex G-200 column (1.5 cm X 31

cm) equilibrated and eluted with PBS. The head pressure was maintained to achieve a flow rate of 35 ml/h. Fractions of 3 ml each were collected and each fraction was read at 280 nm. Fractions showing blockage of agglutination activity were pooled and concentrated with PEG 6000 at 4°C (Figure 7a). The homogeneity of the preparations was checked by PAGE and the molecular weight of the purified receptor was estimated by SDS-PAGE. The molecular weight of the receptor for SAF from *E. coli* was approximately, 125 kDa (Figure 7b) [24].



7a 7b

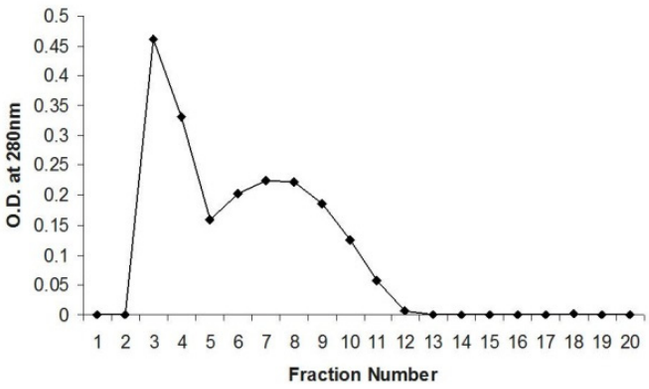
Figure 7. **(a)** Elution pattern of receptor from human sperm on Sephadex G-200 column. **(b)** SDS-PAGE (molecular weight determination) **Lane 1**: Standard proteins markers, **Lane 2**: Purified receptor protein

10. Isolation and purification of receptor for SIF isolated from *E. coli*

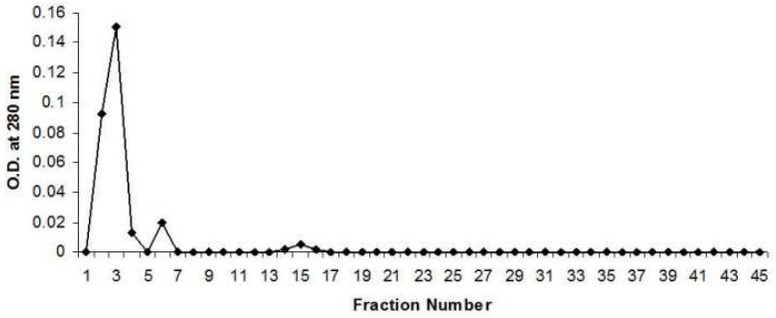
The spermatozoa were washed twice with PBS (50 mM, pH 7.2) and then resuspended in same buffer. The washed spermatozoa were treated with different concentrations of NaCl i.e.1, 2, 3 and 4 M for different time intervals in order to optimize the best concentration and time combination for receptor isolation. These were incubated at 37°C under shaking conditions (150 rpm). The salt treated sample was centrifuged at 1500 g for 15 min. Cell debris was suspended in minimum amount of PBS. Both the cell debris and supernatant after dialysis and concentration (against PEG 6000) were checked for blocking of immobilization induced by SIF. Preliminary results showed that SIF receptor could be efficiently extracted by 2 M NaCl from spermatozoa when incubated for 20 h at 37°C under shaking conditions.

Purification of the receptor was further done by filtration through a Sephadex G-200 column (1.5 cm X 31 cm) equilibrated and eluted with PBS. The head pressure was maintained to achieve a flow rate of 35 ml/h. Fractions of 3 ml each were collected and each fraction was read at 280 nm (U.V. spectrophotometer). The column chromatographic pattern showed that the receptor could be eluted in the fractions 3-4 which could block the SIF induced sperm immobilization (Figure 8a). These fractions were pooled and concentrated with polyethylene glycol (PEG 6000) at 4°C and were applied to ion exchange column. First of all, 80 ml of elution buffer (PBS) was allowed to run down the column. Final elution was done with 0.05, 0.1, 0.2, 0.4 and 0.6 M NaCl dissolved in PBS. Fractions of 4 ml each were collected

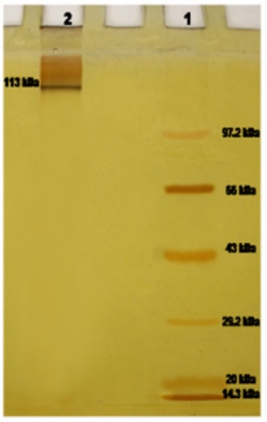
and read at 280 nm on U.V. spectrophotometer. The fractions showing blockage of sperm immobilization i.e. 2-4 were pooled and concentrated (Figure 8b). Molecular weight of the purified SIF receptor was estimated by SDS-PAGE. The purified SIF receptor was first denatured and then loaded onto the gel along with the standard protein markers. After the gel was run, silver staining was done and molecular weight was estimated to be ~113 kDa (Figure 8c) [25].



8a



8b



8c

Figure 8. **(a)** Elution pattern of receptor from human sperm on Sephadex G-200 column. **(b)** Elution pattern of receptor from human sperm on DEAE cellulose column. **(c)** SDS-PAGE (molecular weight determination) **Lane 1**: Standard proteins markers, **Lane 2**: Purified receptor protein

11. Conclusion

This study identifies receptor-ligand interaction between bacteria and spermatozoa that results in sperm immobilization/agglutination.

12. Further research

Understanding bacteria-spermatozoa interactions at receptor-ligand level may hold potential for infertility treatment.

13. Abbreviations

~	:	approximately
%	:	percentage
°C	:	degree celcius
BHI	:	brain heart infusion
DEAE	:	Diethylaminoethyl
h	:	hour
i.e.	:	id est (that is)
kDa	:	kilodalton
µg	:	microgram
µl	:	microlitre
µm	:	micrometre
mA	:	milliampere
min	:	minutes
ml	:	millilitre
M	:	molar
mM	:	millimolar
mm	:	millimetre
cm	:	centimetre
nm	:	nanometre
mg	:	milligram
NaCl	:	sodium chloride
O.D.	:	optical density
PAGE	:	polyacrylamide gel electrophoresis
PBS	:	phosphate buffered saline
PEG	:	polyethylene glycol
rpm	:	revolutions per minute
s	:	seconds
SDS	:	sodium dodecyl sulphate
SAF	:	sperm agglutinating factor
SIF	:	sperm immobilization factor
U.V.	:	ultraviolet
V	:	volts

Author details

Vijay Prabha and Siftjit Kaur
Department of Microbiology, Panjab University, Chandigarh, India

Acknowledgement

This work was supported by funds from University Grants Commission, New Delhi and Indian Council of Medical Research, New Delhi, India.

14. References

[1] Bonde JP, Ernst E, Jensen TK, Hjollund NH, Kolstad H, Henriksen TB, Scheike T, Giwercman A, Olsen J, Skakkebaek NE (1998) Relation between semen quality and fertility: a population-based study of 430 first-pregnancy planners. Lancet 352: 1172-1177.

[2] Karpuz V, Gokturk A, Meral Koyuturk M (2007) The effects of sperm morphology and motility on the outcome of intracytoplasmic sperm injection. Marmara med. j. 20: 92-97.

[3] Hafez ESE (1980) Physiology of spermatozoal insemination in the female reproductive tract. In: Emperiare JC, Audebent A, Hafez ESE editors. Homologous artificial Insemination. The Hague, Mastinus Nijhoff Publishers .pp.328-334.

[4] Golshani M, Taheri S, Eslami G, Suleimani Rehber AA, Fallah F, Goudarzi H (2006) Genital tract infection in asymptomatic infertile men and its effect on semen quality. Iranian j. publ. health 35: 81-84.

[5] Pellati D, Mylonakis I, Bertoloni G, Fiore C, Andrisani A, Ambrosini G, Armanini D (2008) Genital tract infections and infertility. Eur. j. obstet. gynecol. reprod. biol. 140: 3-11.

[6] Menkveld R, Wong WY, Lombard CJ, Wetzels AMM, Thomas CMJ, Merkus HMWM, Steegers-Thuenissen RPM (2001) Semen parameters, including WHO and strict criteria morphology, in a fertile and subfertile population: an effort towards standardization of in-vivo thresholds. Hum. reprod. 16: 1165-1171.

[7] Cunningham KA, Beagley KW (2008) Male genital tract chlamydial infection: implications for pathology and infertility. Biol. reprod. 79:180-189.

[8] Bartoov B, Ozbonfil D, Maayan MC, Ohad E, Nitzan Y (1991) Virulence characteristics of male genital tract *Escherichia coli* isolated from semen of suspected infertile men. Andrologia 23: 387-394.

[9] Comhaire F, Verschraegen G, Vermeulen L (1980) Diagnosis of accessory gland infection and its possible role in male infertility. Int. j. androl. 3: 32-45.

[10] Huwe P, Diemer T, Ludwig M, Liu J, Schiefer HG, Weidner W (1998) Influence of different uropathogenic microorganisms on human sperm motility parameters in an *in vitro* experiment. Andrologia 301: 55-59.

[11] Khalili MB, Sharifi-Yazdi MK (2001) The effect of bacterial infection on the quality of human's spermatozoa. Iranian j. publ. health 30: 119-122.

[12] Paulson JD, Polakoski KL (1977) Isolation of a spermatozoal immobilization factor from *Escherichia coli* filtrates. Fertil. steril. 28: 182-185.

[13] Diemer T, Weidner W, Michelmann HW, Schiefer HG, Rovan E, Mayer F (1996) Influence of *Escherichia coli* on motility parameters of human spermatozoa in vitro. Int. j. androl. 19: 271-277.

[14] Fowler JE Jr, Mariano M (1984) Difficulties in quantitating the contribution of methral bacteria to prostatic fluid and seminal fluid cultures. J. urol. 132:471-473.

[15] Emokpae NA, Uadia PO, Sadiq NM (2009) Contribution of bacterial infection to male infertility in Nigerians. OJHAS 8:6. Available at: http://openmed.nic.in/3277/01/2009-1-6.pdf

[16] Ohri M, Prabha V (2005) Isolation of sperm agglutinating factor from *Staphylococcus aureus* isolated from a woman with unexplained infertility. Fertil. steril. 84: 1539-1541.

[17] Laemmeli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685.

[18] Prabha V, Gupta T, Kaur S, Kaur N, Kala S, Singh A (2009) Isolation of a spermatozoal immobilization factor from *Staphylococcus aureus* filtrates. Can. j. microbiol. 77: 874-878.

[19] Lowry OH, Rosebrough Al, Randall RJ (1951) Protein measurement with Folin-Phenol reagents. J. biol. chem. 193: 265-275.

[20] Prabha V, Thakur N, Kaur S, Kaur N, Singh A, Kala S (2009) Agglutination of human spermatozoa due to human semen culture bacterial isolates bearing sperm ligand. Am. j. biomed. sci. 1: 126-132.

[21] Prabha V, Sandhu R, Kaur S, Kaur K, Sarwal A, Mavuduru RS, Singh SK (2010) Mechanism of sperm immobilization by *Escherichia coli*. Adv. urol. 2010:1-6. doi: 10.1155/2010/240268.

[22] Kaur S, Prabha V, Sarwal A (2010) Receptor mediated agglutination of human spermatozoa by spermagglutinating factor isolated from *Staphylococcus aureus*. J. urol. 184: 2586-2590.

[23] Prabha V, Chaudhary N, Kaur S (2011) Molecular mimciry between bacteria and spermatozoa. J. urol.186: 2442-2447.

[24] Kaur K, Kaur S, Rishi P, Singh SK, Prabha V (2012) Evidence for the occurrence of receptor in sperm for spermagglutinating factor isolated from *Escherichia coli.* Gynecol. endocrinol.-int. j. gynecol. obstet. 34: 207-209.

[25] Kumar V, Prabha V, Kaur S, Kaur K, Singh SK (2011). Receptor dependent immobilization of spermatozoa by sperm immobilization factor isolated from *Escherichia coli:* Proof of evidence. Int. j. urol. 18: 597–603.