Contraceptive Sperm Agglutinating Proteins Identified in *Staphylococcus warneri*, Natural Microflora of an Infertile Woman

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Abstract *Staphylococcus warneri*, isolated from the cervix of an adult female with unexplained infertility, was found to agglutinate human spermatozoa in vitro leading to their death. A genomic library of *S. warneri* was generated using pSMART-*Escherichia coli* vector-host system. Approximately 3500 transformants were screened and four showed sperm agglutinating activity. Sperm agglutinating proteins (SAPs) were partially purified from the positive transformants and were found to agglutinate sperms in vitro. Cloned ORFs in positive transformants were sequenced and ORF finder identified them as endonuclease, accessory secretory protein-Asp1, accessory secretory protein-Asp2 and signal transduction protein. Mannose was found to competitively inhibit sperm agglutination, indicating that SAPs in *S. warneri* bind to mannose in glycoprotein receptors on the surface of sperms for agglutination. This is the first report on identification of SAPs which may be responsible for unexplained infertility in women and may be used as contraceptive agents.

Keywords *Staphylococcus warneri* · Spermicide · SAPs · Sperm agglutination · Contraceptive

Introduction

The world population has risen to an alarming level, which in turn has left nations bursting at the seams in terms of population density thus affecting the society and environment severely [1]. It is estimated that each year there are approximately 210 million pregnancies worldwide, of which 80 million are unintended and over 46 million end in abortions [2]. Besides population explosion, unintended pregnancies and elective abortions are major public health issues. A safe and effective female contraceptive method is a promising way out. The characteristics of an ideal contraceptive should have certain characteristics which include effectiveness, no side effects, no loss of natural feeling, being relatively inexpensive, easy to use and reversible. The contraceptive methods available for women include oral contraceptive (OC) pills, vaginal ring, barrier methods, surgical sterilization, injectable contraceptives, intrauterine devices, but is associated with many side effects which include irregular bleeding, weight increase, queasiness, and mood alterations. Vaginal contraceptives may be considered as the key alternative to all these problems. Advantages of this approach include no loss of natural feeling as compared to condoms, no adverse side effects like those coupled with hormonal contraceptives, no bleeding or pain as is the case with IUDs, no need of professional intervention because it is do-it-yourself method and is inexpensive, reversible and easy to use. Spermicidal agents from natural sources such as plants (saponins) [3], animals (Immotilin, Magainin-A) [4–6] and microorganisms (Nisin, Lacticin 3147, Gramicidin D, Subtilisin) [7–12] have been identified. Various studies have shown the role of diverse recombinant proteins like rZP3, rFA-1, rhESP, rDE, repin, rTSA-1, rNZ-1 and rNZ-2 to obstruct the pregnancy in different in vivo studies. Nonetheless, all these proteins are

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in preliminary phases of advancement. In this way, the hunt for more effective and safer alternatives is still on. In this context, sperm agglutinating *Staphylococcus warneri* was isolated from the cervix of an infertile woman. Its genomic library was generated and sperm agglutinating proteins (SAPs) were identified. These SAPs were found to interact with mannose containing glycoprotein receptors on the surface of sperms for agglutination.

Material and Methods

Bacterial Strain and Plasmid

Staphylococcus warneri was isolated from the cervix of an adult woman with unexplained infertility and identified by MALDI Microflex LT mass spectrometer [13]. Sample profiles were compared with the MALDI Biotyper database. It was maintained in Brain Heart Infusion broth. Plasmid pSMART and *Escherichia coli* DH10 β strains were used for the cloning and expression of recombinant proteins.

Semen Samples

Semen samples were obtained from healthy donors from Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh, India. Ejaculates were obtained by masturbation following a 24 h continence period. Only those ejaculates showing normal sperm parameters (motility and count) according to WHO [14] criteria were used. The sperm count was determined with a hemocytometer and was adjusted to 40×10^6 spermatozoa ml $^{-1}$ with sterile PBS. The experimental protocols were approved by the Institutional Ethics Committee, PGIMER, Chandigarh and were carried out in accordance with the committee's guidelines. An informed consent was obtained from all the subjects before participation in the study.

Sperm Agglutination by *S. warneri*

Staphylococcus warneri was grown in Brain Heart Infusion (BHI) at 37 °C/180 rpm for 72 h, centrifuged at $10,000 \times g$ for 10 min at 4 °C and the supernatant was passed through a 0.22 μ m Millipore filter to ensure that it was cell free. The bacterial cells in pellet were washed twice and resuspended in sterile PBS. Equal volumes of semen sample (40×10^6 spermatozoa ml $^{-1}$) and whole cell culture or washed cells (10^7 cells ml $^{-1}$) or cell free supernatant were mixed and incubated at 37 °C for 0, 15, 30, 60, 120 and 240 min. 10 μ l aliquot was observed for agglutination at 400 $\times$ magnification under light

microscope after each time interval. Sterile BHI was used as control.

Construction of Genomic Library

Chromosomal DNA was isolated [15] and partially restricted with HaeIII. The digest was run on a preparative gel and the agarose gel, containing fragments (2–6 kb), was excised with sterile blade to extract DNA using the commercial QIAquick Gel Extraction kit (QIAGEN). Ligation was carried out with the linear pSMART vector (Lucigen) and transformation was done in electrocompetent *E. coli* DH10 β cells (Lucigen).

Screening of Transformants

Each transformant was grown in LB-Kanamycin (25 μ g ml $^{-1}$) for 72 h at 37 °C/150 rpm. The cells were centrifuged, washed with and suspended in PBS, sonicated and the extracellular, intracellular fractions and cell debris in PBS (100 μ l each) were incubated with 100 μ l of semen sample containing 40×10^6 spermatozoa ml $^{-1}$ at 37 °C for 0, 15, 30, 60, 120 and 240 min. After each time interval, 10 μ l aliquot was observed for sperm agglutination.

Partial Purification of Recombinant Sperm Agglutinating Proteins (rSAPs)

The recombinant cells were grown in 1 L LB-kanamycin for 72 h at 37 °C/150 rpm, washed twice and suspended in PBS, sonicated and centrifuged at $10,000 \times g$ for 20 min at 4 °C. Both the extracellular and intracellular supernatants were checked for sperm agglutinating activity. Further, fractionation of intracellular milieu was done with ammonium sulphate to get 20, 40, 60, 80 and 100% saturation and dialysed fractions were checked for activity. The bioactive fractions were further purified by Sephadex G-200 gel permeation chromatography. Partially purified rSAPs were checked for sperm agglutination and by spermicidal activities. To evaluate the spermicidal effect, 10 μ l of the (100 μ g) rSAP was mixed with 10 μ l of eosin (0.5%) and 10 μ l sperms (40×10^6 spermatozoa ml $^{-1}$) and examined under the light microscope (400 $\times$). The results were analyzed as live spermatozoa that appeared unstained and dead spermatozoa that were stained pink due to the damaged membrane that allowed the dye to enter.

In Silico Analysis

Inserts from transformants showing sperm agglutinating activity were sequenced and searched for ORFs by NCBI ORF finder. Multalin and BLAST searches were used to find their identity and relatedness to other known proteins.

Physicochemical parameters were calculated by ProtParam online tool. Secondary and tertiary structures of SAPs were predicted by GOR IV and SWISS MODEL online tools respectively. RAMPAGE was used to validate the three-dimensional structure.

Effect of Sugars on Agglutination

Effect of mannose, glucose, galactose, sucrose, fructose and dextrose on sperm agglutination was examined [16]. Briefly, partially purified recombinant protein (20 µg) was incubated with sugar (2–20 mM) for 30 min at room temperature before mixing with 0.1 ml of semen sample (40×10^6 spermatozoa ml^{-1}) at 37 °C for 2 h. As controls, 0.1 ml of semen sample (40×10^6 spermatozoa ml^{-1}) was mixed with 0.1 ml of PBS or 20 mM sugar (negative controls) or with partially purified recombinant protein (positive controls). On completion of incubation, 10 µl sample was checked for agglutination at $\times 400$ magnification using light microscope.

Results

Staphylococcus warneri Agglutinated Human Sperms

When semen samples were mixed with 72 h old whole cell culture, washed cells or cell supernatant, continuous decline in the percentage of motile sperm and concomitant increase in the percentage of agglutinated sperm was observed in case of cell culture and washed cells, while the cell free supernatant failed to do so. Complete sperm agglutination was recorded with 72 h old whole cell culture and washed cells after 60 min of incubation (Fig. 1).

Cloning of Sperm Agglutinating Proteins in *E. coli*

Approximately 3500 transformants were screened for sperm agglutinating activity in vitro and four were found to give positive results in intracellular fractions (Fig. 3). No activity was found to be associated with extracellular supernatant or cell debris. Plasmids from the positive transformants were isolated, restricted with EcoRI and HindIII to check the insert size (Suppl. Fig. S1) and the inserts were sequenced. ORF finder and BLAST results revealed the ORFs encoding endonuclease, accessory secretory protein-Asp1, accessory secretory protein-Asp2 and signal transduction protein (Table 1).

Partial Purification of Recombinant Sperm Agglutinating Proteins

The results showed that most of the sperm agglutinating activity was present in precipitates from 40 to 60% saturation. The precipitated proteins were re-dissolved and dialyzed against PBS (50 mM, pH 7.2) and subjected to further purification through Sephadex G-200 column (Suppl. Fig. S2). Agglutinating activity was present in the partially purified recombinant proteins from all the four transformants (Fig. 2). The effect of SAPs on sperm viability was also evaluated by eosin staining and observing under light microscope. All the partially purified proteins at a concentration of 100 µg induced agglutination along with complete loss of viability of spermatozoa within 30 min of incubation (Fig. 3). The proteins encoded by ORFs found in positive transformants were analyzed for their physicochemical parameters (Suppl. Table S1), secondary structures (Suppl. Table S1) and tertiary structures of all these proteins were also predicted (Table 1).

Fig. 1 Representative photomicrograph showing ($40\times$ magnification) **a** Normal human spermatozoa incubated with Phosphate buffer saline **b** Agglutinated spermatozoa incubated with *Staphylococcus warneri* whole cell culture/washed cells

Table 1 Prediction of three dimensional structure of SAPs

SAP in <i>S. warneri</i> (Accession ID)	Template	Seq. Identity (%)	GMQE	QMEAN	Structure	RAMPAGE validation
Endonuclease (WP_019235868.1)	Crystal structure of a taq muts-dna-adp ternary complex from <i>Thermus aquaticus</i> (1fw6)	21.60	0.40	−3.45		Residues in most favoured region: 839 (88.5%) Residues in additional allowed regions: 94 (9.9%) Residues in generously allowed region: 11 (1.2%) Residues in disallowed regions: 4 (0.4%)
Accessory secretory protein-Asp1 (AGC89469.1)	Crystal structure of accessory Sec system protein 1 <i>Streptococcus gordonii</i> (5vaf.1.D)	33.2	0.67	−5.11		Residues in most favoured region: 371 (82.3%) Residues in additional allowed regions: 72 (16%) Residues in generously allowed region: 5 (1.1%) Residues in disallowed regions: 3 (0.7%)
Signal transduction protein (Q8GQQ1.1)	Structure of a major regulator of <i>Staphylococcal pathogenesis</i> (4AE5)	68.90	0.77	−1.21		Residues in most favoured region: 140 (95.2%) Residues in additional allowed regions: 7 (4.2%) Residues in generously allowed region: 0 (0%) Residues in disallowed regions: 0 (0%)
Accessory secretory protein Asp2 (AGC89468.1)	S-formylglutathione hydrolase <i>Saccharomyces cerevisiae</i> (4flm.1.A)	19.8	0.17	−7		Residues in most favoured region: 155 (76%) Residues in additional allowed regions: 40 (19.6%) Residues in generously allowed region: 6 (2.9%) Residues in disallowed regions: 3 (1.5%)

Effect of Sugars on Sperm Agglutination

Mannose has been reported to mediate *E. coli* and spermatozoa interactions resulting in sperm agglutination [16]. Out of all the sugars tested, mannose alone was found to inhibit human sperm agglutination (Table 2). Sperms incubated with mannose showed no agglutination (Fig. 4a). However, partially purified recombinant proteins

(endonuclease shown as representative) incubated with human spermatozoa showed significant agglutination (Fig. 4b) but recombinant protein incubated with mannose for 30 min and then adding sperms showed significantly decreased agglutination (Fig. 4c).

Fig. 2 Representative photomicrograph showing (40X magnification) **a** Normal human spermatozoa incubated with intracellular fraction of *Escherichia coli* DH10 β host culture; **b** Agglutinated spermatozoa after incubation with intracellular fraction of positive

transformants; **c** Agglutinated spermatozoa with partially purified sperm agglutinating protein from positive transformant (endonuclease)

Fig. 3 Representative photomicrograph showing eosin staining of human spermatozoa after incubation with **a** Phosphate buffer saline (live spermatozoa remain unstained) **b** Partially purified sperm agglutinating protein (endonuclease)-Pink stained dead spermatozoa

Table 2 Effect of mannose on inhibition of sperm agglutinating protein (endonuclease)—mediated sperm agglutination

Inhibition of agglutination after different incubation periods				
Mannose (mM)	10 min	30 min	1 h	2 h
2	—	—	—	+
4	—	—	—	++
8	—	—	—	++
10	—	+	++	+++
20	—	++	+++	+++

— represents no inhibition of agglutination; + represents 20–40%, ++ 40–60% and +++ 60–80% inhibition of agglutination

Discussion

Many contraceptive routes are available but still a better, safe and efficient method is required. Other than physical and chemical, biological agents (from plants, animals or microorganisms) are gaining much attention in this field. Microbial agents like nisin [6–9], lactacin [10], gramicidin D [11] and subtilisin [12] have shown excellent spermicidal activity. These microbial peptides affect the sperm

motility and availability by either agglutinating or immobilizing them. In this study, *S. warneri* was isolated from the cervix of an adult female with unexplained infertility and identified by MALDI [13] which is emerging as a highly accurate method for bacterial identification and is used besides other than bacterial identification procedures like biochemical assays and use of biomarkers such as *rrs* (16S rRNA) and other species-specific genes e.g. *recA* [17]. Though *Staphylococcus* is a natural microflora, yet it is a source of potentially dangerous nosocomial infections caused by antibiotic resistant strains that form rigid biofilms which can be targeted by exploring quorum quenching mechanism [18–23]. *Staphylococcus warneri* contained multiple proteins responsible for complete sperm agglutination. The spermatozoa were agglutinated by whole culture and washed cells but not by supernatant showing that the agglutinating capacity was not associated with any microbial metabolite but either with the surface or intracellular proteins. As the fertilization potential of spermatozoa majorly depends upon their motility, so factors affecting this parameter have huge potential to be exploited as contraceptive. To identify the proteins

Fig. 4 Effect of mannose on human sperm agglutination. **a** sperms incubated with 20 mM mannose; **b** sperms incubated with partially purified recombinant protein (20 µg)—endonuclease shown as

representative; **c** sperms incubated with mannose followed by partially purified recombinant protein (endonuclease)

responsible, genomic library of *S. warneri* was generated and transformants were screened (whole culture, washed cells and intracellular soluble fraction) for sperm agglutinating activity. The library yielded four positive transformants. Partially purified, intracellular proteins from positive transformants agglutinated sperms completely and also showed complete (100%) spermicidal effect. The results indicate that *S. warneri* produces multiple proteins that can interact with human sperms in vagina and agglutinate them. *S. warneri* possibly interacts with sperms and agglutinates via (1) cell surface proteins which are yet to be identified (as indicated by agglutination by washed *S. warneri* cells) and (2) intracellular proteins (as identified in case of transformants obtained in the present study; which might be released following lysis of *S. warneri* cells in vagina). The extensive literature survey revealed various studies which have indicated a direct interaction between spermatozoa and *E. coli* i.e. adhesion of *E. coli* to spermatozoa and subsequent agglutination along with structural damage [24–27]. This has been reported to be mediated by mannose-binding structures present on both spermatozoa and *E. coli* [16]. We have also observed similar results i.e. mannose upon incubation with human spermatozoa and partially purified SAP prevented agglutination further providing an evidence for the above hypothesis, that mannose present in glycoproteins on human sperm surface could possibly be interacting with SAPs to cause agglutination. This is the first report providing the insights to the proteins responsible for sperm agglutination in vitro. Further in vivo evaluation in mice is going on in our laboratory that can establish their vaginal contraceptive potential.

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