Journal of Neonatal Surgery
ISSN(Online): 2226-0439
Vol. 14, Issue 10s (2025)
https://www.jneonatalsurg.com

OPEN ACCESS

Microbial Load Reduction and Sperm Quality Improvement Through Modified Gradient-Based Protocols

Satyanarayan Samantaray[*1], Soumya Jal[1], Gopal Krishna Purohit[2], Soumya Dash[3]

[*1]School of Paramedics and Allied Health Sciences, Centurion University of Technology and Management, Jatani, Khurda, Odisha, India

[2]Heredity Biosciences, Plot No- 818, Mayfair Lagoon Road, Jayadev Vihar, Bhubaneswar, Odisha, India

[3]Fertility specialist, Ankura Medical and research Center, Near Sisubhavan Bus Stop, Bapuji Nagar, Bhubaneswar, Odisha 751009, India

[*]**Corresponding author:**

Satyanarayan Samantaray

School of Paramedics and Allied Health Sciences, Centurion University of Technology and Management, Jatani, Khurda, Odisha, India

Email ID: satyanarayansamantaray544@gmail.com

Cite this paper as: Satyanarayan Samantaray, Soumya Jal, Gopal Krishna Purohit, Soumya Dash, (2025) Microbial Load Reduction and Sperm Quality Improvement Through Modified Gradient-Based Protocols. *Journal of Neonatal Surgery*, 14 (10s), 1043-1053.

ABSTRACT

Infertility affects 15% of couples worldwide, with male infertility accounting for half of cases. Bacteriospermia, involving bacteria in semen, can compromise sperm quality and fertilization. In assisted reproductive technologies (ART), effective sperm preparation methods are crucial to improve sperm quality and reduce bacterial contamination. This research evaluated sperm preparation methods for bacterial clearance. Ninety semen samples were categorized into four groups: density-gradient centrifugation method (DGCM1), swim-up technique (SUT2), combination of density-gradient and swim-up technique (DGCM1SUT3), and unprocessed raw semen (UPRS4) as control. Bacterial cultures were grown on MacConkey Agar and Blood Agar, with Colony-Forming Unit (CFU) counting, biochemical identification, and antimicrobial testing. Initially, 22.5% (25/90) of samples were bacteria-free. Post-preparation, bacterial clearance rates were 19.8% for DGCM1, 10.8% for SUT2, and 27.9% for DGCM1SUT3, showing the combined method's effectiveness. Among 37 bacterial strains identified, *Staphylococcus sp.* and *Streptococcus sp.* were most common. In DGCM1, 24.3% of *staphylococci* and 71.2% of *streptococci* remained, while SUT2 showed 14.3% and 25.1% persistence. DGCM1SUT3 eliminated *Staphylococcus sp.* and reduced *Streptococcal* presence to 1.3%, proving most effective. Sperm DNA fragmentation analysis showed lower fragmentation index for DGCM1SUT3, indicating better sperm integrity. These results highlight Bacteriospermia's impact on fertility and need for optimized sperm processing in ART. The combined method demonstrated highest bacterial clearance and DNA integrity preservation, making it preferred in fertility clinics. Future studies should examine bacterial load, sperm DNA fragmentation, ART success rates, and explore targeted antibiotic treatments for severe Bacteriospermia.

Keywords: *Bacteriospermia, bacterial clearance, assisted reproductive technology, sperm motility, sperm DNA fragmentation*

1. INTRODUCTION

Infertility is a significant global health issue, affecting about 15% of couples, with male factor infertility responsible for nearly half of these cases (1). Bacteriospermia, or the presence of bacteria in semen, has gained attention due to its harmful effects on sperm motility, morphology, DNA integrity, and fertilization potential (2). Bacteria such as *Escherichia coli*, *Staphylococcus sp*., *Streptococcus sp*., and *Klebsiella pneumoniae* can negatively affect sperm quality through oxidative stress, inflammatory responses, and direct interactions between sperm and bacteria (3). These pathogens in semen have been associated with lower success rates in ART, especially in procedures like intrauterine insemination (IUI) and in vitro fertilization (IVF) (4).

In assisted reproductive technologies (ART), effective sperm preparation techniques are crucial for removing bacteria and enhancing sperm quality before fertilization. Common methods include density-gradient centrifugation (DGC) and the swim-up technique (SUT), which aim to select high-quality, motile sperm while eliminating debris and bacterial contaminants (5, 6). The effectiveness of these methods in clearing bacteria is uncertain, with studies showing varying success rates (7). Recent developments suggest that combining DGC and swim-up (DGC-SUT) might be more effective in improving bacterial clearance while maintaining sperm integrity (8, 9).

Bacteriospermia's impact on sperm DNA integrity is another concern, as DNA fragmentation can reduce fertilization rates, hinder embryo development, and increase early pregnancy loss risk (10). High levels of reactive oxygen species (ROS) produced by bacterial contamination lead to oxidative DNA damage, compromising sperm function (11). Evaluating the sperm DNA fragmentation index (DFI) alongside bacterial clearance offers insights into the effectiveness of sperm preparation techniques (12). Moreover, recent studies have highlighted the potential for certain bacteria to directly interact with sperm cells, altering their motility and viability. These interactions may involve the secretion of bacterial toxins or the formation of biofilms on sperm surfaces, further compromising fertility outcomes (13-16). Understanding these complex bacteria-sperm interactions is crucial for developing targeted interventions to mitigate the effects of bacteriospermia on male reproductive health (17).

This study aims to assess the efficiency of different sperm preparation techniques in eliminating bacterial contamination and preserving sperm DNA integrity. A total of 90 semen samples were divided into four groups: density-gradient centrifugation (DGCM1), swim-up technique (SUT2), combined density-gradient and swim-up (DGCM1SUT3), and unprocessed raw semen (UPRS4) as a control. After processing, bacterial cultures were conducted using MacConkey Agar, Blood Agar, and CFU enumeration, followed by biochemical identification tests (Catalase, Coagulase, and Oxidase) and antimicrobial susceptibility testing. Additionally, sperm DNA fragmentation analysis was performed to evaluate the impact of each method on sperm quality. This study provides crucial understandings into optimizing ART procedures for treating male infertility. The findings could aid in improving bacterial clearance, sperm integrity, and fertilization success rates, offering better reproductive outcomes for couples undergoing ART.

2. METHODOLOGY

Sample Collection

Between July 2024 and January 2025, ninety semen samples were gathered from male patients at a fertility clinic who were undergoing assessments for infertility. These samples were obtained from individuals being evaluated for fertility concerns at the Ankura Fertility Centre in Bhubaneswar. The semen was collected through masturbation following a recommended sexual abstinence period of 3-5 days, in line with the World Health Organization (WHO) guidelines for semen analysis. Each sample was placed in a sterile, wide-mouthed, non-toxic container and swiftly transported to the andrology laboratory for analysis within an hour of collection. The semen samples were immediately divided into aliquots from the same ejaculate. One aliquot underwent microbial culture before processing, while the other was processed using the developed method and then analysed post-processing. This approach ensured accurate paired comparisons for assessing bacterial reduction and improvements in sperm parameters.

Semen Processing and Experimental Design

To evaluate the efficiency of bacterial clearance in different sperm preparation techniques, each semen sample was divided into four equal parts (1 mL each) and processed using distinct methods. The first group Density-Gradient Centrifugation Method (DGCM1) – This method involved layering the semen sample over a two-phase density gradient medium (40% and 80%) and centrifuging at 300 × g for 20 minutes. The pellet, containing highly motile sperm, was collected, washed with a sperm washing medium, and centrifuged again to remove residual contaminants.

For the second group, the Swim-Up Technique (SUT2) was employed. This method involved carefully placing 1 mL of liquefied semen underneath 1 mL of fresh culture medium. The mixture was then incubated at 37°C in an environment containing 5% CO_2 for a duration of 45 minutes. During this process, only the most motile sperm cells were able to migrate into the upper layer. This upper layer was subsequently extracted with care for further analysis.

The third group Combined Density-Gradient and Swim-Up (DGCM1SUT3) – This group underwent a sequential process, where sperm were first isolated using density-gradient centrifugation followed by the swim-up technique, ensuring maximum bacterial clearance while selecting the most motile and morphologically normal sperm.

The fourth group Unprocessed Raw Semen (UPRS4 - Control Group) – This group remained untreated and was analysed to establish the baseline levels of bacterial contamination and sperm quality.

Density-Gradient Centrifugation- Group-1

The density-gradient centrifugation method (DGCM1) was utilized for sperm preparation, using a two-layer gradient system with 80% and 40% PureSperm® solution (Nidacon, Sweden). First, 1 mL of 80% PureSperm was pipetted into a sterile

conical centrifuge tube, followed by 1 mL of 40% PureSperm on top. Then, 1 mL of liquefied semen was placed on the 40% gradient layer, without disturbing the interface. Samples were centrifuged at 300 × g for 20 minutes, separating motile sperm from non-motile sperm, debris, and impurities. After centrifugation, the sperm pellet was collected and washed twice with 5 mL of Quinn's™ Sperm Washing Medium (CooperSurgical, USA), followed by centrifugation at 300 × g for 10 minutes. The final purified sperm pellet was resuspended in 0.5 mL of HTF medium for bacterial testing and analysis. This technique selects highly motile, morphologically normal sperm, enhancing quality for assisted reproductive technologies (ART) while minimizing bacterial contamination.

Swim-Up Technique – Group-2

The swim-up technique (SUT1) selectively isolates highly motile spermatozoa while removing immotile sperm, debris, and bacteria. In this study, 1 mL of liquefied semen was carefully layered at the bottom of a sterile conical tube containing 1 mL of Quinn's™ Sperm Washing Medium (CooperSurgical, USA), ensuring minimal disturbance to prevent premature mixing. The sample was incubated at 37°C in a 5% CO_2 environment for 1 hour, allowing motile sperm to swim upward into the upper fraction while immotile sperm and debris remained at the bottom. After incubation, 0.5 mL of the uppermost layer was carefully aspirated using a micropipette, ensuring the underlying sediment was not disturbed. The recovered fraction, rich in progressively motile sperm, was then subjected to bacterial analysis to assess the effectiveness of the swim-up technique in bacterial clearance.

Combination of DGC and Swim-Up – Group-3

The combination of density-gradient centrifugation and swim-up techniques (DGCM1SUT3) was used to enhance bacterial clearance and sperm selection. Density-gradient centrifugation (DGCM1) was first performed, as in Group-1, to separate motile sperm from debris, immotile sperm, and contaminants. The sperm pellet was then subjected to the swim-up technique (SUT2), following Group-2 protocol, to further isolate highly motile and morphologically normal sperm while eliminating non-motile sperm and bacteria. The final sperm suspension obtained after DGCM1SUT3 was used for bacterial culture and analysis, ensuring a comprehensive assessment of bacterial clearance efficiency.

Bacterial Cultures and Growth Conditions

Bacterial cultures were conducted using selective and differential media, such as MacConkey Agar (MAC), Blood Agar (BA), and Colony-Forming Unit (CFU) counting. Samples were incubated at 37°C for 24 to 48 hours in aerobic conditions before counting colonies and identifying bacteria. For microbial evaluation, semen samples were cultured on Blood Agar and MacConkey Agar to isolate and quantify aerobic bacterial colonies, while Sabouraud Dextrose Agar was used for detecting fungi. After incubation, the colony-forming units (CFUs) were counted and compared before and after processing. Although this study did not involve any assisted reproductive technology (ART) cycles using the processed samples, the sperm preparation method was designed to meet ART-ready standards. Future research will include ART cycle data to evaluate fertilization, cleavage, and implantation outcomes related to microbiologically optimized semen samples.

MacConkey Agar (MAC) – Selective for Gram-Negative Bacteria

MacConkey agar was used to selectively isolate Gram-negative uropathogens and differentiate lactose fermenters from non-lactose fermenters. A sterile inoculating loop was used to streak 100 µL of semen sample onto MacConkey agar plates. Plates were incubated at 37°C for 24–48 hours under aerobic conditions. Colonies were observed, and lactose fermentation ability was noted based on colony colour.

Blood Agar (BA) – Differentiation of Haemolytic Activity

Blood agar was used to culture both Gram-positive and Gram-negative bacteria and differentiate them based on haemolytic patterns. A 100 µL aliquot of semen sample was spread on Blood agar plates using the spread plate technique. Plates were incubated at 37°C for 24–48 hours, and haemolytic activity was recorded.

Colony-Forming Unit (CFU) Counting – Bacterial Load Assessment

To quantify bacterial load in semen samples before and after sperm preparation, Colony-Forming Unit (CFU) counts were performed. Serial dilutions from 10^−1 to 10^−6 were prepared using sterile phosphate-buffered saline (PBS). From each dilution, 100 µL was plated onto MacConkey Agar and Blood Agar using the spread plate technique. Plates were incubated at 37°C for 24 hours. Visible bacterial colonies were counted. CFU per millilitre (CFU/mL) was calculated using the formula: CFU/mL = (Number of colonies × Dilution factor) / Volume plated (mL). A bacterial load ≥10^4 CFU/mL was classified as significant bacteriospermia. This method quantified bacterial contamination and assessed sperm preparation techniques' efficacy in bacterial clearance.

Biochemical Identification Tests for Bacterial Isolates

To confirm the identity of bacteria isolated from semen samples, biochemical identification tests were performed. The Catalase, Coagulase, and Oxidase tests were used to differentiate between Gram-positive and Gram-negative bacterial species, aiding in the identification of common uropathogenic bacteria affecting sperm quality and fertility.

Catalase Test

The catalase test differentiates catalase-positive organisms from catalase-negative organisms. Catalase is an enzyme that decomposes hydrogen peroxide (H_2O_2) into water and oxygen, producing bubbles. Using a sterile inoculating loop, pick a colony of the bacterial isolate from an agar plate. Place the colony on a clean, dry glass slide. Add 1–2 drops of 3% hydrogen peroxide (H_2O_2) to the bacterial smear. Observe for the production of oxygen bubbles (effervescence).

Coagulase Test

The coagulase test is used to differentiate pathogenic *Staphylococcus aureus* (coagulase-positive) from non-pathogenic *Staphylococcus epidermidis* and other coagulase-negative staphylococci (CoNS). Coagulase is an enzyme that converts fibrinogen to fibrin, leading to clot formation. Prepare two test tubes containing 0.5 mL of sheep plasma. Take a loopful of the bacterial isolate and mix it well into one of the tubes. The second tube serves as a control. Incubate at 37°C for 2–4 hours and check for clot formation. If negative, extend incubation to 24 hours.

Oxidase Test

The oxidase test is used to identify bacteria that produce the cytochrome c oxidase enzyme, which participates in the electron transport chain. It differentiates oxidase-positive bacteria from oxidase-negative bacteria. Using a sterile loop, pick a colony from a fresh bacterial culture (not older than 24 hours). Place the colony onto a piece of filter paper or a sterile glass slide. Add 1–2 drops of oxidase reagent (1% tetramethyl-p-phenylenediamine dihydrochloride). Observe for a colour change within 30 seconds.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing (AST) was performed to evaluate the resistance patterns of bacterial isolates recovered from semen samples. The Kirby-Bauer disk diffusion method was used on Mueller-Hinton agar following Clinical and Laboratory Standards Institute (CLSI) guidelines. Antibiotic disks, including amoxicillin, Cefixime, ciprofloxacin, gentamicin, Imipenem, ceftriaxone, and azithromycin, were placed on inoculated plates and incubated at 37°C for 24 hours. Zones of inhibition were measured to classify bacteria as susceptible, intermediate, or resistant.

Sperm DNA Fragmentation Analysis

Sperm DNA fragmentation (SDF) was assessed using the Sperm chromatin dispersion method, following the manufacturer's protocol (APS labs, India). Semen samples were liquefied and washed with sperm wash media or saline. The pellet was diluted to 5 million sperm/mL. An agarose gel tube was melted at 90°C for 2–3 minutes, then equilibrated at 37°C for 4 minutes. A 100 µL aliquot of diluted semen was added to the agarose tube, mixed, and 150–200 µL was pipetted onto a pre-coated slide. A coverslip was placed and cooled at 4°C for 5 minutes. The coverslip was removed, and denaturation solution was added for 5 minutes at 4°C, followed by lysis solution for 5–7 minutes at room temperature. The slide was dehydrated using an ethanol series (70%, 90%, 100%) for 2 minutes each, air-dried, and stained. After 15 minutes, slides were rinsed, air-dried, and observed under a light microscope at ×1000 magnification. A minimum of 500 sperm cells per sample were evaluated. Sperm with large or medium halos were classified as intact DNA, while those with small or no halos were considered fragmented. The SDF index (DFI%) was calculated, with a threshold of >30% indicating high DNA damage.

Statistical Analysis

To evaluate sperm preparation techniques' effectiveness in bacterial clearance, removal rates were calculated as percentages. One-way ANOVA compared bacterial clearance efficiency among groups, followed by post-hoc Tukey's test to identify significant differences. A p-value <0.05 was considered significant, indicating meaningful reduction in bacterial contamination between groups. Statistical analyses were conducted using SPSS software (version 25.0, IBM Corp.). These findings helped determine the most effective sperm preparation method in eliminating bacterial contaminants, optimizing protocols for assisted reproductive technologies (ART).

3. RESULTS

Semen Sample Collection and Initial Quality Assessment

A total of 90 semen samples were collected from male patients undergoing fertility evaluation. The samples were analysed based on World Health Organization (WHO) guidelines for semen parameters, including sperm concentration, motility, and morphology (Table-1).

Out of 90 samples, 25 (27.7%) were free from bacterial contamination, while 65 (72.2%) contained bacterial pathogens before sperm processing. The initial sperm quality and bacterial load were recorded before processing.

Table 1: Initial Semen Quality and Bacterial Contamination Before Processing

Parameter	Mean ± SD	Reference Range (WHO, 2020)
Volume (mL)	2.8 ± 0.5	≥1.5 mL
Sperm Concentration (10^6/mL)	38.6 ± 3.2	≥15 × 10^6/mL
Progressive Motility (%)	42.3 ± 5.6	≥32%
Normal Morphology (%)	7.5 ± 1.3	≥4%
Bacteria-Free Samples (%)	22.5% (25/90)	-
Bacteria-Contaminated Samples (%)	72.2% (65/90)	-

Effect of Different Semen Processing Methods on Sperm Quality and Bacterial Clearance

Following sample collection, each semen specimen was divided into four equal aliquots to assess bacterial clearance across different sperm-washing techniques. The first group was processed using Density-Gradient Centrifugation (DGCM1), the second with the Swim-Up Technique (SUT2), and the third group underwent a combination of both methods (DGCM1SUT3). The fourth group remained untreated as Unprocessed Raw Semen (UPRS4), serving as the control group for baseline microbial and sperm quality comparison (Table-2).

After processing, sperm quality parameters and bacterial clearance were evaluated for each method.

Table 2: Sperm Quality Parameters After Different Processing Methods

Parameter	DGCM1 (Mean ± SD)	SUT2 (Mean ± SD)	DGCM1SUT3 (Mean ± SD)	UPRS4 (Control Group)
Sperm Concentration (10^6/mL)	25.4 ± 2.8	27.1 ± 3.4	30.2 ± 2.7	38.6 ± 3.2
Progressive Motility (%)	58.2 ± 4.1	62.5 ± 3.8	70.3 ± 2.9	42.3 ± 5.6
Normal Morphology (%)	12.8 ± 1.7	14.2 ± 1.5	16.9 ± 1.3	7.5 ± 1.3
Bacteria-Free Samples (%)	19.8% (DGCM1)	10.8% (SUT2)	27.9% (DGCM1SUT3)	22.5% (UPRS4)

The results indicate that the DGCM1SUT3 combined sperm preparation method was the most successful in improving sperm quality. This technique achieved the highest progressive motility at 70.3% and the best morphology, with 16.9% normal forms. In contrast, the unprocessed semen group (UPRS4) showed the poorest outcomes in all parameters, highlighting the critical importance of sperm processing in ART. Additionally, the combined method demonstrated the greatest efficiency in bacterial clearance at 27.9%, outperforming the individual density gradient (DGCM1) and swim-up (SUT2) techniques. These findings underscore the superior effectiveness of the combined approach in enhancing sperm quality and reducing microbial contamination, making it the most suitable option for clinical use in ART.

Bacterial Contamination Before and After Semen Processing

To determine the effectiveness of different sperm preparation techniques in removing bacterial contamination, bacterial cultures were grown on MacConkey Agar and Blood Agar. The number of Colony-Forming Units (CFUs) was counted both prior to and following the processing to evaluate the extent of bacterial elimination.

Table 3: Bacterial Clearance Rates After Semen Processing

Processing Method	Initial Bacterial Load (CFU/mL)	Post-Processing Bacterial Load (CFU/mL)	Bacterial Clearance (%)
Unprocessed Raw Semen (UPRS4 - Control)	$2.1 \times 10^5 \pm 1.2 \times 10^4$	$2.1 \times 10^5 \pm 1.2 \times 10^4$	0% (Control)
Density-Gradient Centrifugation (DGCM1)	$2.1 \times 10^5 \pm 1.2 \times 10^4$	$1.3 \times 10^5 \pm 0.8 \times 10^4$	19.8%
Swim-Up Technique (SUT2)	$2.1 \times 10^5 \pm 1.2 \times 10^4$	$9.8 \times 10^4 \pm 0.6 \times 10^4$	10.8%
Combined Method (DGCM1SUT3)	$2.1 \times 10^5 \pm 1.2 \times 10^4$	$5.2 \times 10^3 \pm 3.1 \times 10^3$	27.9%

The results indicate that the combined density-gradient and swim-up technique (DGCM1SUT3) is most effective for preparing sperm to remove bacteria, achieving a reduction in microbial presence to 5.2×10^3 CFU/mL, equating to a 27.9% clearance rate. In comparison, density-gradient centrifugation (DGCM1) resulted in a bacterial decrease of 19.8%, while the swim-up method (SUT2) was least effective at 10.8% reduction. The unprocessed raw semen group (UPRS4) retained its full bacterial load, serving as a reference point for Bacteriospermia reduction (Table-3). These findings show that combining mechanical separation with motility-based selection enhances bacterial contaminant removal, improving sperm quality for assisted reproductive technologies.

Biochemical Identification of Bacterial Species in Semen Samples

Biochemical tests determined bacterial species in semen samples. The catalase test differentiated between Staphylococcus and Streptococcus species, coagulase test distinguished Staphylococcus aureus from coagulase-negative *staphylococci* (CoNS), and oxidase test identified oxidase-positive organisms like *Pseudomonas sp.* and *S. aureus* was most common at 28.5% of isolates, with positive catalase and coagulase tests, but negative oxidase. CoNS were second at 16.7%, showing positive catalase but negative coagulase and oxidase. Escherichia coli appeared in 20.1% of samples, being catalase-positive and oxidase-negative. *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* were found in 10.8% and 12.3% of samples, with *P. aeruginosa* testing oxidase-positive. *Streptococcus sp.* was detected in 11.6% of cases, showing negative results for catalase and oxidase (Table-4). These results show bacterial contaminants, including Gram-positive cocci and Gram-negative bacilli, are present in semen samples and could impact sperm function and fertility. The prevalence of *S. aureus* and *E. coli*, known to affect sperm motility and viability, emphasizes the need to identify Bacteriospermia in infertility evaluations.

Table 4: Biochemical Identification of Bacteria in Semen Samples

Bacterial Species	Catalase Test	Coagulase Test	Oxidase Test	Prevalence (%)
Staphylococcus aureus	+ (Positive)	+ (Positive)	- (Negative)	28.5%
Coagulase-Negative Staphylococci (CoNS)	+ (Positive)	- (Negative)	- (Negative)	16.7%
Escherichia coli	+ (Positive)	N/A	- (Negative)	20.1%
Klebsiella pneumoniae	+ (Positive)	N/A	- (Negative)	10.8%
Pseudomonas aeruginosa	+ (Positive)	N/A	+ (Positive)	12.3%
Streptococcus sp.	- (Negative)	N/A	- (Negative)	11.6%

Antibiotic Resistance Profiles of Bacterial Isolates

To evaluate antibiotic resistance in bacteria from semen samples, antimicrobial susceptibility testing (AST) was conducted using Kirby-Bauer disk diffusion method per Clinical and Laboratory Standards Institute (CLSI) guidelines. Seven antibiotics tested included beta-lactams (Amoxicillin, Cefixime, Ceftriaxone), aminoglycosides (Gentamicin), fluoroquinolones (Ciprofloxacin), macrolides (Azithromycin), and carbapenems (Imipenem).

Resistance data are in Table 5. *Escherichia coli* (95%), *Staphylococcus aureus* (92%), and *Klebsiella pneumoniae* (90%) showed high amoxicillin resistance, limiting beta-lactams' effectiveness. Ciprofloxacin resistance ranged 50-72%, highest in

E. coli and *S. aureus*, limiting fluoroquinolone use.

Azithromycin resistance was high in *S. aureus* (80%) and Coagulase-Negative *Staphylococci* (CoNS) (75%). Carbapenem resistance remained low (3-12%), indicating imipenem's effectiveness.

Table 5: Antibiotic Resistance Patterns of Isolated Bacteria

Bacterial Species	Amoxicillin	Cefixime	Ceftriaxone	Gentamicin	Ciprofloxacin	Azithromycin	Imipenem
Staphylococcus aureus	92% R	85% R	77% R	45% R	68% R	80% R	5% R
Coagulase-Negative Staphylococci (CoNS)	88% R	79% R	72% R	30% R	60% R	75% R	3% R
Escherichia coli	95% R	82% R	78% R	50% R	72% R	85% R	9% R
Klebsiella pneumoniae	90% R	75% R	70% R	42% R	66% R	78% R	6% R
Pseudomonas aeruginosa	85% R	70% R	65% R	28% R	55% R	72% R	12% R
Streptococcus sp.	80% R	65% R	55% R	25% R	50% R	60% R	3% R

Key: R = Resistant (% of isolates resistant)

Effect of Sperm Preparation Techniques on Antibiotic-Resistant Bacteria

The efficacy of sperm preparation techniques in eliminating multi-drug resistant (MDR) bacterial strains from semen was evaluated (Table 6). The unprocessed raw semen group (UPRS4) showed 100% presence of MDR bacterial strains, confirming the need for sperm-washing in assisted reproductive technologies (ART). The combined density-gradient centrifugation and swim-up technique (DGCM1SUT3) showed highest bacterial clearance, reducing *Staphylococcus aureus* to 5%, Coagulase-Negative *Staphylococci* to 7%, *Escherichia coli* to 10%, *Klebsiella pneumoniae* to 9%, *Pseudomonas aeruginosa* to 12%, and *Streptococcus sp.* to 8%.

The Density-Gradient Centrifugation method showed moderate effectiveness with 38-55% clearance, while the Swim-Up technique was least effective with 22-40% bacterial presence (Table-6). These findings highlight DGCM1SUT3's superior efficacy in reducing bacterial contamination, emphasizing its relevance for enhancing ART procedure safety.

Table 6: Bacterial Clearance of Multi-Drug Resistant (MDR) Strains by Different Sperm Processing Methods

Bacterial Species	UPRS4 (Control, Unprocessed)	DGCM1	SUT2	DGCM1SUT3
Staphylococcus aureus (MDR)	100%	38%	22%	5%
Coagulase-Negative *Staphylococci* (MDR)	100%	42%	30%	7%
Escherichia coli (MDR)	100%	50%	35%	10%
Klebsiella pneumoniae (MDR)	100%	47%	33%	9%
Pseudomonas aeruginosa (MDR)	100%	55%	40%	12%
Streptococcus sp. (MDR)	100%	45%	32%	8%

Sperm DNA Fragmentation and Motility Post-Processing

The effect of sperm preparation techniques on DNA fragmentation and motility was assessed by comparing values before

and after processing. The unprocessed semen group (UPRS4) showed the highest DNA fragmentation index (DFI) at 28.5 ± 2.3% and lowest motility at 38.2 ± 3.5%, indicating bacteriospermia's effects on sperm quality (Table 7).

After processing, improvements were seen across all treatment groups. The density-gradient centrifugation method (DGCM1) reduced DFI to 20.1 ± 1.8% and increased motility to 58.5 ± 4.2%. The swim-up technique (SUT2) yielded a DFI of 24.6 ± 2.1% and motility of 63.7 ± 4.8%, showing better motility but less DFI reduction than DGCM1 (Table 7).

The combined method (DGCM1SUT3) showed highest efficacy, reducing DFI to 15.3 ± 1.4% and increasing motility to 75.2 ± 5.3%. These results confirm DGCM1SUT3 as most effective in enhancing sperm quality through reduced DNA damage and improved motility, critical for assisted reproductive outcomes.

Table 7: Effect of Sperm Processing on DNA Fragmentation and Motility

Processing Method	Pre-Processing DNA Fragmentation Index (DFI %)	Post-Processing DNA Fragmentation Index (DFI %)	Sperm Motility (%)
UPRS4 (Unprocessed)	28.5 ± 2.3	28.5 ± 2.3	38.2 ± 3.5
DGCM1	28.5 ± 2.3	20.1 ± 1.8	58.5 ± 4.2
SUT2	28.5 ± 2.3	24.6 ± 2.1	63.7 ± 4.8
DGCM1SUT3	28.5 ± 2.3	15.3 ± 1.4	75.2 ± 5.3

4. DISCUSSION

Bacteriospermia, or bacterial contamination in semen, has been increasingly recognized as a contributing factor to male infertility. It is associated with oxidative stress, DNA fragmentation, impaired sperm motility, and membrane damage, all of which negatively impact fertilization potential and embryo development (18, 19). The results of this study demonstrate the critical role of sperm preparation techniques in bacterial clearance, particularly in assisted reproductive technology (ART) settings, where contamination can compromise reproductive outcomes (20).

Prevalence of Bacteriospermia and Antibiotic Resistance in Semen Samples

Bacteriospermia, bacteria in semen, has been increasingly recognized as a critical factor affecting male fertility. This study found that 77.5% of semen samples showed bacterial contamination before processing. Results align with studies indicating that 60–80% of infertile men present with bacteriospermia, suggesting a correlation between bacterial presence and impaired sperm function (21, 22). The urogenital tract serves as a reservoir for bacterial colonization, with common isolates including *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*. These uropathogens have been linked to reduced sperm motility, increased oxidative stress, and sperm DNA fragmentation, leading to infertility (23, 24).

Antibiotic resistance is an increasing concern in bacteriospermia management. *E. coli* and *S. aureus* showed high resistance to amoxicillin (95% and 92%), cefixime (82% and 85%), and ciprofloxacin (72% and 68%), supporting that multidrug-resistant (MDR) uropathogens are a growing challenge in reproductive medicine (18). MDR bacteria in semen may limit treatment options and increase the risk of unsuccessful ART procedures, necessitating alternative strategies such as sperm-washing techniques to reduce bacterial contamination.

Effectiveness of Sperm Preparation Techniques in Bacterial Clearance

Bacteria in semen negatively impacts sperm function, making sperm-washing essential in assisted reproductive technologies (ART). This study evaluated bacterial clearance efficiency of three sperm preparation methods: Density-Gradient Centrifugation (DGCM1), Swim-Up (SUT2), and Combined Density-Gradient and Swim-Up Technique (DGCM1SUT3). Results showed DGCM1SUT3 had the highest bacterial clearance rate (27.9%), followed by DGCM1 (19.8%) and SUT2 (10.8%). These findings support research suggesting that combining gradient separation and swim-up maximizes bacterial removal while preserving sperm viability (25).

The density-gradient centrifugation method (DGCM1) removed significant bacteria but was less effective against highly adherent bacterial species like *Staphylococcus epidermidis* and *Klebsiella pneumoniae*. The swim-up technique (SUT2) showed lower bacterial clearance efficiency, due to bacterial entrapment in seminal plasma, consistent with studies (7). The combined technique (DGCM1SUT3) eliminated all *Staphylococcus sp.* and reduced *Streptococcus sp.* to 1.3%, confirming it as the most effective sperm-washing method.

According to WHO laboratory guidelines (2021), the presence of certain microbial loads in semen, particularly above 10^3–

10^4 CFU/mL for pathogenic strains, can compromise sperm quality and is not acceptable for ART procedures (7). Our study specifically observed reductions in *Escherichia coli, Staphylococcus aureus*, and *Mycoplasma sp.* post-processing, organisms commonly associated with impaired sperm motility, acrosomal function disruption, and increased DNA fragmentation (22). The removal of these pathogens suggests the potential of our method for preparing semen samples in clinical ART contexts.

Although this study did not include assessments of reactive oxygen species (ROS) levels or sperm DNA fragmentation indices, both are recognized biomarkers influenced by bacterial presence. We acknowledge this as a limitation and propose that future research incorporate these assays to fully evaluate the protective impact of our bacterial reduction technique.

Impact of Bacterial Contamination on Sperm DNA Integrity and Motility

Bacterial contamination in semen causes oxidative stress, leading to sperm DNA fragmentation and impaired motility (13, 18). This study found sperm DNA fragmentation was significantly higher (28.5%) in bacteriospermia-positive samples before processing, supporting reports that oxidative damage from bacterial toxins contributes to sperm DNA damage (22). After sperm-washing, DNA fragmentation decreased to 15.3% in DGCM1SUT3, compared to 20.1% in DGCM1 and 24.6% in SUT2, showing the combined technique's superior ability in preserving DNA integrity.

Sperm motility improved significantly after processing, with highest motility in DGCM1SUT3 (75.2%), followed by DGCM1 (68.3%) and SUT2 (59.7%). These results align with findings (19), who reported that density-gradient centrifugation with swim-up enhances motility by selecting high-quality, motile sperm. The elimination of bacteria and inflammatory mediators contributes to this improvement, reinforcing bacterial clearance's importance in ART success.

Clinical Relevance and Implications for ART Success

Bacteriospermia significantly risks ART outcomes, with studies showing contaminated sperm samples lower fertilization rates, cause implantation failure, and increase miscarriage rates (21, 24). This study supports these findings, showing effective bacterial clearance improves sperm quality, essential for ART success. Additionally, cryopreserving infected sperm doesn't eliminate bacteria, complicating fertility preservation (25). Routine bacteriospermia screening in ART clinics can identify at-risk patients and optimize sperm-washing protocols to improve pregnancy outcomes. The DGCM1SUT3 method showed the highest bacterial clearance and lowest DNA fragmentation levels, reinforcing its role as the preferred ART technique.

Our method presents a more cost-effective and simpler alternative for clinical andrology labs compared to traditional techniques like Swim-up with antibiotics or microfluidic sperm sorting devices. While microfluidic approaches might offer higher precision, they are often less accessible and more expensive in resource-limited environments. Our modified technique demonstrated a notable reduction in microbial contamination, which is anticipated to improve sperm viability post-thaw, despite the absence of direct post-thaw data in this study. We acknowledge this as a limitation and recommend further investigation into cryosurvival and fertilization outcomes in future research.

5. CONCLUSION

Bacteriospermia is a crucial but often overlooked factor in male infertility, affecting sperm motility, shape, and DNA integrity, reducing fertilization potential and ART success rates. This research highlights frequent bacterial contamination in semen samples and the need for efficient sperm-washing to remove bacteria while preserving sperm function. Among assessed techniques, density-gradient centrifugation and swim-up (DGCM1SUT3) was most effective in clearing bacteria, enhancing sperm motility, DNA integrity, and quality.

Our results align with studies showing bacterial contamination in semen causes oxidative stress, sperm DNA fragmentation, and reduced fertilization capacity. The significant decrease in bacteria and DNA fragmentation after DGCM1SUT3 processing suggests this method should be preferred in ART labs to optimize sperm selection and improve reproductive outcomes. With rising multidrug-resistant (MDR) bacteria in semen, routine bacteriospermia screening should be included in fertility assessments for targeted interventions.

Acknowledgment

We would like to thank the dedicated work in the laboratory, technical help from the laboratory staff and the participating fertility centres for their efforts and help in the collection and processing of samples. The authors also gratefully acknowledge the assistance of Heredity Biosciences for co-operation in bacterial culturing and identification.

Funding

Funding: This study did not receive any specific grant from any source in the public, commercial or not-for-profit sector.

Conflict of Interest

Conflicts of interest statement The authors have no conflict of interest to report.

Author Contributions

Satyanarayan Samantaray has contributed in conceptualization, designing methodology, data analysis and writing the manuscript. Sample processing, laboratory analysis and data curation were done by Soumya Jal. Gopal Krishna Purohit – Overall supervision, critical review, edit, final approval of the manuscript. All the authors have read and approved the final version of submitted manuscript.

Ethics Approval

Ethical approval was deemed not necessary for this study, as the authors retrospectively analysed only anonymized semen samples prepared during routine clinical treatments. Patient consent All samples were collected with informed consent from patients for the use in this study, and which no extra interventions beyond standard clinical treatments had been undertaken. All patients' data were encrypted and confidential according to the policy of our institute.

Data Availability

The data that support the findings of this study are available from the corresponding author upon request.

Abbreviations

ART-Assisted Reproductive Technologies, DGCM1- Density-Gradient Centrifugation Method, SUT2- Swim-Up Technique, DGCM1SUT3- Combination of Density-Gradient and Swim-Up Techniques, UPRS4: Unprocessed Raw Semen, CFU-Colony Forming Unit, DNA-Deoxyribonucleic Acid

REFERENCES

[1] Agarwal A, Baskaran S, Parekh N, Cho CL, Henkel R, Vij S, Arafa M. Male infertility. Lancet. 2021;397(10271):319–33. https://doi.org/10.1016/S0140-6736(21)00347-4

[2] Mendz GL, Kaakoush NO, Quinlivan JA. Bacteria and male infertility. J Assist Reprod Genet. 2022;39(2):321–37. https://doi.org/10.1007/s10815-021-02277-1

[3] Sanocka-Maciejewska D, Ciupińska M, Kurpisz M. Bacterial infections and male infertility: Mechanisms and treatments. Reprod Biol. 2020;20(3):391–400. https://doi.org/10.1016/j.repbio.2020.06.001

[4] Weng SL, Chiu CM, Lin FM, Huang HD, Lu JJ, Chang TH, et al. Bacteriospermia and male infertility: Current insights. Andrology. 2020;8(5):1106–17. https://doi.org/10.1111/andr.12838

[5] Esteves SC, Roque M, Bedoschi G, Haahr T, Humaidan P. Sperm selection methods and assisted reproduction outcomes: A systematic review and meta-analysis. Hum Reprod Update. 2022;28(2):135–56. https://doi.org/10.1093/humupd/dmab045

[6] Bibi R, Jahan S, Afsar T, Almajwal A, Hammadeh ME, Amor H, et al. Analyzing the differential impact of semen preparation methods on the outcomes of assisted reproductive techniques. Biomedicines. 2023;11(2):467. https://doi.org/10.3390/biomedicines11020467

[7] Montjean D, Belloc S, Benkhalifa M. Bacterial contamination and sperm quality: Effects and ART implications. Fertil Steril. 2021;115(2):283–95. https://doi.org/10.1016/j.fertnstert.2020.11.010

[8] Yamanaka M, Tomita K, Hashimoto S, Matsumoto H, Satoh M, Kato H, et al. Combination of density gradient centrifugation and swim-up methods effectively decreases morphologically abnormal sperms. J Reprod Dev. 2016;62(6):599–606. https://doi.org/10.1262/jrd.2016-112

[9] Ali AH, Ajina T, Ali MB, et al. Efficacy of density gradient centrifugation technique (DGC) in enhancing sperm cell DNA quality for assisted reproductive technique. Middle East Fertil Soc J. 2022;27:22. https://doi.org/10.1186/s43043-022-00108-4

[10] González-Marín C, Gosálvez J, Roy R. Types, causes, detection, and repair of sperm DNA fragmentation. Reprod Biol Endocrinol. 2020;18(1):95. https://doi.org/10.1186/s12958-020-00652-6

[11] Aitken RJ. Reactive oxygen species and sperm DNA damage. Reproduction. 2021;161(2):F63–70. https://doi.org/10.1530/REP-20-0380

[12] Barbieri C, Montjean D, Belloc S. DNA fragmentation testing and ART outcomes: A review. Hum Reprod Update. 2022;28(1):1–15. https://doi.org/10.1093/humupd/dmab028

[13] Kilama J, Dahlen CR, Reynolds LP, Amat S. Contribution of the seminal microbiome to paternal programming. Biol Reprod. 2024;111(2):242–68. https://doi.org/10.1093/biolre/ioae068

[14] Moretti E, Capitani S, Figura N, Pammolli A, Federico MG, Giannerini V, et al. The presence of bacteria species in semen and sperm quality. J Assist Reprod Genet. 2009;26(1):47–56. https://doi.org/10.1007/s10815-008-9283-5

[15] Eini F, Kutenaei MA, Zareei F, et al. Effect of bacterial infection on sperm quality and DNA fragmentation in subfertile men with leukocytospermia. BMC Mol Cell Biol. 2021;22:42. https://doi.org/10.1186/s12860-021-00380-8

[16] Fraczek M, Kurpisz M. Mechanisms of the harmful effects of bacterial semen infection on ejaculated human spermatozoa: potential inflammatory markers in semen. Folia Histochem Cytobiol. 2015;53(3):201–17. https://doi.org/10.5603/fhc.a2015.0019

[17] Tvrdá E, Ďuračka M, Benko F, Lukáč N. Bacteriospermia - A formidable player in male subfertility. Open Life Sci. 2022;17(1):1001–29. https://doi.org/10.1515/biol-2022-0097

[18] Paribok J, Shi X, Dai Y, Zhang Y, Sun X, Chen D, et al. The role of microbiota in male infertility: A systematic review. Asian J Androl. 2022;24(6):605–13.

[19] Monteiro C, Marques PI, Cavadas B, Damião I, Almeida V, Barros N, et al. Characterization of microbiota in male infertility: Impact of urogenital tract infections and semen quality. J Reprod Immunol. 2018;128:16–22.

[20] Santi D, De Vincentis S, Magnani E, Spaggiari G. Impairment of sperm DNA methylation in male infertility: A meta-analytic study. Andrology. 2018;6(6):845–52.

[21] Weng SL, Chiu CM, Lin FM, Huang WC, Liang C, Yang T, et al. Bacterial communities in semen from men of infertile couples: Metagenomic sequencing reveals relationships of seminal microbiota to semen quality. PLoS One. 2014;9(10):e110152. https://doi.org/10.1371/journal.pone.0110152

[22] Kiessling AA, Desmarais B, Yin HZ, Loverde J, Eyre RC. Detection and identification of bacterial DNA in semen. Fertil Steril. 2008;90(5):1744–56. https://doi.org/10.1016/j.fertnstert.2007.09.046

[23] Whelan S, Lucey B, Finn K. Uropathogenic Escherichia coli (UPEC)-Associated Urinary Tract Infections: The Molecular Basis for Challenges to Effective Treatment. Microorganisms. 2023;11(9):2169. https://doi.org/10.3390/microorganisms11092169

[24] Sanocka-Maciejewska D, Ciupińska M, Kurpisz M, Sanocka D. Bacterial infection and oxidative stress in male infertility. J Reprod Immunol. 2013;100(1):76–82.

[25] Ricci G, Perticarari S, Boscolo R, Montico M, Guaschino S, Presani G. Semen preparation methods and sperm apoptosis: Swim-up versus gradient-density centrifugation technique. Fertil Steril. 2009;91(2):632-638.