BACTERIOSPERMIA IN MEN AMONG INFERTILE COUPLES IN NEPALESE COMMUNITY AND ITS RELATION TO SPERM DNA INTEGRITY

A DISSERTATION SUBMITTED TO THE

CENTRAL DEPARTMENT OF MICROBIOLOGY

INSTITUTE OF SCIENCE AND TECHNOLOGY

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NEPAL

FOR THE AWARD OF

DOCTOR OF PHILOSOPHY

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BY

ANIMA SHRESTHA

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DECLARATION

Dissertation entitled "**Bacteriospermia in men among infertile couples in Nepalese community and its relation to sperm DNA integrity**" which is being submitted to the Central Department of Microbiology, Institute of Science and Technology (IoST), Tribhuvan University, Nepal for the award of the degree of Doctor of Philosophy (Ph.D.), is a research work carried out by me under the supervision of **Prof. Dr. Anjana Singh** of Central Department of Microbiology, Tribhuvan University and co-supervised by **Associate Prof. Dr. Dev Raj Joshi** of Central Department of Microbiology, Tribhuvan University.

This research work is original and has not been submitted earlier in part or in full in this or any other form to any university or institute, here or elsewhere, for the award of any degree. I hereby declare that approximately 1% of my dissertation content have been used with artificial intelligence help.

.....

Anima Shrestha

RECOMMENDATION

This is to recommend that **Ms. Anima Shrestha** has carried out research entitled "**Bacteriospermia in men among infertile couples in Nepalese community and its relation to sperm DNA integrity**" for the award of Doctor of Philosophy (Ph.D.) in **Microbiology** under our supervision. To our knowledge, this research work has not been submitted for any other degree.

She has fulfilled all the requirements laid down by the Institute of Science and Technology (IoST), Tribhuvan University, Kirtipur for the submission of the thesis for the award of a Ph.D. degree.

.....

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LETTER OF APPROVAL

Date: 16th September, 2025

On the recommendation of Prof. Dr. Anjana Singh and Associate Prof. Dr. Dev Raj Joshi, this Ph.D. dissertation submitted by Ms. Anima Shrestha, entitled "**Bacteriospermia in men among infertile couples in Nepalese community and its relation to sperm DNA integrity,**" is forwarded by Central Department Research Committee (CDRC) to the Dean, IoST, T.U.

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EXAMINERS

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Anima Shrestha
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शोधसार

विवाहित जोडीहरुमा निसन्तानपनाको समस्या संसारभर बढ्दो अवस्थामा छ। यसमा स्त्री र पुरुष दुवै बराबर जिम्मेवार हुन्छन। पुरुषमा निसन्तानपना प्रजनन अंगमा समस्या, हर्मोनमा गडबडी, शुक्राणु उत्पादनमा असर पुर्याउने कारकहरुको सम्पर्क र मूत्र तथा प्रजनन मार्ग वा सहायक प्रजनन ग्रन्थीको संक्रमणका कारण हुन सक्छ। मूत्र तथा प्रजनन मार्ग वा सहायक प्रजनन ग्रन्थीको संक्रमणको कारण वीर्यमा जीवाणु संक्रमण हुन सक्छ, जसले गर्दा वीर्यको गुणस्तरमा ह्रास आउन सक्छ। वीर्यको गुणस्तर पत्ता लगाउन शुक्राणुको संख्या, गतिशिलता, आकार र जीविता जाँच गरिन्छ। शुक्राणुको गुण र कार्यह्रासको लागि शुक्राणुको डीएनए खण्डीकरण पनि एउटा कारण मानिएको छ। शुक्राणुको डीएनए खण्डीकरणले सामान्य निषेचन र विकास, गर्वाधान, भ्रूण प्रत्यारोपण र विकासमा महत्वपूर्ण भूमिका खेल्छ। शुक्राणुको डीएनए खण्डीकरण विभिन्न कारणले हुन सक्छ। शुक्राणुको अन्य गुणका साथै डीएनएलाई वीर्यमा भएको जीवाणुले असर गर्न सक्छ। तर नेपालका पुरुषहरुको वीर्यको गुणमा असर गर्ने जीवाणुको तथ्याड़क सीमित छ। त्यसैले यस अध्ययनको उद्देश्य वीर्यमा पाइने जीवाणुको मूल्याङ्कन गर्नु र निसन्तान दम्पतीका पुरुषहरूमा ती जीवाणुको शुक्राणुको डीएनए खण्डीकरणसँगको सम्बन्ध निर्धारण गर्नु हो। प्रस्तुत अध्ययन जून २०२१ देखी अगस्ट २०२४ सम्म गर्दा कृयटर्स आइ. भी. एफ. नेपाल प्रा. लि., ललितपुरमा परामर्शका लागी आएका दम्पति मध्येका पुरुषबाट २१७ वटा वीर्यको नमूनाहरु संकलन गरिएको थियो। नमूनाको प्रयोगशाला परिक्षण कृयटर्स आइ.भी. एफ. नेपालको एन्ड्रोलोजी प्रयोगशाला तथा त्रिभुवन विश्वविद्यालयको माइक्रोवायोलोजी केन्द्रिय विभागको प्रयोगशालामा गरियो। विश्व स्वास्थ्य सङ्गठनको मानव वीर्य विश्लेषण २०२१ को मापदण्ड अनुसार वीर्यको मात्रा, शुक्राणुको घनत्व, गति, आकार्, जीविता, श्वेत रक्तकोष संख्या, वीर्यको डीएनएको खण्डिकरण परिक्षण गरियो। वीर्यमा जीवाणुको अध्ययन कल्चर (culture) तथा १६एसआरआरएनए (16S rRNA) जीनमा आधारित विधिबाट गरियो। कल्चर बिधिबाट परिक्षण गर्दा ५५ वटा (२५.३५%) तथा १६एसआरआरएनए जीनको अनुक्रम (Sequencing) मा आधारित विधिबाट १४८ वटा (७०.८१%) नमूनामा जीवाणु (ब्याक्टेरिओस्पेर्मिया) भएको पत्ता लगाइयो। कल्चर गर्दा सबैभन्दा बढी भेटिएको जीवाणु *स्टाफैलोकोक्कस अरियस* (*Staphylococcus aureus*) थियो जसमा प्रतिजैविक जीन *मेक*ए (*mec*A) तथा विषमताका जीनहरु *सिएलेफ*ए (*clf*A) र *सिएलेफ*बी (*clf*B) उल्लेखनीय मात्रामा भेटिए। १६एसजीनका अनुक्रम बिष्लेषण गर्दा विविध जीवाणुहरु

- *प्रेभोटेल्ला* (*Prevotella)* (१४.२%), *फेनोल्लेरिया* (*Fenollaria*) (१२.२%), *लक्टोबेसिलस* (*Lactobacillus*) (९.६%), *भिलोनेला* (*Veillonella*) (८.४%), *कोरिनिबेक्टेरियम* (*Corynebacterium*) (७.८%), *एसिनेटोबेक्टर* (*Acinetobacter*) (६.७%), *स्नेथिया* (*Sneathia*) (५.७%), *गार्देनेरेला* (*Gardnerella*) (५.१%), *पर्फाइरोमोनास* (*Porphyromonas*) (४.२%), *इजकिएला* (*Ezakiella*) (३.०%), *डिअलिस्तर* (*Dialister*) (२.८%), *स्त्रेप्टोकोकस* (*Streptococcus*) (२.७%), *पेप्टोनिफिलस* (*Peptoniphilus*) (२.४%), *नेगेटिभिकोकस* (*Negativicoccus*) (१.७%), *फाइनगोल्दिया* (*Finegoldia*) (१.६%), *एनएरोकोकस* (*Anaerococcus*) (१.४%), *स्टेफाइलोकोकस* (*Staphylococcus*) (१.०%), *मेगास्फेरा* (*Megasphaera*) (१.०%), *मोरिएल्ला* (*Moryella*) (०.९%), र *कम्पाइलोबाक्टर* (*Campylobacter*) (०.६%) पत्ता लगाइए। धेरै जस्तो जीवाणुहरु अवायवीय थिए। वीर्य परिक्षणमा १८८ वटा नमूनाको शुक्राणु घनत्व सामान्य थिए, जसमा ३८ वटा (२०.२१%) नमूनाको डिएन्ए खण्डिकरण उच्च थियो। बाक्टेरियोस्पर्मियाको वीर्यका गुणहरुसँगको तथ्यगत विश्लेषण कल्चरमा आधारित बाक्टेरियोस्पर्मियाको वीर्यको मात्रासँग तथा १६एस जीनका आधारमा शुक्राणु घनत्वसँग विपरित सम्बन्ध रहेको पाइयो। तर बाक्टेरियोस्पर्मिया र शुक्राणुको डिएनए खण्डिकरणसँग तथ्यगत सम्बन्ध भेटिएन। १६एस जीन सिक्वेन्सिङ परिणामको भिन्नता प्रचुरताको विश्लेषण गर्दा *एसिनेतोबाक्टर* (*Acinetobacter*) वीर्यका धेरै गुणहरुसँग नकारात्मक सम्बन्ध थियो भने *लेक्टोबेसिलस* (*Lactobacillus*) सामान्य गुण भएको र शुक्राणुको डिएनए कम खण्डिकरण भएको वीर्यमा धेरै पाइएको थियो, जसले लेक्टोबेसिलसको वीर्यमा सुरक्षात्मक भूमिका रहेको संकेत गर्छ। वीर्यमा भएको जीवाणुको समुदायले वीर्यको गुणहरुमा असर गर्न सक्छ भन्ने यस अध्ययनले देखाएको छ। यस अध्ययनले भविष्यमा वीर्यमा पाइने जीवाणु र तिनले पार्ने प्रभावबारे बिस्तृत खोज गर्नको निम्ती आधारभूत तथ्यांक पेश गरेको छ जसले नेपाली पुरुषहरुको प्रजनन स्वास्थ्य सुधार गर्न मद्दत गर्न सक्छ।

मुख्य शब्दहरु: *पुरुष निसन्तानपना-वीर्य-बाक्टेरियोस्पर्मिया-शुक्राणु डिएनए खण्डिकरण-वीर्यमाइक्रोबायोम-लेक्टोबेसिलस*

ABSTRACT

Infertility has been an increasing problem in married couples worldwide. Both partners may contribute equally to the infertility. Male infertility can be caused by anatomical defects, hormonal imbalance, exposure to various other factors affecting spermatogenesis, and infection of the urogenital tract or accessory reproductive glands. Genitourinary or accessory gland infection results in the presence of bacteria in semen that may affect the semen quality. The fertility potential of semen is investigated by analysis of semen for sperm concentration, motility, morphology, and vitality. Sperm DNA fragmentation has now been known as a significant defect affecting the diagnostic sperm characteristics and function. Sperm DNA integrity plays a crucial role in normal fertilization, healthy embryo development and successful implantation, pregnancy, as well as in fetal development. The fragmentation of sperm DNA might be caused due to several factors. Bacteriospermia is considered one of the factors affecting sperm DNA integrity and other sperm characteristics. However, there is limited data available on bacteria causing sperm defects in Nepalese men. Therefore, this study aimed to assess the seminal bacteria and to determine their relation with sperm DNA integrity in men among infertile Nepalese couples. A cross-sectional study was carried out from June 2021 to August 2024, in which 217 semen samples were collected from male partners of couples for fertility consultation at Creator's IVF Nepal, Lalitpur. The laboratory analysis of samples was done in the andrology laboratory of Creator's IVF Nepal and the laboratory of the Central Department of Microbiology, Tribhuvan University. Semen analysis for ejaculate volume, sperm concentration, motility, morphology, vitality, leucocyte count, and sperm DNA fragmentation (SDF) was done following WHO guidelines for human semen analysis, 2021. Bacteria in semen were studied by culture-based and 16S rRNA gene-based approaches. Bacteriospermia was detected in 55 (25.35%) samples by semen culture and 148 (70.81%) samples by 16S rRNA gene amplification. *Staphylococcus aureus* was the most frequently isolated bacterium. Virulence genes for clumping factors (*clf*A, *clf*B) and the methicillin resistance gene (*mec*A) were detected in a significant proportion (66.67%) of *S. aureus* isolates. The sequencing of the 16S rRNA gene amplicon revealed diverse bacterial genera including *Prevotella* (14.2% relative abundance), *Fenollaria* (12.2%), *Lactobacillus* (9.6%), *Veillonella* (8.4%), *Corynebacterium* (7.8%), *Acinetobacter* (6.7%), *Sneathia* (5.7%), *Gardnerella* (5.1%), *Porphyromonas* (4.2%), *Ezakiella* (3.0%), *Dialister* (2.8%),

Streptococcus (2.7%), *Peptoniphilus* (2.4%), *Negativicoccus* (1.7%), *Finegoldia* (1.6%), *Anaerococcus* (1.4%), *Staphylococcus* (1.0%), *Megasphaera* (1.0%), *Moryella* (0.9%), and *Campylobacter* (0.6%). Most of the bacterial genera were anaerobes. Semen analysis showed that 188 out of 217 had normal sperm concentration, among which 20.21% had high DNA fragmentation index (DFI ≥ 30%). The association of bacteriospermia with semen characteristics, including sperm DNA fragmentation, was studied. Culture-based bacteriospermia was significantly associated with decreased ejaculate volume (p = 0.001), whereas 16S rRNA-based bacteriospermia was significantly associated with lower sperm concentration (p = 0.02). However, no significant associations were found between bacteriospermia and sperm DNA fragmentation. Differential abundance analysis of 16S rRNA sequencing results depicted that *Acinetobacter* was negatively associated with multiple semen parameters, while *Lactobacillus* was consistently enriched in semen with normal parameters and lower sperm DNA integrity, indicating a potentially protective role. The bacterial community composition of semen obtained in this study concludes that a rich seminal microbiota may influence the characteristics of semen. This study provides baseline data for future in-depth studies on bacteria and their clinical implications in the seminal function that may help in improving the reproductive health of Nepalese men.

Keywords: *Male infertility-Semen-Bacteriospermia-Sperm DNA fragmentation-Semen microbiome-Lactobacillus*

LIST OF ACRONYMS AND ABBREVIATIONS

AMR : Antimicrobial Resistance
ART : Assisted Reproductive Technology
ATCC : American Type Culture Collection
BA : Blood Agar
BHI : Brain Heart Infusion
BMI : Body Mass Index
CA : Chocolate Agar
CI : Confidence Interval
CLSI : Clinical and Laboratory Standards Institute
CoNS : Coagulase Negative *Staphylococcus*
CPM : Counts Per Million
Ct : Threshold Cycle
DFI : DNA Fragmentation Index
DLC : Differential Leukocyte Count
DNA : Deoxyribonucleic Acid
FDR : False Discovery Rate
IL : Interleukin
IVF : In Vitro Fertilization
LOG2FC : Log Two Fold Change
MA : MacConkey Agar
MAGI : Male Accessory Gland Infection
MR : Methicillin Resistance
MRSA : Methicillin Resistant *Staphylococcus aureus*
NCBI : National Center for Biotechnology Information
NGS : Next Generation Sequencing
NHRC : Nepal Health Research Council
OATS : Oligoasthenoteratospermia
OS : Oxidative Stress
OUT : Operational Taxonomic Unit
PCOA : Principal Co-ordinate Analysis
PCR : Polymerase Chain Reaction

QIIME : Quantitative Insights Into Microbial Ecology

qPCR : Quantitative Polymerase Chain Reaction

ROS : Reactive Oxygen Species

rRNA : ribosomal Ribonucleic Acid

RT-PCR : Real-time Polymerase Chain Reaction

SAF : Sperm Agglutination Factor

SCD : Sperm Chromatin Dispersion

SD : Standard Deviation

SDF : Sperm DNA Fragmentation

SIF : Sperm Immobilization Factor

SPSS : Statistical Package for the Social Sciences

TE : Tris-EDTA

TFR : Total Fertility Rate

TLC : Total Leukocyte Count

TNF : Tumour Necrosis Factor

TSB : Tryptone Soya Broth

TUNEL : Terminal deoxynucleotidyl transferase-mediated dUTP Nick-End Labeling

UGC : University Grant Commission

WHO : World Health Organization

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CHAPTER 1

1. INTRODUCTION

1.1 Introduction

Infertility is defined as the inability to achieve pregnancy after a year of regular unprotected sexual intercourse (WHO, 2023). Globally, about 15% of couples are infertile, about 20-30% of the infertility cases are due to male factors alone, and 30-40% of the cases are due to a combination of both male and female factors (Huang *et al.*, 2023). Similar infertility rates have been reported in Nepal in previous studies (Subedi *et al.*, 2016; Tamrakar & Bastakoti, 2019). Male infertility may be affected by various factors, including the age of the patient, hormonal imbalance, testicular temperature, exposure to toxic chemicals and environmental pollutants, predisposing disease, and infections (Okonofua *et al.*, 2022).

Infections of the genitourinary tract account for about 15% of cases of male infertility (Hou *et al.*, 2016; Rusz *et al.*, 2012). Male infertility has been linked to genital tract infection, leading to the presence of bacteria in semen (Domes *et al.*, 2012; Weng *et al.*, 2014; Zeyad *et al.*, 2017), and some studies concluded that seminal bacteria did not influence the potency of sperm (Baud *et al.*, 2019; Hou *et al.*, 2016; Kaur & Prabha, 2012; Vilvanathan *et al.*, 2016). However, the role of bacteria in sperm function is still controversial (Gachet *et al.*, 2022). An area of the semen microbiome is of growing interest due to the potential implications of semen microbiota for the reproductive health of male and female partners (Altmäe *et al.*, 2019; Baud *et al.*, 2019).

Genital tract infection is one of the common causes of infertility in men (Henkel *et al.*, 2021). Various sites of the male genitourinary system, such as the prostate, the epididymis, the testis, and the urethra, may be infected by bacteria, leading to the occurrence of bacteria in semen (Rusz *et al.*, 2012; Zuber *et al.*, 2023). The presence of bacteria in concentrations greater than 10^3 bacteria/mL ejaculate is regarded as bacteriospermia (WHO, 2021). Bacteriospermia, together with leukocytospermia (≥1million leukocytes per mL of ejaculate), is considered a result of acute male genital tract infection (WHO, 1996). The bacterial infections harm the concentration, motility, morphology, and DNA fragmentation of spermatozoa (Farahani *et al.*, 2021;

Pergialiotis *et al.*, 2018), affecting male fertility (Tvrdá *et al.*, 2022). The male genitourinary tract may be infected by sexually transmitted or non-sexually transmitted microorganisms (Pellati *et al.*, 2008). Sexually transmitted bacteria *Chlamydia trachomatis, Neisseria gonorrhoeae*, *Mycoplasma* spp., *Ureaplasma* spp., and *Treponema pallidum* were found to reduce semen quality (Gimenes *et al.*, 2014). Other common non-sexually transmitted bacteria like *Escherichia coli*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Bacteroides* spp, coagulase-negative *Staphylococcus* (CoNS), *Pseudomonas aeruginosa*, and *Streptococcus* spp may also cause male genitourinary tract infection, showing negative effects on various spermatozoa parameters (Fraczek & Kurpisz, 2015; Gholami *et al.*, 2022).

Genital tract or male accessory gland infection leads to bacteriospermia. However, bacteriospermia does not necessarily indicate an active genital tract infection. Bacteria in the semen of asymptomatic individuals may be present due to colonization of commensals, contamination, or genital tract microbiome dysbiosis (Alqawasmeh *et al.*, 2022). A diverse bacterial community within semen affecting the male reproductive health is the semen microbiome, which can be revealed by the advanced molecular profiling techniques (Vajpeyee *et al.*, 2024). Semen microbiome is a less investigated area of research than other body fluids (Altmäe *et al.*, 2019). Semen was previously believed to be sterile fluid in healthy men; however, metagenomic studies of semen revealed the presence of a high number of diverse types of bacteria in the semen of healthy men (Hou *et al.*, 2016). The application of next-generation sequencing (NGS) of previously uncultured and unrecognized organisms has contributed to a better understanding of the semen microbiome (Skrzypczak-Zielinska & Fraczek, 2021). Metagenomic analysis of semen has changed the knowledge of microbial density and diversity in semen (Skrzypczak-Zielinska & Fraczek, 2021). It has been observed that the quality of semen depends on the composition of seminal microbial communities (Gachet *et al.*, 2022; Vajpeyee *et al.*, 2024). Specific bacteria present in the semen tend to have a significant negative impact on semen quality. For example, *E. faecalis* shows a negative impact on total motility (Farahani *et al.*, 2021); *Mycoplasma hominis* harms sperm concentration, motility, morphology, and vitality (Farahani *et al.*, 2021; Le *et al.*, 2022); *S. aureus* decreases sperm motility (Kaur *et al.*, 2010), as well as increases abnormalities in sperm morphology (Berjis *et al.*, 2018).

The number of infertile couples seeking medical treatment has been increasing year by year, in the global scenario as well as in Nepal (Tamrakar & Bastakoti, 2019). According to a study done in Nepal, about half of the infertility cases are attributed to male factors (Subedi *et al.*, 2016). The study regarding male infertility in Nepal is mainly confined to semen parameters in infertile men (Gautam *et al.*, 2021; Lamichhane *et al.*, 2014; Pant, 2013; Tamrakar & Bastakoti, 2019). Only limited studies from Nepal report on data on seminal bacteria (Bhatt *et al.*, 2015; Pokhrel *et al.*, 2020), eliciting the association of seminal bacteria with semen parameters affecting male fertility. Analysis of the composition of bacteria in semen, along with its association with semen parameters, is important to understand the etiology of genitourinary infections, which aids in managing male infertility due to bacteriospermia. Few studies on semen microbiota in infertile men have been carried out in Nepal, which are only based on cultivable bacteria (Bhatt *et al.*, 2015; Pokhrel *et al.*, 2020). Identified bacteria were aerobes or facultative anaerobes. To date, non-cultivable and anaerobic seminal bacteria have not been reported from Nepal. Therefore, this study aims to investigate the bacteriological profile of semen in infertile men by applying a culture-based approach and a metagenomic 16S rRNA gene amplicon sequencing approach. The outcome of the study could reflect the presence of cultivable, non-cultivable, and difficult-to-cultivate bacteria, as well as anaerobic bacteria in semen that affect the fertility potential of men.

1.2 Rationale

Infertility in couples is a subject of concern worldwide, including Nepal, and is contributed to by the male and female factors almost equally. Bacterial infection of the male reproductive tract and accessory glands is one of the factors contributing to male infertility. The presence of bacteria in semen may be either due to infection or contamination from the commensals of the genitourinary tract. Bacteria present in semen in significant numbers can influence the conventional parameters, such as sperm count, motility, morphology, vitality, and sperm DNA fragmentation negatively. However, semen with a normal range of conventional parameters may have fragmented sperm DNA. The fragmentation of sperm DNA may affect its fertilization potential, the development of the embryo after fertilization, and pregnancy outcomes.

Exploration of the semen microbiome and its implications on semen quality and male fertility has gained growing interest in the research arena globally. This study was focused on exploring the cultivable and non-cultivable bacteria in the semen of men among infertile couples. The study findings provide data on bacteria in the semen of Nepalese men, helping to reduce the existing gap in the data from Nepal. An understanding of the virulence and antimicrobial resistance properties of the commonly isolated bacteria in this study signifies the detection of bacteria in infertility diagnosis. There is controversial evidence from various regions of the world explaining the link between bacteriospermia with semen characteristics and sperm DNA integrity. This study further investigates the relationship between bacteriospermia and the integrity of sperm DNA. An analysis and culture of semen are the common diagnostic practices in the examination of semen-related male infertility. However, issues of sperm DNA fragmentation and the presence of non-cultivable bacteria have been overlooked. Understanding of the bacterial community present in semen and its linkage with sperm DNA integrity may provide significant help in identifying problems in the diagnosis of male infertility. This knowledge supports the development of targeted diagnostic and therapeutic strategies.

1.3 Objectives

General objective

To assess the bacterial species in semen and their association with sperm DNA integrity in infertile men of Nepal

Specific objectives

1. To assess the rate of bacteriospermia in men among infertile couples

2. To determine the antibiotic resistance profile of the bacterial isolates

3. To estimate the sperm DNA fragmentation index (DFI) in Nepalese men

4. To correlate bacterial types in semen and sperm DNA integrity

CHAPTER 2

2. LITERATURE REVIEW

2.1 Nepal's population dynamics and fertility rate

The total fertility rate (TFR) has been dropping swiftly in many countries for decades. It has been projected that 77.4% of the 195 countries by 2050 AD, and 93.8% by 2100 AD will have decreased TFR below the replacement level (Vollset *et al.*, 2020). The TFR of Nepal has dropped from 4.8 births per woman in 1996 to 2.1 births per woman in 2022 (Ministry of Health and Population, 2022). The decline in the fertility rate might be due to accessibility to family planning, enhancement in the education level, urbanization, economic growth, gender equity, or successful implementation of the governmental family planning policy program (Fauser *et al.*, 2024; Thapa, 2019). This global demographic change may have a positive effect on the environment; however, it may have negative economic, environmental, geopolitical, and societal implications in the future (Fauser *et al.*, 2024; Vollset *et al.*, 2020). The problem of demographic change also contributes to the incidence of infertility in the population (Rutstein & Shah, 2004). Amidst the decrease in fertility rate in the global context, it is also important to emphasize that about 48 million couples of reproductive age are having the problem of infertility (Fauser *et al.*, 2024; WHO, 2020).

2.2 Global prevalence of infertility

The World Health Organization (WHO) defines infertility as a disease of the male or female reproductive system defined by the failure to achieve a pregnancy after 12 months or more of regular unprotected sexual intercourse (WHO, 2023). If pregnancy has not occurred even once, it is termed primary infertility, whereas if pregnancy has been achieved previously at least once, it is termed secondary infertility (WHO, 2024). Infertility can be an important component of sexual and reproductive health and rights (SRHR) that helps in addressing sustainable development goals (SDGs) 3 and 5 (WHO, 2023).

WHO estimates the highest lifetime prevalence of infertility (23.2%) in the WHO Western Pacific Region, and the lowest (10.7%) in the Eastern Mediterranean Region, whereas it did not identify any study for the WHO South-East Asia Region (WHO,

2023). A review study done in 2007 estimates the prevalence rate of infertility in more developed nations to be 3.5% to 16.7%, while from 6.9% to 9.3% in less-developed countries (Boivin *et al.*, 2009). A study from one decade on infertility in a hospital in Nepal showed an increasing number of infertile couples seeking medical care (Tamrakar & Bastakoti, 2019).

2.3 Causes of infertility

Reproductive disorders in either one or both partners might be the cause of infertility in a couple. The cause of infertility may not be identified in either of the partners in some cases. Various factors may affect the fertility of a couple. Factors such as the age of the partners, length of sexual exposure, and coitus frequency determine fertility (Anwar & Anwar, 2016). Female factors contribute to about 40% of the total infertility cases, factors affecting the migration of semen contribute to 10% of the cases, and male factors to 20-35% of the infertility cases, while 10-20% of the cases may not have any explained causes (Brugo-Olmedo *et al.*, 2001; Makar & Toth, 2002). Disorders in reproductive functions, such as tubal, uterine, ovarian, and endocrine hormone imbalance, are the major reasons for female infertility. Men may contribute to infertility due to the problem of semen ejection and deterioration of semen quality (WHO, 2024).

2.4 Consequences of infertility

Infertility is considered a disease of the reproductive system of a male or female partner, causing ruinous societal as well as health consequences (WHO, 2023). One-third of infertile women in Nepal were living under high levels of stress (Sharma *et al.*, 2014). Infertile couples undergo stressful situations in the family and society, which may create marital problems, feelings of anger, anxiety, depression, and unworthiness (Ara *et al.*, 2022). These negative emotions created by infertility lead to a low quality of life (Rooney & Domar, 2018). The quality of life of infertile women is affected by the type of reasons and duration of infertility, particularly in women with primary infertility (Shrestha *et al.*, 2020). Blaming one partner on the other for impotency and the breakdown of marital status is also common in infertile couples (Rutstein & Shah, 2004). Although infertility in couples may be attributed to any of the partners, it has a more significant societal impact on women (WHO, 2024). It is considered in most cultures that it is the task of women to conceive and give birth to a child, and women suffer from anxiety and depression more than their male partners (Luk & Loke, 2015).

A married woman in several communities of Nepal defines her identity and sets up her role in family and society after childbirth (Khanal, 2019). However, Nepalese infertile men also undergo psychosocial stress, which relates to the masculine identity of men (Lamichhane, 2022).

2.5 Male infertility

2.5.1 Global status of male infertility

Male infertility is a global health issue that has not been considered as a disease (Agarwal *et al.*, 2015). The global prevalence of male fertility has increased by 76.9% within twenty-nine years. Male fertility has received less attention compared to female fertility, although both partners contribute equally to infertility (Huang *et al.*, 2023). However, accurate data on male infertility might be lacking in several countries due to differences in culture and patriarchy. The global data on male infertility shows that the highest prevalence of 60-70% in the Middle East, 50% each in Europe, Latin America, and North America, while 30% each in Oceania, Africa, and Asia (Agarwal *et al.*, 2015).

In Nepal, infertility due to the male factor was 40% (Subedi *et al.*, 2016; Tamrakar & Bastakoti, 2019). Nepal's range of male factor infertility in 2009 in five different districts were Sindhupalchowk (25%), Manang (20.8%), Darchula (19.7%), Baitadi (18.9%), and Rautahat (19.2%) (Pant, 2009). The study done at Tribhuvan University Teaching Hospital, Kathmandu, in 2020 reported male factor infertility alone (28.7%), and in combination with female factor (6.7%) (Rijal & Maskey, 2020).

2.5.2 Causes of male infertility

Infertility in males may be due to some known causes or may be unexplained or idiopathic. Infertility is unexplained if a patient is physically normal without any abnormal clinical history and has normal semen characteristics, and idiopathic if the etiology of the abnormal semen characteristics of a man is unidentifiable (Pozzi *et al.*, 2023). In a broad sense, known causes of infertility in men may be congenital or acquired (Agarwal *et al.*, 2021). Congenital causes include bilateral absence of vas deferens, anorchia (absence of one or both testes), cryptorchidism (undescended testes), microdeletion of the Y-chromosome, and imbalance of hormones produced by pituitary glands, hypothalamus, and testicles (Agarwal *et al.*, 2021). Infertility in men can be due

to several factors that are acquired after the birth during the lifetime and include varicocele, germ cell tumors, testicular injury, infection of the urogenital tract and male accessory gland, reproductive hormonal imbalance, inflammations, systemic diseases, production of anti-sperm antibodies, sexual dysfunction, or exposure to external factors like medications, chemotherapy, radiation, and heat. There are certain idiopathic factors, including behavioral and lifestyle factors like smoking, alcohol consumption, recreational drugs, diet, obesity and lack of exercise, exposure to toxins, and psychological stress, which may contribute to the risk of developing conditions that may affect the fertility potential.

Male sexual dysfunction and male infertility often coexist and are associated with each other. The sexual dysfunctions in men may lead to infertility and vice versa, which may include a lack of sexual desire, erectile dysfunction, ejaculatory dysfunction-premature, delayed, or retrograde ejaculation, orgasm disorder, and penile conditions (Chung *et al.*, 2023).

2.5.3 Assessing male infertility

Either or both partners may attribute infertility to a couple. Thus, evaluation of the couple in parallel is required. Female partners are regarded as care-seekers in the treatment of infertility, whereas the assessment of male partners is often neglected (Culley *et al.*, 2013). The evaluation of infertility in males is done to identify the conditions that can be corrected, any irreversible conditions that are not correctable, but to opt for assisted reproductive techniques or accept insemination from donor semen, any life-threatening condition, or any genetic abnormalities (Jarow *et al.*, 2010).

Infertility in men should be assessed with a complete reproductive clinical history, physical examination, and analysis of semen. The reproductive history includes the frequency and timing of coitus, duration and type of infertility, any childhood illness, chronic illness, respiratory or sexually transmitted infections, prior surgeries, and gonadal exposure to toxins and heat (Schlegel *et al.*, 2021). Information on medical and reproductive history helps identify risk factors influencing men's fertility. Semen analysis is the backbone of the diagnostic work-up for every infertile man, which helps to determine the severity of male infertility (Pozzi *et al.*, 2023). Semen parameters may vary with each ejaculation of a person. Thus, two semen analyses in a month are

recommended if the first semen analysis shows abnormal characteristics (Schlegel *et al.*, 2021).

2.5.4 Semen characteristics

Semen or seminal fluid, released during ejaculation, is a suspension of sperm cells that are produced in the testes, processed in the epididymis, and mixed with fluid secreted from accessory sex organs-prostate, seminal vesicles, bulbourethral glands, and epididymis. The major quantifiable attributes of semen are the total number of spermatozoa, which reflects the testicular function in sperm production, and the volume of seminal fluid that reflects the functioning of accessory sex organs (WHO, 2010). An investigation of semen also includes the examination of the nature of sperm (motility, morphology, and vitality) and the analysis of the seminal fluid composition. Semen is considered normal if the values of all the semen parameters are above the lower reference limit given by the WHO (2021). According to the WHO, the recommended reference limits for the semen parameters are tabulated in **Table 1**.

Table 1: Reference limit of semen parameters for routine semen analysis

Semen parameters	Lower reference limit
Volume of ejaculate	1.4 mL
Sperm concentration (per mL)	16 million
Total sperm count (per ejaculate)	39 million
Total motility	42%
Progressive motility	30%
Morphology (normal forms)	4%
Vitality	54%
Leucocyte (per mL)	<1 million

Source: WHO, 2021

Routine analysis of semen is the initial laboratory investigation of male infertility, but it is not adequate for predicting men's fertility potential (Agarwal *et al.*, 2020). Idiopathic infertility requires extended semen analysis for sperm DNA fragmentation, reactive oxygen species (ROS), and anti-sperm antibodies.

2.5.5 Sperm DNA integrity

The spermatozoal head contains a haploid genome, which is delivered to the oocyte during fertilization. Unlike somatic cells, sperm DNA possesses unique structural and compositional properties that are vital for its ability to merge with the maternal genome post-fertilization. These differences ensure the proper integration of paternal genetic material, a crucial step in embryonic development (Panner Selvam *et al.*, 2021). The integrity of sperm DNA is thus crucial for successful fertilization and embryo development in both natural conception and assisted reproduction (Sakkas & Alvarez, 2010).

Damages in sperm DNA include DNA fragmentation, mitochondrial DNA damage, telomere attrition, Y-chromosome microdeletion, and epigenetic abnormalities (Panner Selvam *et al.*, 2021). Sperm DNA damage can be caused by six key factors during sperm production or transport. These include: 1) apoptosis during spermatogenesis, 2) DNA breaks during chromatin remodeling in spermiogenesis, 3) fragmentation from oxygen radicals during sperm transport, 4) damage from endogenous enzymes like caspases and endonucleases, 5) harm from radiotherapy and chemotherapy, and 6) exposure to environmental toxins (Sakkas & Alvarez, 2010; Valipour *et al.*, 2024).

DNA integrity of spermatozoa can be reflected by laboratory tests of semen. Sperm DNA fragmentation (SDF) test has been cited as a promising biomarker and included as an extended semen assessment test in the sixth edition of the WHO manual for the laboratory examination and processing of human semen (WHO, 2021). Sperm DNA fragmentation index (DFI) is a diagnostic measure that determines the proportion of sperm with fragmented DNA among the total sperm counted. DFI <15% denotes low fragmentation, 15–29% moderate, and ≥30% high (Li *et al.*, 2024). DFI ≥30% significantly impairs pregnancy outcomes (Evenson & Wixon, 2006).

Assessment of sperm DNA fragmentation can be done either by a direct method that measures the breaks in the single or double strands of DNA, or by an indirect method that measures the susceptibility of DNA to acid denaturation (Sharma *et al.*, 2021). The presence of breaks in DNA strand(s) is directly detected by terminal deoxynucleotidyl transferase nick end labelling (TUNEL) and single-cell gel electrophoresis (Comet) assays. The susceptibility of chromatin to acid treatment can be detected by acridine

orange flow cytometry and sperm chromatin dispersion test (SCD) assays (WHO, 2021).

2.6 Bacteriospermia

It was previously thought that human semen in the normal state is free of bacteria, and the presence of bacteria in the semen would adversely affect the reproductive health of a person (Onemu & Ibeh, 2001). Studies have shown that the presence of bacteria in semen can cause significant changes in the normal properties and functioning of spermatozoa that may reduce the fertility potential of men (Baud *et al.*, 2019; Yang *et al.*, 2020). Bacteria present in semen as a consequence of infections of the male genitourinary tract and male accessory gland can result in the formation and development of spermatozoa, leading to abnormalities of spermatozoal characteristics (Oghbaei *et al.*, 2020). Seminal bacteria might not be only a result of infection. Microbial flora of skin and urethral opening may contribute to the non-pathogenic bacteria in semen (Cottell *et al.*, 2000; Willén *et al.*, 1996). However, non-pathogenic bacteria occurring as contaminants may be problematic during assisted reproductive technology (ART), wherein seminal bacteria do not need to combat the natural defense *mec*hanisms of the female genital tract, such as lysozyme, lactoferrin, and phagocytosis (Cottell *et al.*, 2000). Furthermore, studies have also shown similar bacterial flora without significant difference in semen of both healthy and infertile men (Bukharin *et al.*, 2022; Hou *et al.*, 2016).

Bacteria in semen may be pathogenic due to infection in the male genital tract or male accessory gland, or may be non-pathogenic as a result of contamination from urethral or skin flora. Therefore, there may be a usual occurrence of bacteria in low numbers in semen. Bacteriospermia is considered when the number of bacteria in semen exceeds 10^3 colony-forming units per mL of the ejaculate (Rusz *et al.*, 2012). This cut-off value differentiates the bacterial occurrence in semen and the occurrence of bacteria from non-semen sources (Weidner *et al.*, 2013).

2.6.1 Bacteria in semen

The presence of bacteria in the human body in large numbers significantly influences human health and disease. The semen microbiome, though less studied than other microbiomes, is gaining attention for its potential impact on male reproductive health,

couple fertility, and even offspring health due to the transfer of microorganisms (Altmäe *et al.*, 2019).

Semen culture has been used as a routine technique for detecting bacteria in semen. Commonly urinary tract pathogens such as *E. coli*, *S. aureus*, *E. faecalis*, *Proteus* spp, *Klebsiella* spp., CoNS, *Pseudomonas* spp, and *Streptococcus agalactiae* in semen of infertile men (Alzaidi & Kareem, 2024; Weidner *et al.*, 2013). Sexually transmitted pathogenic bacteria, including *Mycoplasma hominis, M. genitalium*, *Ureaplasma urealyticum*, *C. trachomatis,* and *Neisseria gonorrhoeae,* are also commonly detected in the semen of infertile men (Gimenes *et al.*, 2014; Kim *et al.*, 2017). The microbiota of the male reproductive tract was not adequately described for a long time, as culture was considered only a test to detect microorganisms in semen, and was considered free of microbes if no bacteria in culture were detected (Wang *et al.*, 2022). However, semen culture could not detect anaerobic bacteria, difficult-to-culture bacteria, and non-cultivable bacteria present in semen (Mändar *et al.*, 2017).

Recent advances in genomic sequencing have further explored the complex bacterial diversity in human semen. The NGS technique for the detection of 16S rRNA genes in semen with a metagenomic approach has helped to study even non-cultivable bacteria present in semen (Hou *et al.*, 2016). The earlier metagenomic study of semen identified *Ralstonia, Lactobacillus, Corynebacterium, Streptococcus, Prevotella, Staphylococcus, Anaerococcus,* and other bacterial genera in both fertile and infertile men (Hou *et al.*, 2016). Semen microbiota of leukocytospermic and non-leukocytospermic populations showed the presence of *Streptococcus, Lactobacillus, Burkholderia-Caballeronia-Paraburkholderia, Staphylococcus, Gardnerella, Ralstonia, Corynebacterium, Veillonella, Acinetobacter, Rhodococcus, Finegoldia, Peptoniphilus, Enterococcus, Prevotella,* and *Haemophilus*, with a decrease in the number of *Lactobacillus* and an increase of *Streptococcus* in the semen with leukocytospermia (Yao *et al.*, 2022).

2.6.2 Possible sources of bacteria in semen

Semen contains nutrients such as proteins, lipids, fructose, and inorganic ions derived from the male accessory glands. The nutrient-rich environment of semen supports the growth of microorganisms acquired from different sources (Tomaiuolo *et al.*, 2020).

Seminal bacteria may not always indicate an infection, and might result from contamination from the other parts of the male body or female sexual partner (Koedooder *et al.*, 2019). The possible sources of bacteria in semen are described below.

2.6.2.1 Urinary tract infection

Semen during ejaculation may harbor microorganisms present in men's urinary tracts, as both the semen and urine have the urethral opening as a portal of exit from the body. Therefore, if a man has a urinary tract infection, semen contains urinary pathogens. However, semen may also include normal genitourinary microbial flora since the distal urethra is not normally sterile in a healthy human. Semen culture of a healthy man may yield the growth of bacteria that are normal flora of the genitourinary tract (Solomon & Henkel, 2017). The human genome project revealed that about 30% of the human microbiome is confined to the urethra (Yang *et al.*, 2020)**.** Contaminating bacteria of the male urogenital tract can negatively affect the sperm parameters that may influence the reproductive health of men (Fraczek & Kurpisz, 2015).

2.6.2.2 Male accessory gland infection (MAGI)

The accessory glands of the male reproductive system are the prostate, seminal vesicle, and bulbourethral gland that play significant roles in the formation and functioning of the semen. The secretions of these glands not only nourish and protect the spermatozoa but also maintain the fluidity of semen, helping in the mobility of sperm cells, which is important for successful fertilization. Infection of these glands begins from the urethra to the prostate, seminal vesicle, vas deferens, and epididymis (Krause, 2008).

Prostatitis is a condition with the inflammation of the prostate gland. Bacterial infection of the prostate is one of the major causes of prostatitis. Semen culture is considered a diagnostic tool for bacterial prostatitis (Panackal & Panackal, 2017). The bacterial diversity and density in semen of men with prostatitis are higher than those in semen of men without prostatitis (Mändar *et al.*, 2017). Bacterial genera such as *Streptococcus, Escherichia, Actinomyces, Bacillus, Pseudomonas, Georgenia,* and *Propionibacterium* that were not present in the semen of healthy men were detected in the semen of men with prostatitis (Bukharin *et al.*, 2022).

2.6.2.3 Sexually transmitted infection

Semen contamination may be caused by the transmission of pathogens from the infected sexual partner. Bai and colleagues detected a high rate of sexually transmitted pathogens in the semen of asymptomatic sub-fertile men (Bai *et al.*, 2021). The major sexually transmitted pathogens in semen are *N. gonorrhoeae*, *C. trachomatis, M. hominis,* and *U. urealyticum* (Solomon & Henkel, 2017).

2.6.2.4 Skin flora

Semen may contain commensal bacteria as contaminants of the skin, glans penis, and lower urethra. The presence of significant bacteriospermia in combination with leukocytospermia in the semen suggests a genital tract bacterial infection requiring antimicrobial treatment. However, the presence of bacteria in semen collected for assisted reproductive technology may cause deleterious effects on the sperm (Cottell *et al.*, 2000). The WHO has also recommended the precautionary steps to be followed to avoid microbial contamination during the collection of a semen sample (WHO, 2010).

2.6.2.5 Hematogenous transmission

Urogenital organs may acquire bacterial infection through hematogenous spread of the infectious agent from other infected sites of the body. However, seminal bacteria from the hematogenous transmission are less common (Sykes & Westropp, 2022).

2.6.3 Role of bacteriospermia in infertility

The reproductive health of men can be affected by various factors, including genetic issues, diseases, infection, and injuries of the reproductive tract or organs, lifestyle factors, and exposure to adverse environmental conditions (Tvrdá *et al.*, 2022). Infection of the genitourinary tract and bacteriospermia may compromise the fertility potential of men, either directly by degrading the structure and function of sperm or indirectly by triggering the inflammatory reaction (Henkel, 2024).

2.6.3.1 Direct attachment of bacteria on spermatozoa

It was known for a long time that bacteria such as *N. gonorrhoeae* can attach to sperm cells (Gomez *et al.*, 1979; James *et al.*, 1976). Diemer and his colleagues demonstrated agglutination of spermatozoa due to direct adherence of *E. coli* on sperm cells by

scanning electron microscopy (Diemer *et al.*, 1996). Siftjit and her colleagues also showed a difference in the adherence property of live and killed bacteria. An *in vitro* experiment was conducted to study the agglutination of sperm by *S. aureus* isolated from the cervix of a female patient after co-incubation of sperm cells with live and killed bacteria separately. Light microscopy revealed the agglutination of sperm cells after co-incubation with live *S. aureus*, whereas scanning electron microscopy revealed the attachment of live *S. aureus* on sperm cells, distorting the morphology of sperm (Kaur *et al.*, 2010).

Bacteria may damage spermatozoa, reducing their fertility potency by the secretion of exotoxins. A protein of approximately 70kD termed sperm immobilization factor (SIF) was isolated from *S. aureus* that caused agglutination and immobilization of spermatozoa (Kaur *et al.*, 2010). Sperm agglutination factor (SAF), a protein of about 71kD, isolated from *E. coli*, capable of agglutinating sperm cells, could induce agglutination, morphological damage, and apoptosis in spermatozoa (Kaur & Prabha, 2013).

2.6.3.2 Inflammatory response and cytokine release

Bacteriospermia due to infection of the genital tract may cause local inflammation, causing an accumulation and activation of leukocytes. The activated leukocytes release a large amount of reactive oxygen species (ROS) (Fraczek & Kurpisz, 2007), and also cytokines such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), or interleukin-8 (IL-8), causing damage to spermatozoa, which leads to a reduction in the quality of semen (Henkel, 2024).

2.6.3.3 Oxidative stress and ROS

ROS is produced by spermatozoa in a required concentration to allow the capacitation of spermatozoa, due to which the fluidity of their plasma membrane increases, necessary for successful fertilization to occur (de Lamirande & Cagnon, 1993). Various factors, including infection, leukocytes, chronic diseases, and environmental stress, increase the production of ROS in semen (Agarwal *et al.*, 2018). Oxidative stress (OS) is a result of excessive production of ROS or limitation of antioxidants. OS stimulates premature capacitation, making spermatozoa unsuitable for fertilization, which results in infertility (Velando *et al.*, 2008). OS significantly contributes to infertility in men,

especially during bacterial infections. Pathogens such as *S. aureus*, *C. trachomatis*, and *N. gonorrhoeae* induce inflammation, excessive ROS production, and damage to reproductive organs. This leads to impaired spermatogenesis, poor sperm quality, DNA damage, mitochondrial dysfunction, and cell apoptosis, ultimately disrupting male fertility (Wang *et al.*, 2021).

2.6.4 Mechanism of bacteria-induced sperm DNA fragmentation

Breaks or abnormalities in the genetic material of sperm result in sperm DNA fragmentation. DNA fragmentation of spermatozoa may be contributed to by various clinical and environmental factors. Clinical factors include aging, obesity, hormonal imbalance, chronic diseases like diabetes and cancer, systemic infection, and infection of the male genital tract, whereas environmental factors include diet, drug abuse, smoking, alcohol, environmental toxins, radiation, and high testicular temperature (Agarwal *et al.*, 2020). Bacterial presence in the male genital tract, whether from infection or colonization, induces sperm DNA fragmentation. This impairs sperm-oocyte fertilization, disrupts embryo development, and contributes to infertility (Farkouh *et al.*, 2023). The role of bacterial infection in inducing damage to DNA has gained increasing attention as it can trigger a cascade of events and compromise sperm DNA integrity (Garcia-Segura *et al.*, 2023). Bacteria induce sperm DNA fragmentation through multiple mechanisms, including direct toxin-mediated damage, indirect effects driven by inflammatory responses, and the production of oxidative stress.

2.6.4.1 Bacterial adhesion and toxin production

Gram-negative bacteria can adhere to sperm surfaces and release toxins like lipopolysaccharides, whereas *E. coli* and *Enterococcus* are capable of producing toxins like α-hemolysin and β-hemolysin, respectively. These toxins directly disrupt the membranes and DNA of spermatozoa (Tvrdá *et al.*, 2022). Some bacteria can directly cause the fragmentation of sperm DNA by releasing the enzyme restriction endonuclease (Gosálvez *et al.*, 2024). *S. aureus* produces clumping factors A and B (regulated by the genes *clf*A and *clf*B, respectively) that act as adhesins that help the bacteria bind to the host fibrinogen proteins. This binding helps in colonization, biofilm formation, and evasion of the host immune system by making the bacteria too large for efficient macrophage phagocytosis (Abbas & Ghareeb, 2023). *S. aureus* isolated from

the cervix of a woman with unexplained infertility has been known to produce a protein SAF that agglutinates human spermatozoa (Ohri & Prabha, 2005).

2.6.4.2 Inflammatory response

Bacterial infection also triggers an inflammatory response in the reproductive tract. This causes infiltration of immune cells, mostly leukocytes, and the release of pro-inflammatory cytokines (Zeyad *et al.*, 2017). Lipopolysaccharides present in Gram-negative bacteria activate Toll-like receptor signaling, which stimulates the secretion of pro-inflammatory cytokines such as TNF-α, IL-6, and IL-8, and activates nuclear factor kappa B. This cascade elevates ROS levels, ultimately causing damage to both the sperm membrane's structural integrity and DNA fragmentation (Wang *et al.*, 2021). An experiment with normal and abnormal leukocyte levels in the semen revealed that the fragmentation of sperm DNA was significantly higher in the semen with abnormal leukocyte levels (Liu *et al.*, 2021). Leukocytes in the plasma of semen produce ROS, like superoxide anions, hydrogen peroxide, and hypochlorous acid, during infection or inflammation. These ROS, along with inflammatory factors released by activated immune cells, increase OS, disrupting the balance with antioxidants. This triggers inflammatory chemokines that deteriorate sperm function and contribute to male infertility. Additionally, high levels of inflammatory cytokines can damage the blood-testis barrier, alter the testicular environment, and increase sperm DNA damage (Dutta *et al.*, 2021).

2.6.4.3 Apoptosis

Apoptosis is a mechanism that plays a significant role in spermatogenesis. However, higher levels of apoptosis result in damage to spermatozoa, leading to the problem of infertility (Weng *et al.*, 2002). Increased production of ROS by leukocytes may increase the rate of sperm apoptosis (Lopes *et al.*, 1998). Moreover, it has also been demonstrated in an *in vitro* experiment co-incubating sperm cells with *E. coli*, *S. aureus*, and *E. faecalis* that apoptosis of sperm cells could also be stimulated directly by bacteria, even without production of ROS, resulting in changes in motility, vitality, and DNA integrity of spermatozoa (Villegas *et al.*, 2005). Additionally, porins and lipopolysaccharides extracts from *Salmonella enterica* and *Pasteurella multocida* increased spontaneous apoptosis of human spermatozoa after co-incubation (Gorga *et*

al., 2001). Thus, bacterial infection, even without the production of ROS, can induce apoptosis, resulting in damage to sperm DNA integrity.

2.6.5 Bacteriospermia and semen characteristics

The occurrence of bacteria in significant numbers in semen might be a result of male genital tract infection or colonization of bacteria originating from the urinary tract or sexual transmission. Bacteria can either directly damage sperm cells or indirectly, after the production of toxins and metabolites, exhibiting changes in semen parameters (Oghbaei *et al.*, 2020). However, the impact of colonization of non-pathogenic bacteria in the male genitourinary tract versus active infection on the impaired sperm characteristics is a controversy in andrology research (Fraczek & Kurpisz, 2015). The effect of bacteria on semen parameters depends on the type and concentration of bacteria present in semen (Fraczek *et al.*, 2007). The semen parameters like ejaculate volume, sperm concentration, motility, and morphology are the key indicators of semen analysis of semen, and the presence of bacteria can significantly confound these assessments (García-Ferreyra, 2015; Wang & Swerdloff, 2014).

2.6.5.1 Sperm concentration

Bacterial infection in the male reproductive tract can damage the sperm producing cells or may also block the reproductive ducts, interfering with the release of sperm (Farsimadan & Motamedifar, 2020). Studies comparing sperm concentration in men with infected and uninfected semen have revealed significantly lower sperm concentration in semen with bacteria (Fraczek *et al.*, 2016; Osadchiy *et al.*, 2024). A meta-analysis study of 18 studies, including the population with bacteriospermia and healthy controls, concluded that sperm concentration is significantly affected by bacteriospermia (Pergialiotis *et al.*, 2018). Facultative bacteria in semen were also associated with oligozoospermia in men (Enwuru *et al.*, 2016). The abundance of *Prevotella* and *Haemophilus* in semen also showed decreased concentration of spermatozoa (Gachet *et al.*, 2022).

2.6.5.2 Sperm motility

The complex connection between bacteriospermia and sperm motility has become a key focus in reproductive biology research. Sperm motility, which plays a vital role in successful fertilization, may be negatively impacted by bacterial contamination through

multiple pathways. Bacteria can directly hinder sperm movement, either by agglutinating the sperm cells or releasing substances that impair function, ultimately reducing the ability to travel through the reproductive tract of the female to fertilize an egg. *E. coli*, a frequent isolate of semen, has been found to agglutinate sperm cells by directly attaching to bacteria (Diemer *et al.*, 1996). Several studies have proved the agglutination of sperm cells in the presence of *S. aureus* or the sperm agglutinating factor, hindering the spermatozoal motility (Kaur *et al.*, 2010; Kaur & Prabha, 2012; Prabha *et al.*, 2009). Pathogenic bacteria of the genitourinary tract of men, like *Ureaplasma* and *Mycoplasma*, also significantly affect the motility of spermatozoa (Stojanov *et al.*, 2018). *Prevotella* and *Haemophilus* in semen also affected the spermatozoal motility (Gachet *et al.*, 2022).

2.6.5.3 Sperm morphology

Bacteria can negatively affect the morphology of sperm cells. Eini and colleagues obtained more morphologically abnormal sperm cells in semen with bacterial infection. A significant decrease in normal morphology of sperm cells was observed in the semen infected with *E. coli*, *S. aureus*, *Proteus* spp, and *K. pneumoniae* (Eini *et al.*, 2021). SAFs produced by *S. aureus* and *E. coli*, which agglutinate sperm cells, also cause damage to sperm morphology (Tvrdá *et al.*, 2022). *U. urealyticum* has also been found to impart morphological damage to sperm cells (Pebdeni *et al.*, 2022; Xianchun *et al.*, 2023). Morphological abnormality of sperm was associated with various other bacteria, like *Streptococcus* (Hannachi *et al.*, 2018) and *Mycoplasma* (Rybar *et al.*, 2012). However, lactobacilli were present in abundance in semen with higher normal forms of sperm (Baud *et al.*, 2019).

2.6.5.4 Sperm vitality

A study on the effect of common Gram-negative bacteria on semen resulted in the adverse impact of *E. coli* on the viability of sperm cells due to apoptosis (Marchiani *et al.*, 2021). The semen infected with *M. hominis* showed diminished spermatozoal vitality (Tam Le *et al.*, 2022). Contrarily, *Lactobacillus* was positively associated with the vitality of sperm (Baud *et al.*, 2019).

2.6.5.5 Sperm DNA fragmentation

Sperm DNA integrity is one of the important predictors of male infertility, and numerous bacteria are associated with the fragmentation of sperm DNA (Pagliuca *et al.*, 2021). Semen with bacterial infection contained sperm with higher DNA fragmentation in past studies (Eini *et al.*, 2021; Fraczek *et al.*, 2016; Zeyad *et al.*, 2018). Higher sperm DFI was observed in semen infected with *U. urealyticum* (Reichart *et al.*, 2000) and *Mycoplasma* (Rybar *et al.*, 2012). Gram-negative bacteria produce outer membrane vesicles, which can damage sperm cells, and it was experimentally proven that outer membrane vesicles produced by *E. coli* damage the acrosome, adversely affecting sperm DNA (Folliero *et al.*, 2022).

2.6.4 Methodological approaches for the detection of bacteriospermia

Detection of bacteriospermia is crucial during the evaluation of male infertility and before opting for the appropriate treatment. WHO, in its manual for the investigation and diagnosis of infertile couples, mentioned criteria for detecting prostatitis and significant bacteriospermia (WHO, 1996). The exploration of the bacterial composition of semen helps to understand the etiology of urogenital tract infections, their pathogenesis, and their association with male infertility (Iovene *et al.*, 2017). Accurately detecting bacteriospermia poses a significant challenge in evaluating male fertility, necessitating a comprehensive approach that integrates both conventional microbiological methods and cutting-edge molecular techniques.

2.6.4.1 Culture method

In 1952, bull semen was cultured for the isolation of bacteria present in the semen of bulls that may affect the fertility (Wu *et al.*, 1952). Bacteria in the semen of men by the culture method were documented back in 1956 by Riley and Masters (Riley & Masters, 1956). An enrichment of semen in broth before plating on agar media was considered to increase the sensitivity of culture (Alo *et al.*, 2013). An enrichment of sediment of centrifuged seminal fluid in brain heart infusion (BHI) broth before semen culture was also experimented with, and was also considered a more sensitive method than culture without enrichment. This modification in culture method isolated bacteria that are a possible cause of infection and are less frequently isolated by the culture technique without enrichment (Iovene *et al.*, 2017). Semen culture was a commonly

adopted method for detecting the presence of bacteria, and is still in use for routine microbiological assessment of semen. The culture method was considered a gold standard method due to its accuracy, reliability, and affordability (Bunn & Sikarwar, 2016). However, this method is applicable for cultivable bacteria only and cannot be applied to non-cultivable and difficult-to-cultivate bacteria.

2.6.4.2 PCR detection of bacteria

PCR, being a highly sensitive advanced technique used to detect the presence of bacterial DNA in semen, has improved in the diagnosis of bacterial infection responsible for sexually transmitted diseases and male infertility. This methodological approach can detect cultivable, difficult-to-culture, as well as non-cultivable bacteria, even though present in low density (Jain *et al.*, 2016). PCR can detect a wide range of bacteria, with the use of universal 16S rRNA gene primers. Bacteria such as anaerobes that are often missed by standard cultural techniques can be detected by PCR methods (Jarvi *et al.*, 1996).

PCR enables the detection of targeted pathogenic bacteria, usually sexually transmitted pathogens. Moreover, the multiplex PCR technique also simultaneously detects multiple pathogens at a time (Gomgnimbou *et al.*, 2025). However, the conventional PCR technique can be considered as a qualitative technique and cannot reveal the quantity of DNA or bacteria present in the sample. This limitation has been overcome by a real-time quantitative PCR (qPCR) technique, which accurately quantifies the concentration of DNA of targeted bacteria present (Voroshilina *et al.*, 2020).

2.6.4.3 Next-generation sequencing (NGS) of 16S rRNA gene

NGS provides a comprehensive and culture-independent analysis of the seminal microbiome and detects bacteria even missed by the PCR technique. NGS allows millions of DNA fragments to be sequenced at once, making it possible to detect a wide variety of bacterial species, even those that cannot be cultured or exist in very small numbers (Campbell *et al.*, 2023). A widely used method for detecting bacteria involves sequencing the hypervariable regions of the 16S rRNA gene (Weng *et al.*, 2014). This gene is conserved among all bacteria but contains species-specific sequences within these regions. The process involves DNA extraction from semen, amplification of the 16S rRNA regions by PCR, library preparation, high-throughput sequencing (often on

Illumina platforms), and bioinformatics analysis to classify bacterial taxa using reference databases (Davies *et al.*, 2024). This technique throws light on the relative abundance and diversity of bacteria, which helps in a better understanding of the dominance of specific bacteria in a community (Weng *et al.*, 2014).

2.7 Research gaps

The WHO (2023) report on global infertility prevalence reveals regional variations; however, these differences are not deemed statistically significant due to overlapping confidence intervals and limited data in certain areas. Notably, no studies were identified for the WHO South-East Asia Region, highlighting a significant data gap (WHO, 2023). Numerous studies have been conducted on the seminal microbiome in infertile men in different parts of the world; however, it is essential to assess the seminal microbiome and its impact on semen function in different population groups (Baud *et al.*, 2019). The microbiota in different sites of the human body vary with variation in geographical distribution (Suzuki & Worobey, 2014). The geographical variation in microbiome also applies to semen, and also for bacterial etiology concerning male infertility (Gimenes *et al.*, 2014).

As in the global context, infertility is an alarming issue in Nepal, with about half of the cases being contributed by male factor (Subedi *et al.*, 2016; Tamrakar & Bastakoti, 2019). Limited data regarding infertility and seminal bacteria in Nepalese men urges the need for further study in the area of infertility in men. Furthermore, there is no published data on the semen microbiome from Nepal to date.

CHAPTER 3

3. MATERIALS AND METHODS

3.1 Materials

The lists of the different reagents, materials, and equipment used in this study are mentioned in Appendices I and II.

3.2 Methodology

3.2.1 Research design

An observational study based on a single study site was carried out.

3.2.2 Study site

The study site for sample collection was Creator's IVF Nepal Pvt. Ltd. (CIVF), a fertility center located in Lalitpur, Nepal, and the samples were processed in the andrology laboratory of CIVF and the laboratory of the Central Department of Microbiology (CDMi), Tribhuvan University (TU), Kathmandu, Nepal.

3.2.3 Study population and study subjects

A targeted sampling method was applied. The study population was the male partners of the couples trying to conceive for one year and undergoing fertility consultation. The study subjects were recruited based on defined inclusion and exclusion criteria.

3.2.3.1 Inclusion criteria

- Subjects were included in the study with signatures in the consent forms (Appendix III).
- Subjects under fertility consultation were included irrespective of the type of infertility (whether primary or secondary), and the cause of infertility.

3.2.3.2 Exclusion criteria

- The subjects under antibiotic therapy within the past 5 weeks for any disease were excluded (Pajovic *et al.*, 2013).

- The subjects with symptoms of urinary tract infection and a seropositive report for Human immunodeficiency virus (HIV), Hepatitis B surface antigen (HBsAg), and syphilis were excluded from this study.

The data on the demographics of the subjects and history of infertility were also collected in a structured questionnaire (Appendix IV).

3.2.3.3 Sample size

The minimum sample size calculated was 196. However, 217 samples were collected, considering an incomplete data collection and a probable loss of samples during the processing. The minimum sample size was calculated using the formula for sample size calculation.

$n = z^2pq / d^2$

Where,

z = value is assuming a 95% confidence interval that is 1.96

p = prevalence, it is 15% = 0.15 (Diemer *et al.*, 2003; Domes *et al.*, 2012)

d = allowable error is 5% = 0.05

q = 1-p = 1-0.15 = 0.85

n = required sample

Minimum sample size = $(1.96)^2 \times 0.15 \times 0.85 / (0.05)^2$

= 195.9

= 196

3.2.4 Ethical consideration

All the study subjects were provided with the written informed consent form and included in the study only after obtaining their signatures. The study was approved by the ethical review board of the Nepal Health Research Council (NHRC), Kathmandu, Nepal (Approval No. 874/2019) (Appendix V).

3.2.5 Study specimen

Non-repetitive semen samples from study subjects were taken for the study. Semen samples collected in the fertility center by masturbation with abstinence of 2-5 days were included in the study.

3.2.6 Collection of specimens

The study subjects were given a sterile, leak-proof, wide-mouthed plastic container and informed about the aseptic techniques for sample collection for microbiological analysis according to WHO (2021). Before collecting a semen sample, the study subject was asked to wash hands and penis with soap and water, dry the hands and penis with a fresh disposable towel, and finally ejaculate the sample in the sterile container provided (WHO, 2021).

3.2.7 Laboratory processing

After sample collection, the specimen container was immediately transferred to the andrology laboratory, labeled with the name, identification number, and sample collection date and time. The labeled specimen container was kept on a warmer at 37 °C for 30-60 min for liquefaction. Immediately after the liquefaction of the sample, the time taken to liquefy the sample was recorded. Initial macroscopic examination of semen and analysis for sperm concentration, motility, morphology, and vitality were done after the liquefaction of the sample.

3.2.7.1 Initial macroscopic examination

The ejaculate volume, appearance (color and opacity), and pH of the sample were recorded. The viscosity of the sample was examined by gently aspirating the sample into a plastic disposable pipette, allowing the sample to drop. The viscosity of the sample was recorded as normal if the sample left the pipette in small, discrete drops and abnormal if the drop formed a thread more than 2 cm long (WHO, 2021).

3.2.7.2 Semen analysis

Analysis of semen was done within 30 min of semen liquefaction. Semen samples were characterized upon assessing sperm count, sperm motility, sperm vitality, and sperm morphology according to WHO reference values (2021), as mentioned in **Table 1**. The leucocytes present in the samples were also quantified.

Counting of spermatozoa

The number of spermatozoa in a known volume of semen sample (0.1 µL) was counted using a Neubauer’s chamber, expressed as number per mL of sample. The spermatozoal

count and ejaculate volume were used to calculate the sperm concentration per ejaculate.

Assessment of motility

Motility of spermatozoa was assessed in a wet mount preparation of the semen sample in Makler's chamber at 200X total magnification. At least 200 spermatozoa in 5 different microscopic fields were assessed for each sample and were categorized as rapidly progressive, slowly progressive, non-progressive motility, and immotile. Progressive motility includes sperm cells moving actively, either linearly or in a large circle, regardless of speed. Non-progressive motility includes all other patterns of motility with an absence of progression, e.g., swimming in small circles, the flagellar force hardly displacing the head, or when only a flagellar beat can be observed. Immotility includes sperm cells without any movement. Based on the above categories, total motility (rapidly progressive and slowly progressive) was calculated and expressed as a percentage (WHO, 2021).

Assessment of morphology

Spermatozoal morphology was investigated by the Diff-Quik method (WHO, 2010) using a Morphology-D kit (Sperm Processor Pvt. Ltd., India) following the manufacturer's instructions (Appendix VI). At least 200 sperm cells were examined under an oil immersion objective and categorized as normal and abnormal forms. If sperm dimensions for head, neck, and tail were within normal limits, then it was called normal sperm. When any one of the parameters of sperm for head, neck, or tail was not within normal limits, then it was called abnormal sperm. The percentage of sperm of normal forms was calculated for each sample.

Assessment of vitality

Vitality of spermatozoa in semen was assessed using eosin solution (0.5% Eosin Y in normal saline). Equal volumes of sample (5 µL) and eosin solution (5 µL) were mixed well on a glass slide. A cover slip was placed on the mixture and left for 30 s. The slide was then examined under the microscope with a 40X objective, and the dead (stained) and live (unstained) spermatozoa were counted. At least 200 sperm cells were counted, and the vitality percentage was calculated as the percentage of live sperm (WHO, 2010).

Counting of leucocytes in semen

The assessment of leucocytes in semen was done by peroxidase test using a Leukocheck kit (Cryolab International, India) following the manufacturer's instructions (Appendix VII). The leukocheck kit identifies leukocytes in semen based on the cytochemical stain for peroxidase activity. Cells (leukocytes) that contain peroxidase activity stain brown, and other cells counterstain pink. Peroxidase-positive brown round cells in the corner squares of Neubauer's chamber were counted as leucocytes, then the number of leucocytes per mL of the sample was calculated (WHO, 2010).

3.2.7.3 Measurement of sperm DNA fragmentation test

The SDF test was done by a sperm chromatin dispersion assay, which is an indirect method of detecting DNA fragmentation and evaluating the susceptibility of sperm chromatin to denaturation by acid (WHO, 2021). The SDF test was done using a Sperm chroma kit (Cryolab International, India) according to the manufacturer's instructions (Appendix VIII). Five different patterns of sperm characteristics were categorized into two groups: sperms with non-fragmented DNA (sperm cells with large and medium halos), and sperms with fragmented DNA (sperm cells with small or no halo and degraded sperm cells). At least 300 sperm cells were observed under a microscope and categorized into groups. The sperm DNA fragmentation index (DFI) was calculated based on the number of sperm with fragmented DNA and total sperm counted, expressed as a percentage, and categorized as low, moderate, and high SDF (Li *et al.*, 2024).

3.2.7.4 Semen culture

One mL of the liquefied sample was transferred to a sterile microfuge tube immediately after macroscopic examination of the sample and transported to the microbiology laboratory of the CDMi, TU, within 3 h of sample collection, maintaining the cold chain. Samples were inoculated on MacConkey agar (MA), blood agar (BA), and chocolate agar (CA) and incubated at 37 °C for 24 h with the help of a calibrated loop (Hi-media, India). The remainder of the semen samples were transferred to sterile microcentrifuge tubes and stored at -80 °C for DNA extraction.

Inoculated plates with MA and BA were incubated aerobically and with CA in a CO_2-enriched environment in a candle jar. Bacteriospermia was considered if the growth of

bacteria in an agar plate was greater than 10^3 bacteria/mL (Domes *et al.*, 2012; WHO, 1996). The isolates obtained were identified based on their cultural characteristics on MA, BA and CA, Gram reactions, morphological characteristics on microscopic observation, biochemical assays such as catalase test, oxidase test, indole production test, methyl red test, Voges-Proskauer test, citrate utilization test, oxidation-fermentation test, urease production test, triple sugar iron test, nitrate reduction test, etc. and necessary specific tests were performed for the identification of particular bacteria (Tille, 2014).

3.2.7.5 Quality control (Sterility testing and purity plate)

Sterility testing of each batch of media after preparation was done by incubating two sets of media at 37 °C for 48 h to ensure that no growth was obtained on/in the media. The purity plate was checked to ensure that the inoculation used for the biochemical tests contained a pure culture. Before conducting the biochemical assays, a 4 h-incubated broth culture of the isolates was inoculated onto one-half of the nutrient agar plate. Immediately after performing the biochemical test, the remaining half of the nutrient agar plate was inoculated. The plate was incubated at 37 °C for 24 h. The presence of the same organisms' development in both the pre- and post-inoculation sections of the plate indicates consistent maintenance of aseptic conditions throughout the experiment (Tille, 2014).

3.2.7.6 Preservation of bacterial culture

A single bacterial colony from a pure culture of identified bacteria was transferred to a cryovial with 750 µL of sterile trypticase soy broth (TSB) and incubated at 37 °C for 24 h. To the turbid culture after 24 h of incubation, 750 µL of sterile glycerol was added, mixed well, and sealed properly (Fadanka *et al.*, 2022). The sealed cryovials with glycerol stock of bacteria in triplicate were stored at -80 °C for further use.

3.2.7.7 DNA extraction from *S. aureus* isolates

S. aureus was the most predominant bacterium obtained in this study. Thus, DNA from *S. aureus* isolates was extracted to study their antimicrobial and virulence properties. DNA extraction of the *S. aureus* isolates was done by the boiling method as mentioned by Hartas *et al.* (Hartas *et al.*, 1998). A single colony from a 24 h agar plate was picked up and suspended in 100 µL of 50 mM sodium hydroxide in a microcentrifuge tube.

The suspension was incubated at 95 °C for 5 min, cooled to 4 °C for 5 min, and then neutralized with 16 µL of 1 M Tris-HCl (pH 8.0). The suspension was then centrifuged at 10,000 rpm for 3 min, and the supernatant was transferred into another sterile microcentrifuge tube. The purity and concentration of the extracted DNA were assessed by using a Nanodrop spectrophotometer (ThermoScientific, USA). DNA extract with A260/280 value of 1.5-2.0 was considered acceptable and used for PCR amplification.

3.2.7.8 Detection of virulence genes in *S. aureus*

Virulence genes *clf*A, *clf*B, and *tst* were detected in *S. aureus* isolates. Genes encoding two adhesion factors, clumping factor A and clumping factor B, and the *tst* gene encoding toxic shock syndrome toxin were amplified. Conventional PCR amplification was carried out for the detection of the virulence genes in the DNA extracted from *S. aureus* isolates. The primers were used as mentioned in **Table 2**. The composition of the PCR reaction mixture was 1x master mix (SolisBiodyne, Estonia), 0.2 µM forward primer, 0.2 µM reverse primer, 2 µL DNA template, and nuclease-free water to maintain the volume of the reaction mixture to 20 µL. Positive control using the standard gene sequence of the respective gene under study, and negative control without a DNA template were also used as PCR controls. The PCR conditions for amplification of all the genes under study were optimized and are mentioned in **Table 3**. Detection of PCR products was done by agarose gel electrophoresis (2% agarose gel with 0.5 µg/mL ethidium bromide) at 100 V for 1 h, and visualized under a UV transilluminator.

Table 2: Primers used for virulence gene detection in the study

Gene of interest	Primer sequence (5'-3')	Amplicon size (bp)	References
*clf*A	F: CGCCGGTAACTGGTGAAGCT	314	(Stutz *et al.*, 2011)
	R: TGCTCTCATTCTAGGCGCACTT		
*clf*B	F: ATGATCTTGCTTGCGTT	215	(Stutz *et al.*, 2011)
	R: CCGATTCAAGAGTTACACC		
tst	F: ACCCCTGTTCCCTTATCATC	326	(Esmailkhani *et al.*, 2018)
	R: TTTTCAGTATTTGTAACGCC		

Table 3: PCR conditions for the virulence genes under study

Steps in PCR amplification		*clf*A gene	*clf*B gene	*tst* gene
Initial denaturation		95 °C for 5 min	95 °C for 5 min	95 °C for 5 min
Amplification	Denaturation	95 °C for 15 s	95 °C for 30 s	95 °C for 30 s
	Annealing	56 °C for 30 s	50.8 °C for 30 s	52 °C for 60 s
	Extension	72 °C for 40 s	72 °C for 40 s	72 °C for 40 s
	No. of cycles	35	35	35
Final extension		72 °C for 5 min	72 °C for 5 min	72 °C for 5 min

3.2.7.9 Antibiotic susceptibility testing of predominant bacteria *S. aureus*

Antibiotic resistance profile of the predominant bacteria *S. aureus* was determined by antimicrobial susceptibility testing of the isolates using the modified Kirby-Bauer disc diffusion technique (Tille, 2014). A standard dilution (matched with 0.5 McFarland turbidity standard) of the test organism was uniformly swabbed over Mueller Hinton agar and tested against the antibiotics (Hi-media, India) ampicillin (10 mcg), erythromycin (15 mcg), ciprofloxacin (5 mcg), clindamycin (2 mcg), co-trimoxazole (25 mcg), doxycycline hydrochloride (30 mcg), levofloxacin (5 mcg), linezolid (30 mcg), gentamicin (10 mcg), nitrofurantoin (300 mcg) and cefoxitin (30 mcg) as described by Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2021). The diameter of the zone of inhibition (ZOI) in mm, after incubation at 37 °C for 24 h, was measured and interpreted as sensitive, intermediate, and resistant (Appendix IX). The organism tested against the cefoxitin disc was interpreted as sensitive if ZOI ≥ 22 mm, and resistant if ZOI ≤ 21 mm, as described by CLSI guidelines (CLSI, 2021). *S. aureus* resistant to cefoxitin discs were considered as methicillin-resistant *S. aureus* (MRSA). *S. aureus* ATCC 25923 was used as a quality control strain.

3.2.7.10 Detection of the methicillin resistance gene (*mecA*) in *S. aureus* isolates

Conventional PCR amplification was carried out for the detection of the *mec*A in the DNA extracted from *S. aureus* isolates. A forward primer (5'-GTAGAAATGACTGAACGTCCGATAA-3') and a reverse primer (5'-CCAATTCCACATTGTTTCGGTCTAA-3') with the amplicon size of 310 bp were used for PCR (Geha *et al*., 1994) (Sangon Biotech Co. Ltd., China). The composition of the PCR reaction mixture was 1x master mix (SolisBiodyne, Estonia), 0.2 µM forward primer, 0.2 µM reverse primer, 2 µL DNA template, and nuclease-free water

to maintain the volume of the reaction mixture to 20 µL. Positive control using the standard gene sequence of the respective gene under study and negative control without a DNA template were also used as PCR controls. The PCR reaction included an initial denaturation at 95 °C for 5 min, 30 cycles of amplification (denaturation at 95 °C for 30 s, annealing at 56 °C for 30 s, and extension at 72 °C for 40 s) and final extension at 72 °C for 5 min for all four genes. Detection of PCR products was done by agarose gel electrophoresis (2% agarose gel with 0.5 µg/mL) at 100 V for 1 h, and visualized under a UV transilluminator.

3.2.7.11 Genomic DNA extraction from semen

Genomic DNA was extracted from the stored semen samples for the study of bacteria in semen by 16S rRNA gene amplification and sequencing. DNA extraction from 200 µL of semen was performed using a QIAamp DNA mini kit (Qiagen, Germany) following the manufacturer's instructions (Appendix X). In addition, a DNA-free sterile TE buffer was run in parallel as a negative process control. The purity and concentration of extracted DNA were assessed using a Nanodrop Spectrophotometer (Thermo Scientific, USA). DNA extract with A260/280 value of 1.5-2.0 was considered acceptable and used for PCR amplification. The extracted DNA was stored at -80 °C for downstream experiments.

3.2.7.12 Quantitation of 16S rRNA gene in the DNA extract from semen

The occurrence of bacteria in semen samples was evaluated by the detection and quantification of the 16S rRNA gene in the DNA extracted from semen samples. The 16S rRNA gene copy number in semen specimens was quantified using a quantitative real-time PCR system in the total DNA. The primers were designed to amplify the V8-V9 hypervariable region of the 16S rRNA gene. A forward primer (5'-CGGTGAATACGTTCCCGG-3') and a reverse primer (5'-GGTTACCTTGTTACGACTT-3') were used with the amplification product size 148 bp (Sangon Biotech Co. Ltd., China). A reaction mixture contained a master mix of final concentration 1X Hot Firepol Evagreen qPCR Mix Plus ROX (Solis Biodyne, Estonia), 0.1 µM of each forward and reverse primer, 2 µL of DNA template, and nuclease-free water (ThermoFisher Scientific, USA) to make the total volume of 10 µL. Real-time PCR was carried out using Quantstudio™ 5 Real-Time PCR System (ThermoFisher, USA) under the following cycling conditions after optimization: 95 °C

for 12 min of initial incubation, and 40 cycles of denaturation at 95 °C for 15 s, annealing at 53.5 °C for 15 s, and extension at 72 °C for 15 s. The specificity of the amplified products was assessed from the melt curve, which was obtained by increasing the temperature from 60 °C to 95 °C at a rate of 0.1 °C/s.

Each PCR plate included a standard gene sequence for the primers as a positive control and a negative control. A positive control of 2858 bp (Sangon Biotech Co. Ltd., China) was a plasmid construct of the targeted 16S rRNA gene sequence (pUC 57 plasmid vector of 2710 bp and 16S rRNA gene sequence of 148 bp). The standard gene copies in the positive control were calculated (Appendix XI). A known concentration of positive control (1.276×10^{10}) was serially diluted 10-fold to obtain a standard curve after the PCR amplification. Six 10-fold standard dilutions of the positive control (10^7 to 10^2) were prepared to draw a standard curve. Molecular-grade nuclease-free water was taken as a negative control. Triplicates of diluted standards of positive control and negative controls were used in PCR amplification. PCR for each sample was carried out in duplicate to ensure the reliability of the results. After completion of real-time PCR amplification, a standard curve was plotted with obtained threshold cycle (Ct) values and the log of their corresponding gene copy numbers of the dilutions of standard positive controls, resulting in a 0.9806 correlation coefficient (R2), -3.48 slope (m), 93.8% efficiency, and 45.46 y-intercept **(Figure 2)**. This standard curve was used to calculate the gene copy numbers present in the DNA extracted from the samples.

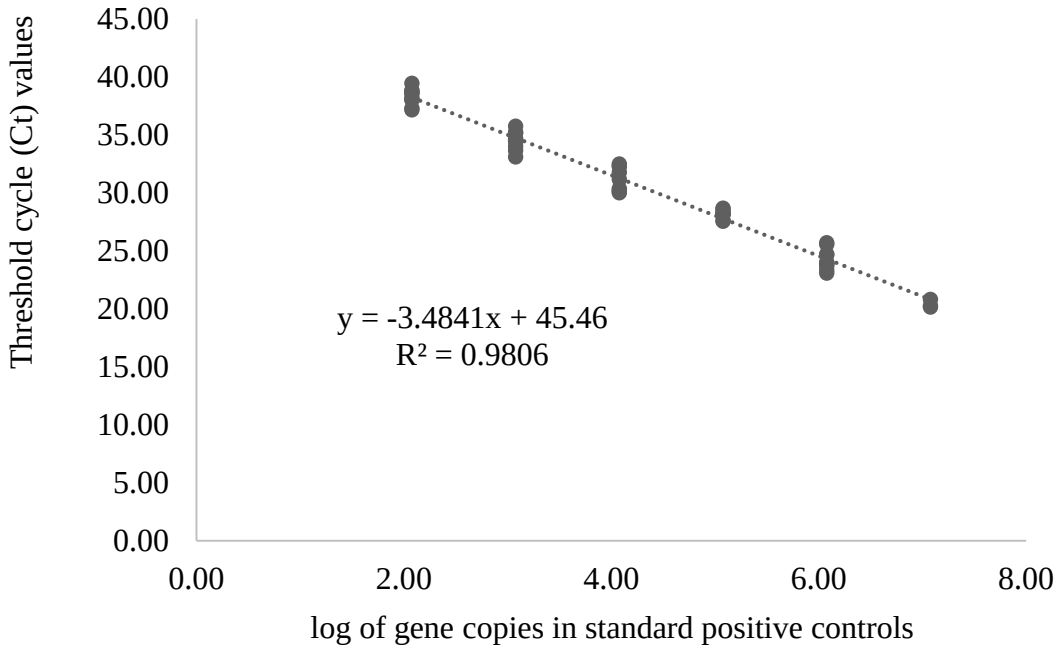

Figure 1: Standard curve of positive control of 16S rRNA gene

3.2.7.13 Sequencing of 16S rDNA in selected semen samples

Semen might contain bacteria that are difficult to culture by the routine culture technique, and also non-cultivable ones. Taking this into consideration, 10% of the samples among those that contained the 16S rRNA gene were further selected for 16S rRNA gene sequencing. Semen samples with diverse semen characteristics were selected for gene sequencing. Genomic DNA extracts were outsourced to Intrepid Nepal, Kathmandu, Nepal, for 16S rRNA gene sequencing. DNA extracts from 21 semen samples were selected based on the confirmed presence of bacteria in samples through PCR amplification of the 16S rRNA gene. The selection also considered the inclusion of semen samples with normal and abnormal sperm parameters. Among the selected samples, 3 were of normal semen characteristics, 3 were azoospermic, 3 were asthenozoospermic, 3 were teratozoospermic, 3 were asthenoteratozoospermic, 2 were oligoasthenozoospermic, 2 were oligoteratozoospermic, and 2 were oligoasthenoteratozoospermic.

PCR amplification of the 16s rDNA gene

The samples were amplified for 16s rDNA using previously designed primers according to the manufacturer's protocol (Illumina) -341F (5'CCTACGGGAGGCAGCAG3') and 805R (5'GACTACHVGGGTATCTAATCC3') targeting the V3-V4 region of 16S rRNA-encoding gene. PCR was performed in a 25µl total volume using 5µl each of 1 pmol/µl forward and reverse primers with 12.5µl of 2x Kapa HiFi Hotstart Ready mix and 2.5µl of template DNA. Thermocycling was performed at 95°C for 3 minutes, followed by 35 cycles of 98°C for 30s, 65°C for 25s, and 72°C for 20s, with final extension at 72°C for 5 minutes. The amplified PCR products were visualized using a 1.5% agarose gel using 1% gel red at 90V for 90 minutes (Caporaso *et al.*, 2011).

Metagenomic library preparation and sequencing

One nanogram (ng) of genomic DNA from each sample was used with the Illumina MiSeq Nextera XT DNA Library Preparation Kit (Illumina, Inc., USA), and the paired-end library was constructed with an insert of 500 bp for all samples. DNA was cleaned by AMPure XP beads (Agentcourt, USA) and tagmented and indexed using Nextera XT Index Kit (Illumina, Inc., USA). Clean DNA was again quantified and evaluated using Qubit (Invitrogen, USA) and Agilent Bioanalyzer DNA 1000 kit (Agilent Technologies, UK). Finally, all samples were pooled at a 4 nM concentration and paired-end [300 bp (2 x 151 bp)] sequenced in the MiSeq platform (Illumina, USA). (Caporaso *et al.*, 2011).

3.2.8 Sequencing data analysis

Sequence data was analyzed using the Quantitative Insights into Microbial Ecology (QIIME) version 2.0 pipeline. Raw sequences were de-multiplexed using demux in QIIME2 (Lappan *et al.*, 2019) and quality filtered with the help of Trimmomatic (Bolger *et al.*, 2014). Clustering was performed with 99% sequence similarity, followed by taxonomic classification from phylum to species level to assign operational taxonomic units (OTUs) using the Silva_132 16S database with a 97% percent identity filter (Quast *et al.*, 2013). FASTQ raw files for sequences of all fifteen DNA samples extracted from each semen were submitted to the NCBI database and were provided with BioProject accession number PRJNA1150943 with BioSample accessions SAMN43297909 – SAMN43297923 (http://www.ncbi.nlm.nih.gov/bioproject/1150943).

3.2.9 Statistical data analysis

All the metadata based on the questionnaire and data obtained on semen characteristics, sperm DNA fragmentation, and detection of bacteria in semen were entered into MS Excel. Statistical Package for the Social Sciences (SPSS version 21.0) was used to calculate frequencies and rates, and to determine the association between the variables. The frequency of samples based on semen parameters was calculated. The rate of bacteriospermia was determined and expressed as a percentage. The Mann-Whitney U test evaluated apparent differences for significance at a 95% confidence level (CI). An association between the occurrences of genes in bacteria was determined by Fisher's exact test. A relationship between bacteriospermia and sperm DNA integrity was determined by performing ordinal regression analysis at 95% CI.

Analysis of data of 16S rRNA gene sequencing was done using the operational taxonomic unit (OTU) table and taxonomy table through the web-based platform Microbiome Analyst 2.0 (Dhariwal *et al.*, 2017). Data filtering was done to remove low-abundance features based on prevalence (20%). Data rarefaction was performed to the minimum library size. Univariate analysis at the genus level was done using the non-parametric Mann-Whitney test or Kruskal-Wallis test, wherever suitable. A correlation between bacterial types isolated from semen and sperm DNA fragmentation, as well as other semen parameters, was estimated by Pearson's correlation test for binary data and Spearman's correlation test for ordinal data at 95% CI. The association between the type of community bacteria and semen characteristics was determined by the statistical tool EdgeR in the web-based platform Microbiome Analyst 2.0. EdgeR is a statistical tool for differential expression analysis, which is designed for count data and commonly used for the analysis of RNA sequences.

The results of all the tests were considered significant if the *p*-value was less than 0.05.

3.2.10 Limitations of the study

- The study was a single-center study, and data and samples were obtained only from one fertility center due to limitations of resources and technical difficulty. The result of this study could not be generalized to the total suspected infertile male population of Nepal.

- The study was limited to the population suspected of infertility. The control group with proven fertility was not included in the study due to the unavailability of consent for the study.
- The samples obtained from the study subjects were non-repetitive. Repetitive specimens from each study subject would standardize the protocol, since semen parameters and microbiome composition may vary over time.
- The samples obtained may be contaminated despite providing the instructions for the aseptic collection of samples to study subjects.
- Semen culture methodology was limited to aerobes and facultative anaerobes only. The study could not confirm the viability of anaerobic bacteria identified by molecular analysis.

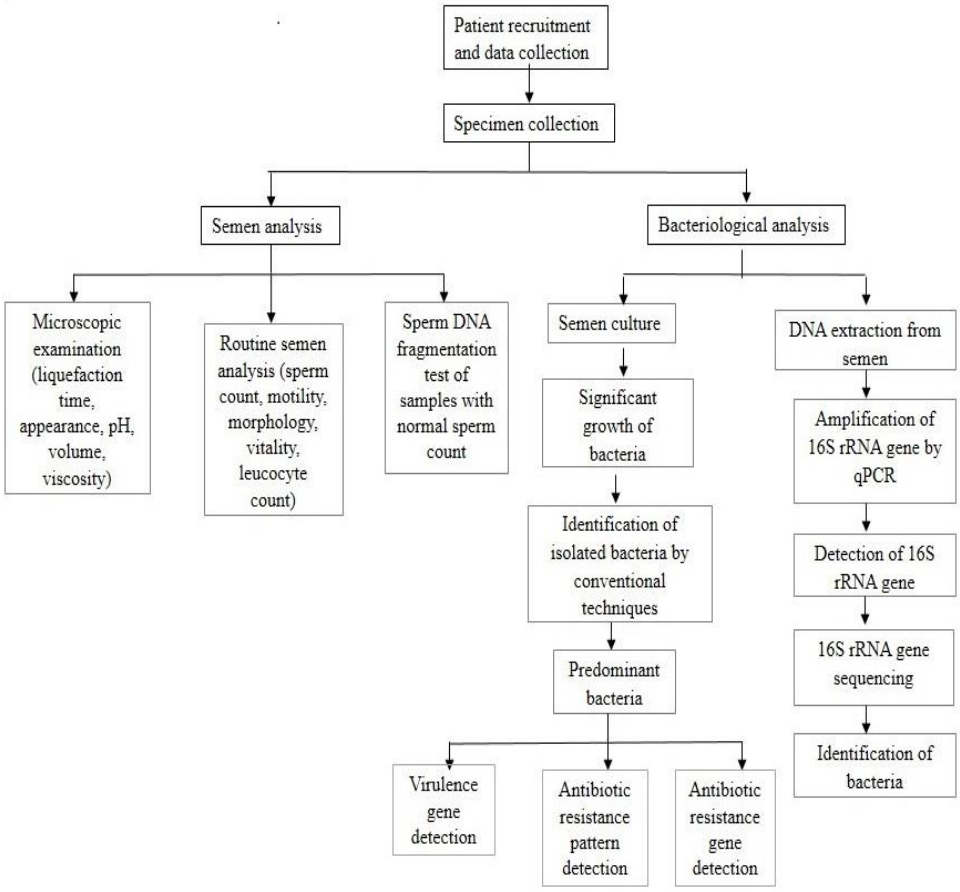

Figure 2: Methodology flowchart of laboratory processing of semen samples

CHAPTER 4

4. RESULT AND DISCUSSION

4.1 Results

4.1.1 Demographic characteristics of study participants

Among the total 217 male partners of couples trying to conceive for more than 12 months, study subjects with primary infertility were 148 (68.2%), and with secondary infertility were 69 (31.8%). The participants' ages ranged from 20 to 55 years, with a median of 35 years. The highest frequency of the participants of age group 35 to 39 years was 68 (31.3%), followed by the age group 30 to 34 years was 66 (30.4%), 40 to 44 years was 33 (15.2%), 25 to 29 years was 28 (12.9%), 45 to 49 years was 16 (7.4%), above 50 years was 4 (1.8%) and 20 to 24 years was 2 (0.9%). The participants from all seven provinces of Nepal were present. The highest number of participants was from Bagmati province, which was 144 (66.4%), whereas the least number was from Karnali province, which was 4 (1.8%). A few study participants did not disclose their data on body weight, smoking, alcohol consumption, and physical activity. According to the Asian BMI category on 211 study participants, most of the participants were obese 125 (59.3%), and the fewest participants were underweight 2 (0.9%). According to the data of 214 study participants on smoking, non-smokers were 86 (40.2%), ex-smokers were 66 (30.8%), occasional smokers were 20 (9.4%), and regular smokers were 42 (19.6%). Among 214 study participants, regular alcohol consumers were 69 (32.3%), whereas the number of participants who were alcohol consumers in the past was 21 (9.8%). Out of 214 study participants, physically active participants were 153 (71.5%), whereas participants without physical activity were 61 (28.5%) as mentioned in **Table 4**.

Table 4. Demographic characteristics of participants

Characteristics	Categories	No. of participants	Percentage
Age (years)	20-24	2	0.9
(n = 217)	25-29	28	12.9
	30-34	66	30.4
	35-39	68	31.3
	40-44	33	15.2
	45-49	16	7.4
	50 and above	4	1.9
Area (Province)	Koshi	15	6.9
(n = 217)	Madhesh	22	10.1
	Bagmati	144	66.4
	Gandaki	12	5.5
	Lumbini	15	6.9
	Karnali	4	1.9
	Sudur-paschim	5	2.3
Type of infertility	Primary	148	68.2
(n =217)	Secondary	69	31.8
Body mass index (kg/m^2)	Underweight (<18.5)	2	0.9
(n = 211*)	Normal (18.5 – 22.9)	43	20.4
	Overweight (23.0 – 4.9)	41	19.4
	Obese (≥25.0)	125	59.3
Smoking	Non-smoker	86	40.2
(n = 214*)	Ex-smoker	66	30.8
	Occasional	20	9.4
	Regular	42	19.6
Alcohol consumption	Never	61	28.5
(n = 214*)	In the past	21	9.8
	Occasional	63	29.4
	Regular	69	32.3
Physical activity	Yes	153	71.5
(n = 214*)	No	61	28.5

*A few participants did not disclose the information

4.1.2 Rate of culture-based bacteriospermia in Nepalese men

Culture of semen samples showed that 55/217 (25.35%) of samples were found to have bacteriospermia.

4.1.2.1 Diversity of cultivable aerobic and facultatively anaerobic bacteria in semen

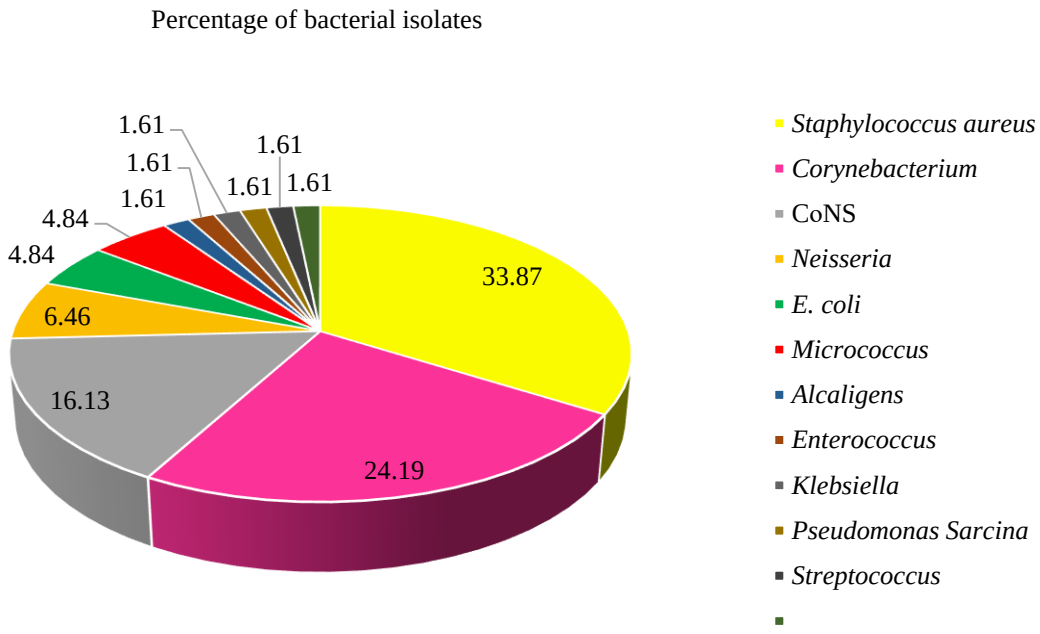

Figure 3: Diversity of bacterial isolates in semen samples

Only one type of bacteria was present in 48 (87.27%) of the total 55 semen samples, whereas two types of bacteria were present in 7 (12.73%) of the samples with bacteriospermia. Altogether 62 bacterial isolates were obtained from a total of 55 samples, out of which 11 genera of bacteria were identified. The most predominant bacteria were *Staphylococcus* spp. Thirty-one isolates (50%) were *Staphylococcus* spp., among which 21 (33.87%) were *S. aureus* and 10 (16.13%) were CoNS. Fifteen (24.19%) isolates were *Corynebacterium*, 4 (6.45%) were *Neisseria* spp., 3 (4.84%) were *Escherichia coli*, and 3 (4.84%) were *Micrococcus* spp. (4.84%), and 1 (1.61%) of each were *Alkaligens* spp., *Enterococcus faecalis*, *Klebsiella pneumoniae*, *Pseudomonas* spp., *Sarcina* spp., and *Streptococcus* spp. (**Figure 3**).

4.1.2.2 Status of occurrence of virulence genes (*clf*A, *clf*B, and *tst* genes) in *S. aureus* isolates

S. aureus was the most commonly isolated bacterium. The virulence genes responsible for the production of adhesion proteins- clumping factor A (*clf*A gene) and clumping factor B (*clf*B gene) were studied in *S. aureus* isolates. The *clf*A gene was detected in 12 (57.14%) isolates, and the *clf*B gene was also detected in 12 (57.14%) isolates. The gene responsible for toxic shock syndrome toxin (*tst* gene) was also studied in *S. aureus* and was detected in only 2 (9.5%) isolates.

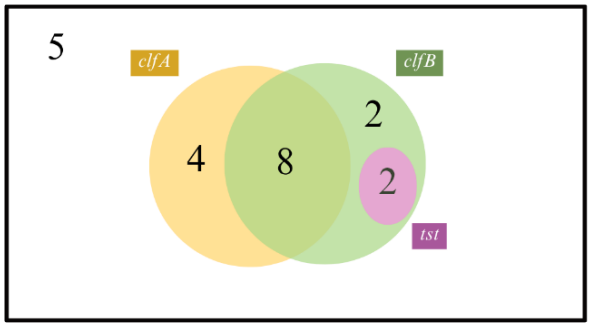

Figure 4: Co-occurrence of *clf*A, *clf*B, and *tst* genes

Co-occurrence of the genes under study showed that two clumping factor genes, *clf*A and *clf*B, were detected in 8 (38.09%) isolates, whereas the *clf*B and *tst* genes were present in 2 (9.52%) isolates. None of the virulence genes under study was detected in 5 (23.81%) isolates (**Figure 4**). However, the co-occurrence of all three genes under study was not detected in any of the isolates of *S. aureus*.

4.1.3 Rate of bacteriospermia based on targeted metagenomic approach by detection of 16S rRNA gene

Due to an inadequate volume of sample in 8 study subjects, gene amplification was carried out only in 209 samples, out of which the 16S rRNA gene was detected in 148 (70.81%) samples.

4.1.3.1 Bacterial diversity based on 16S rRNA gene sequencing

DNA extracts from 15 out of 21 samples were successfully sequenced; 6 samples could not be sequenced successfully. Thus, the sequencing results of only 15 samples were analyzed further. Illumina MiSeq sequencing results revealed a total of 216566 clean sequence reads from 15 semen samples, with 14437 average counts per sample and a

range of 957-55986 counts per sample. Altogether 140 bacterial operational taxonomic units (OTUs) were obtained among 15 samples.

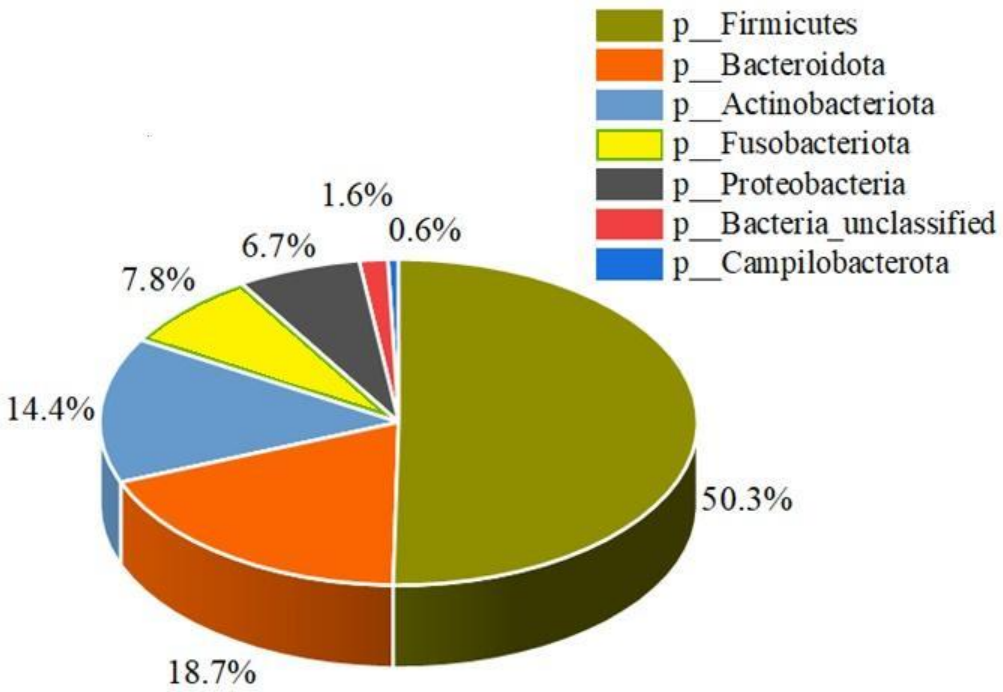

Figure 5: Relative abundance of phyla of bacteria identified based on 16S rRNA gene sequencing

Taxonomic annotation showed the presence of bacteria belonging to six phyla in the semen samples. The most abundant phylum (based on relative abundance) was Firmicutes (50.3%), followed by Bacteroidota (18.7%), Actinobacteriota (14.4%), Fusobacteriota (7.8%), Proteobacteriota (6.7%), and Campilobacteriota (0.6%) (**Figure 5**).

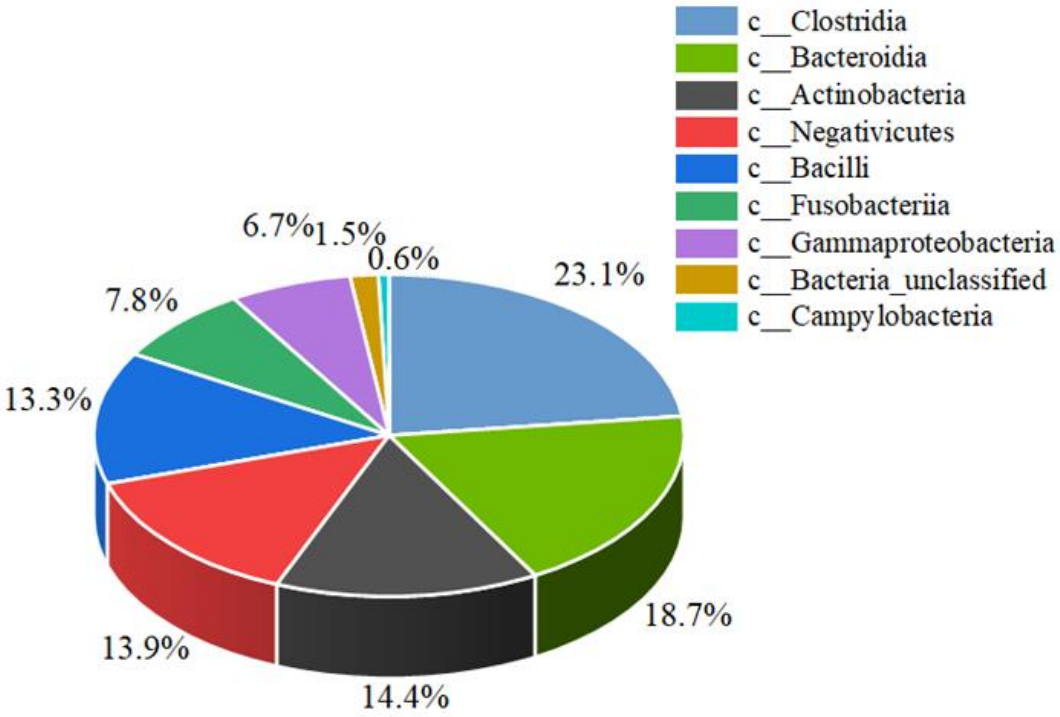

Figure 6: Relative abundance of classes of bacteria identified based on 16S rRNA gene sequencing

The bacteria belonging to the most abundant phylum Firmicutes were classified into classes: Clostridia, Negativicutes, and Bacilli, with relative abundance of 23.1%, 13.9%, and 13.3%, respectively. Other bacterial strains belonged to identified classes as Bacteroidia (18.7%), Actinobacteria (14.4%), Fusobacteria (7.8%), Gammaproteobacteria (6.7%), and Campylobacteria (0.6%) (**Figure 6**).

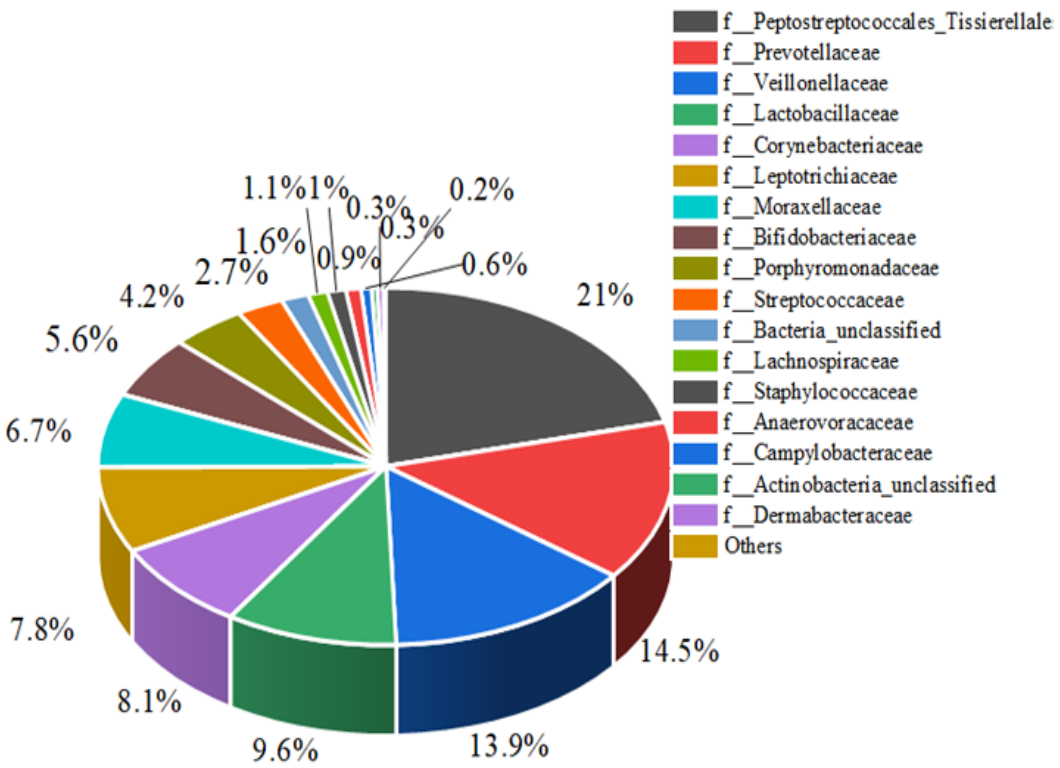

Figure 7: Relative abundance of families of bacteria identified based on 16S rRNA gene sequencing

At the family level, the bacteria were classified based on relative abundance as *Prevotella*ceae (14.5%), Veillonellaceae (13.9%), Lactobacillaceae (9.6%), Corynebacteriaceae (8.1%), Leptotrichaceae (7.8%), Moraxellaceae (6.7%), Bifidobacteriaceae (5.6%), Porphyromonadaceae (4.2%), Streptococcaceae (2.7%), Lachnospiraceae (1.1%), and Staphylococcaceae (1%). Other bacteria belonging to the families - Anaerovoraceae, Campylobacteraceae, unidentified class of Acinobacteria, and Dermabacteraceae were less than 1% (**Figure 7**). However, a group of Peptostreptococcales_Tisierellale contained 21% of the bacteria.

At the lower taxonomic level, the ten most abundant genera identified by 16S rRNA gene sequencing of 15 semen samples included *Prevotella* (14.2%), *Fenollaria* (12.2%), *Lactobacillus* (9.6%), *Veillonella* (8.4%), *Corynebacterium* (7.8%), *Acinetobacter* (6.7%), *Sneathia* (5.7%), *Gardnerella* (5.1%), *Porphyromonas* (4.2%), and *Ezakiella* (3.0%). Among the identified genera of bacteria, the 20 most abundant genera are listed in **Table 5**.

Table 5: Top 20 bacterial genera detected in semen samples as identified by 16S rRNA gene sequencing

Category of bacteria	Identified bacteria	Relative abundance %
Aerobic	*Corynebacterium*	7.8
	Acinetobacter	6.7
Microaerophilic	*Campylobacter*	0.6
Facultatively anaerobic	*Lactobacillus*	9.6
	Gardnerella	5.1
	Streptococcus	2.7
	Staphylococcus	1.0
Anaerobic	*Prevotella*	14.2
	Fenollaria	12.2
	Veillonella	8.4
	Sneathia	5.7
	Porphyromonas	4.2
	Ezakiella	3.0
	Dialister	2.8
	Peptoniphilus	2.4
	Negativicoccus	1.7
	Finegoldia	1.6
	Anaerococcus	1.4
	Megasphaera	1.0
	Moryella	0.9

4.1.3.2 Core microbiome of semen based on 16S rRNA gene

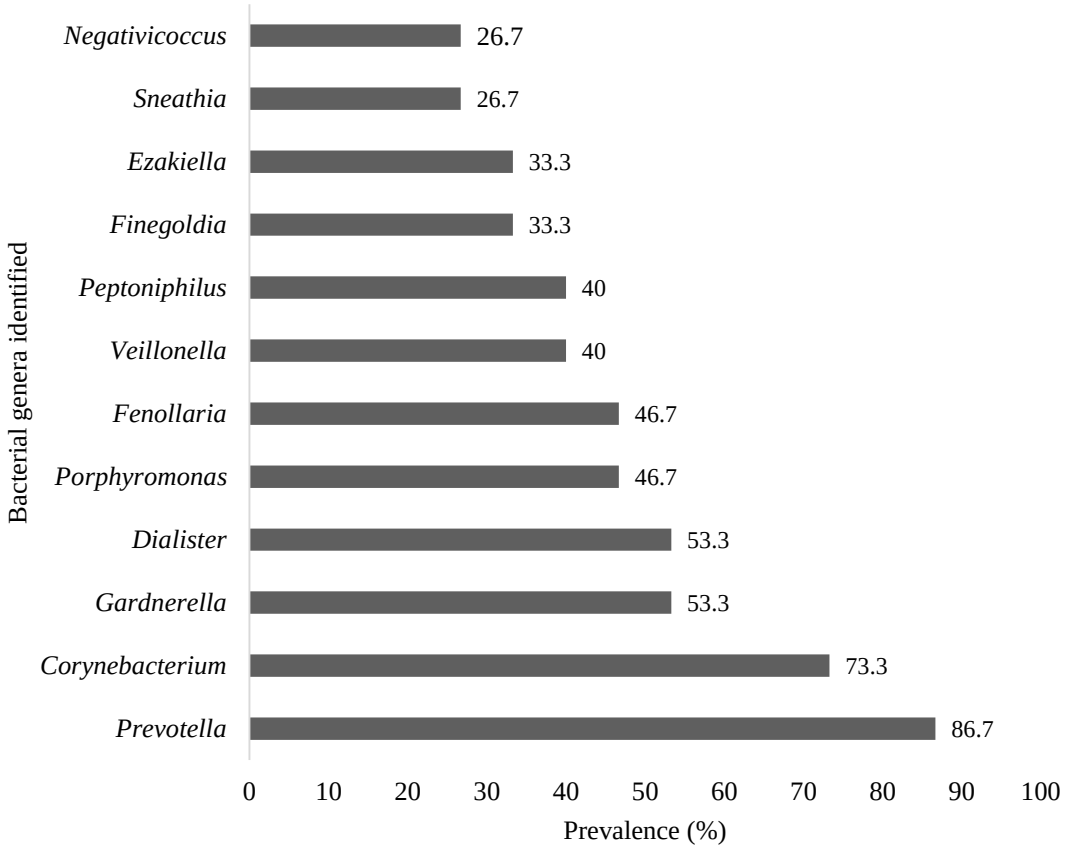

Figure 8: Relative abundance of core microbiome in semen samples

Core microbiome analysis showed that 14 genera of bacteria (prevalence at least 20%) were consistently present in 15 samples at a minimum detection threshold of 0.01% relative abundance. Core microbiome was formed by bacteria of the genera *Prevotella, Fenollaria, Corynebacterium, Gardnerella, Dialister, Veillonella, Porphyromonas, Sneathia*, unclassified member of Leptotrichiaceae, *Peptoniphilus, Ezakiella, Finegoldia, Negativicoccus,* and *Moryella* (**Figure 8**).

4.1.4 Antibiotic resistance profile of predominant bacteria *S. aureus* (21 isolates) in semen

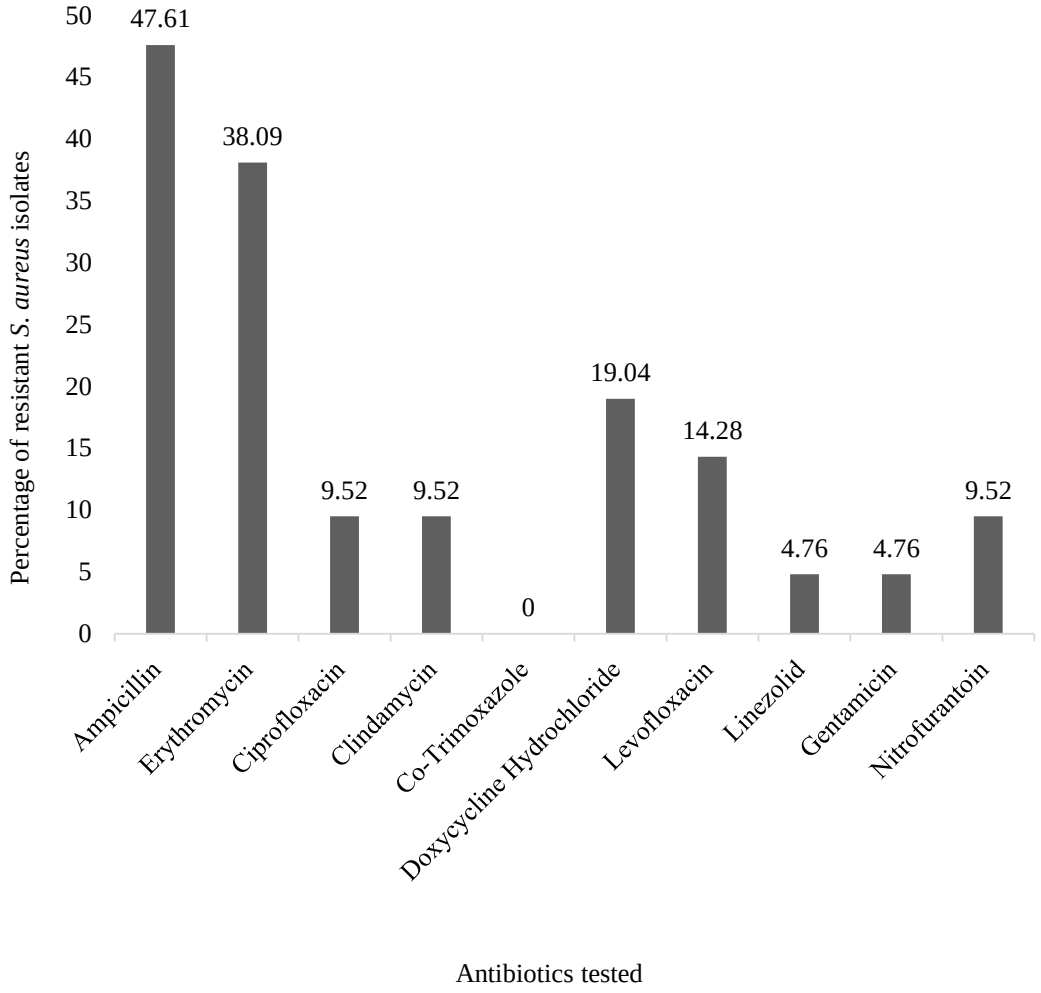

Figure 9: Antibiotic resistance profile of *S. aureus* isolates

Antibiotic susceptibility testing of the predominant bacteria *S. aureus* revealed that ampicillin was found to be the least effective among the ten tested antibiotics. Among 21 isolates of *S. aureus*, 10 (47.61%) were resistant to ampicillin, 8 (38.09%) to erythromycin, 4 (19.04%) to doxycycline hydrochloride, 3 (14.28%) to levofloxacin, 2 (9.52%) to each of ciprofloxacin, clindamycin, and nitrofurantoin, 1 (4.76%) to each of gentamicin and linezolid whereas none of the isolates showed resistance towards co-trimoxazole (**Figure 9**).

4.1.4.1 Occurrence of methicillin-resistant *S. aureus* in semen samples

Out of 21 isolates tested, 12 (57.14%) were resistant to cefoxitin, and 9 (42.86%) were cefoxitin-sensitive. Molecular analysis revealed the detection of the *mecA* gene in 14 (66.67%) isolates, out of which only 10 isolates were resistant, and 4 were sensitive to cefoxitin (Figure 14). There was no association between cefoxitin resistance and the occurrence of the *mec*A gene as determined by Fisher's exact test (*p*-value=0.08, 95% CI).

4.1.4.2 Association of the *mec*A gene and virulence genes in *S. aureus*

The association of the *mec*A gene with the virulence gene was determined by Fisher's exact test. *S. aureus* isolates with the *mec*A gene were detected with *clf*A and *clf*B genes; there is no statistical association between the presence of the *mec*A gene and each of the virulence genes under study (*p*-value >0.05 for all three associations). The results are shown in **Table 6**.

Table 6: Frequency of detection of the *mec*A gene and virulence genes in *S. aureus* isolates

Genes under study	***clf*A gene detection**		***clf*B gene detection**		***tst* gene detection**	
	Present	**Absent**	**Present**	**Absent**	**Present**	**Absent**
*mec*A gene present	9	5	10	4	2	12
*mec*A gene absent	3	4	2	5	0	7
p-value	0.397		0.159		0.533	

4.1.5 Major characteristics of semen samples classified as per the WHO reference range

The macroscopic analysis of semen samples showed that 153 (70.51%) were liquefied within 30 min of incubation, 148 (26.26%) were not viscous, and 193 (88.94%) contained normal ejaculate volume. The classification of semen characteristics was done according to the WHO reference range (2021). It was found that 188 (86.64%) samples had normal sperm concentration, 17 (7.83%) had oligozoospermia, and 12 (5.23%) had azoospermia. Twelve samples with azoospermia were excluded from further analysis of sperm. Out of 205 samples analyzed, 148 (72.20%) had normal motility, 116 (56.59%) had normal morphology, and 80 (39.02%) had normal vitality (**Table 7**). The most common abnormality among the four semen parameters analyzed

was sperm vitality. The majority of the samples analyzed have a leukocyte count in the normal range. Less than 1 million leukocytes per mL of ejaculate was obtained in 190 (87.56%) of the samples (**Table 7**).

Table 7: Semen samples characterization

Characteristics	**Total no. of samples analyzed**	**Categories**	**No. of samples**	**Percentage %**
Time of liquefaction	21	Within 30 min	153	70.51
		30 – 60 min	57	26.26
		Not liquefied till 60 min	7	3.23
Viscosity	217	No	148	68.20
		Yes	69	31.80
Ejaculate volume (mL)	217	Normal (≥1.4)	193	88.94
		Abnormal (<1.4)	24	11.06
Sperm concentration (million/mL)	217	Normal	188	86.64
		Abnormal	29	13.36
Total sperm motility %	205	Normal	148	72.20
		Abnormal	57	27.80
Sperm morphology %	205	Normal	116	56.59
		Abnormal	89	43.41
Sperm vitality %	205	Normal	80	39.02
		Abnormal	125	60.98
Leucocyte count (million/mL of ejaculate)	217	Normal	190	87.56
		Abnormal	27	12.44

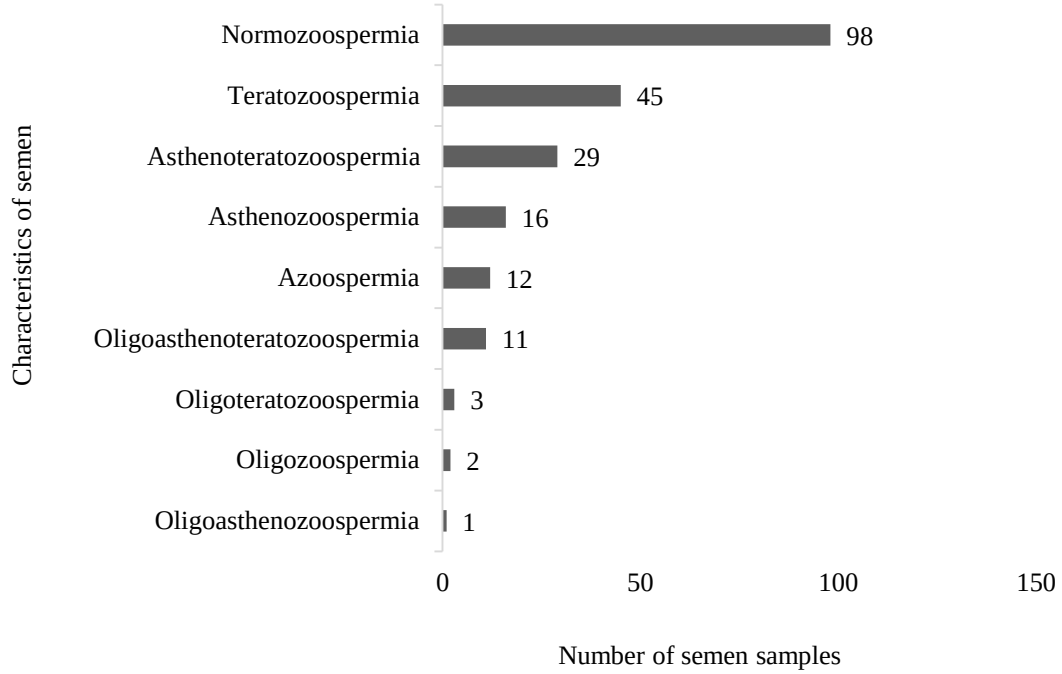

Figure 10: Categories of samples based on semen concentration, motility, and morphology

The quality of semen in 98 (45.16%) samples was normal based on semen concentration, motility, and morphology, and 119 (54.84%) had abnormality in at least one of the above-mentioned parameters (**Figure 10**).

4.1.6 Sperm DNA fragmentation index (DFI) of semen samples

Out of the total 217 semen samples, 188 (86.64%) semen samples had normal sperm concentration (i.e. ≥ 16 million/mL), 17 (7.83%) had low sperm concentration (oligozoospermia), and 12 (5.53%) had no sperm (azoospermia). The sperm DFI% of 188 semen samples were found to be in the range from 4.35 to 76.15. Low DFI was found in 77 (40.96%), moderate in 73 (38.83%), and high in 38 (20.21%) semen samples (**Table 8**).

Table 8: Categories of samples based on sperm DFI.

Category	Sperm DFI	Frequency (%)	Range of sperm DFI%
Low	<15%	77 (40.96)	4.35 – 14.97
Moderate	≥15% and <30%	73 (38.83)	15.02– 29.77
High	≥30%	38 (20.21)	30.08– 76.15

4.1.7 Association of culture-based bacteriospermia with semen parameters

The presence of bacteria in semen samples depicted a significant negative association with the ejaculate volume (*p*-value=0.001, CI 95%). The ejaculate volume was found be significantly less in samples with bacteriospermia based on semen culture. The sperm concentration and total motility were decreased in the semen samples with the presence of bacteria; the associations were statistically insignificant at 95% CI (*p*-value=0.092 for spermatozoa concentration, *p*-value=0.079 for spermatozoa motility). The semen characteristics, morphology, and vitality of the samples tend to have a negative influence on men with bacteriospermia than in those without bacteriospermia; however, the association was statistically insignificant (*p*-value=0.429 for morphology and *p*-value=0.562 for vitality). The concentration of leukocytes in semen was not associated with bacteria in the samples (*p*-value=0.562). Similarly, sperm DFI was not associated with bacteria in the samples (*p*-value=0.573) in this study (**Table 9**).

Table 9: Semen quality with respect to culture-based bacteriospermia

Semen parameters	**Median value (Inter-quartile range)**		***p*-value**
	With bacteriospermia	**Without bacteriospermia**	
Ejaculate volume (mL) (N=217)	2.0 (1.5-3.2) (n=55)	2.8 (2.0-3.5) (n=162)	0.001*
Concentration of sperms (million/mL) (N=217)	75.0 (45.0-80.0) (n=55)	80.0 (70.0-80.0) (n=162)	0.092
Total motility (%) (N=205)	45.0 (40.0-55.0) (n=51)	50.0 (45.0-55.0) (n=154)	0.079
Sperm with normal morphology (%) (N=205)	3.9 (3.0-5.9) (n=51)	4.1 (3.0-6.1) (n=154)	0.391
Vitality of sperm (%) (N=205)	47.2 (40.2-58.6) (n=51)	51.4 (45.0-57.9) (n=154)	0.429
Leukocytes (x10^6 WBC/mL) (N=217)	8.0 (4.0-34.0) (n=55)	13.5 (3.5-36.5) (n=162)	0.562
Sperm DNA fragmentation index (%) (N=188)	14.0 (10.4-26.1) (n=123)	16.8 (12.3-25.8) (n=65)	0.573

*p-value < 0.05, statistically significant at 95% confidence interval

4.1.8 Association of 16S rRNA gene-based bacteriospermia with semen parameters

The comparison between the basic semen parameters, leukocyte count, and sperm DFI of the samples with and without 16S rRNA gene-based bacteriospermia showed that there was a significant difference between the two groups of samples in the concentration of sperm (*p*-value=0.02, 95% CI). The concentration of sperms was found have a significant negative association with 16S rRNA gene-based bacteriospermia. In contrast, no significant difference was observed in the median values of ejaculate volume, total motility, morphology, vitality, leukocyte count, and sperm DFI when grouped on the basis of 16S rRNA gene-based bacteriospermia (**Table 10**).

Table 10: Semen quality with respect to 16S rRNA gene-based bacteriospermia.

Semen parameters	**Median value (Inter-quartile range)**		***p*-value**
	With bacteriospermia	**Without bacteriospermia**	
Ejaculate volume (mL) (N=209)	2.5 (1.7-3.3) (n=144)	3.0 (2.5-3.5) (n=65)	0.080
Concentration of sperm (million/mL) (N=209)	72.5 (55.0-80.0) (n=144)	80.0 (80.0-80.0) (n=65)	0.020*
Total motility (%) (N=198)	50.0 (45.0-55.0) (n=133)	50.0 (45.0-55.0) (n=65)	0.984
Sperm with normal morphology (%) (N=198)	4.3 (2.9-6.1) (n=133)	4.5 (3.4-5.8) (n=65)	0.911
Vitality of sperm (%) (N=198)	52.5 (42.5-58.3) (n=133)	51.1 (46.2-57.2) (n=65)	0.732
Leukocytes (x10^6 WBC/mL) (N=209)	10.0 (4.0-34.0) (n=144)	17.0 (4.0-46.0) (n=65)	0.228
Sperm DNA fragmentation index (%) (N=182)	15.7 (11.3-25.2) (N=123)	20.3 (13.6-29.8) (n=59)	0.092

*p-value < 0.05, statistically significant at 95% confidence interval

4.1.9 Correlation between bacteriospermia and semen characteristics

Pearson's correlation coefficient was calculated for binary data, and Spearman's rho coefficient was calculated for ordinal data. Semen characteristics ejaculate volume, total sperm motility, sperm morphology, sperm vitality, sperm agglutination, leucocyte count, and sperm DNA fragmentation, were analyzed for correlation with culture-based bacteriospermia and 16S rRNA gene-based bacteriospermia. Culture-based bacteriospermia had a significant negative correlation with ejaculate volume only (r = -0.166, *p*-value=0.014). No correlation was observed between culture-based bacteriospermia and sperm concentration, total motility, morphology, vitality, and leucocyte count, and sperm DNA fragmentation (**Table 11**).

Table 11: Correlation between semen parameters and culture-based bacteriospermia

Semen parameters	**Culture-based bacteriospermia**	
	Correlation coefficient	***p*-value**
Ejaculate volume	-0.166	0.014*
Sperm concentration	-0.020	0.767
Total motility	-0.096	0.170
Morphology	-0.020	0.781
Vitality	-0.021	0.766
Agglutination	-0.043	0.542
Leucocytes	0.027	0.692
Sperm DNA fragmentation	-0.116	0.111

*p-value < 0.05, statistically significant at 95% confidence interval

Correlation analysis of 16S rRNA gene-based bacteriospermia with sperm characteristics, leucocyte count, and sperm DNA fragmentation showed that there was no correlation of bacteriospermia with any of the semen characteristics, leucocyte count, or sperm DNA fragmentation (**Table 12**).

Table 12: Correlation between semen parameters and 16S rRNA-based bacteriospermia

Semen parameters	**16S rDNA-based bacteriospermia**	
	Correlation coefficient	***p*-value**
Ejaculate volume	-0.133	0.056
Sperm concentration	-0.074	0.288
Total motility	-0.066	0.357
Morphology	-0.048	0.498
Vitality	0.064	0.367
Agglutination	-0.136	0.056
Leucocytes	-0.003	0.969
Sperm DNA fragmentation	-0.141	0.058

Note: p-value < 0.05, statistically significant at 95% confidence interval

4.1.10 Relationship between bacteriospermia and sperm DNA fragmentation

The relationship of sperm DNA fragmentation with bacteriospermia based on culture and 16S rRNA gene was analyzed by performing ordinal regression and calculating the odds ratio at a 95% confidence interval. In this analysis, bacteriospermia was not a statistically significant predictor of the levels of sperm DNA fragmentation (Wald $\chi 2$=2.869, p=0.09 for culture-based bacteriospermia and Wald $\chi 2$=3.496, p=0.062 for 16S rRNA-based bacteriospermia). The odds ratio (OR=1.72 at 95% CI: 0.920-3.23 for culture-based bacteriospermia and OR=1.74 at 95% CI: 0.970-3.11 for 16S rRNA-based bacteriospermia) suggests that men without bacteriospermia had higher odds of having more fragmented sperm DNA, although there was no statistical significance (**Table 13**).

Table 13: Univariate logistic regression analysis of the association between bacteriospermia and sperm DNA fragmentation

Predictor	Estimate β	Standard error	Wald χ2	*p*-value	Odds ratio (OR)	95% CI for OR
Culture-based bacteriospermia	0.544	0.321	2.869	0.090	1.72	0.92-3.23
16S rRNA-based bacteriospermia	0.554	0.296	3.496	0.062	1.74	0.97-3.11

Note: *p*-value < 0.05, statistically significant at 95% confidence interval

4.1.11 Correlation between bacterial isolates of semen and sperm DNA fragmentation

Spearman's correlation was analyzed between individual types of bacteria isolated from semen and sperm DNA fragmentation index, and no correlation existed between any of the bacterial types isolated by semen culture and sperm DFI in this study (**Table 14**).

Table 14: Correlation between bacterial isolates and sperm DFI

Bacterial isolate	No. of bacterial isolates	Spearman's rho coefficient (ρ)	*p*-value
S. aureus	21	-0.063	0.387
Corynebacterium	15	-0.113	0.122
CoNS	10	-0.060	0.412
Neisseria	4	-0.114	0.119
Micrococcus	3	0.040	0.585
E. coli	3	0.020	0.785
Streptococcus	1	0.109	0.138
Enterococcus	1	-0.080	0.273
Klebsiella	1	-0.080	0.273
Pseudomonas	1	0.028	0.700
Alcaligens	1	-0.080	0.273
Sarcina	1	-0.080	0.273

Note: Correlation analysis was performed using binary coding for bacterial presence (1) or absence (0); p-value < 0.05, statistically significant at 95% confidence interval

4.1.12 Differential abundance of bacteria identified by 16S rRNA gene sequencing from seminal DNA by single-factor analysis

Based on the 16S rRNA gene sequence data of semen samples of this study, *Acinetobacter* was highly abundant in the samples with increased viscosity (Log2FC = -11.32, FDR = 0.0001), decreased sperm concentration (Log2FC = -11.32, FDR = 0.0001), decreased motility (Log2FC = -11.52, FDR = 0.0011), and decreased normal morphology (Log2FC = -11.61, FDR = 0.0412). In this study, *Acinetobacter* was found to be dominant in samples with most of the abnormal semen characteristics (**Table 15**). However, *Acinetobacter* was not associated with vitality and DNA fragmentation of sperm. Another bacterium, *Campylobacter,* was also abundantly present in the semen with decreased normal sperm morphology only (Log2FC = -6.218, FDR = 0.047).

In contrast, a larger number of bacterial genera was positively associated with semen with normal sperm characteristics. *Lactobacillus* was the most abundant genus with a positive influence on all semen characteristics analyzed (**Table 15**). *Lactobacillus* was highly enriched in the semen with the enriched normal viscosity (Log2FC = 8.112, FDR = 0.0033), normal sperm concentration (Log2FC = 8.112, FDR = 0.0033), normal motility (Log2FC = 9.501, FDR = 0.0011), normal morphology (Log2FC = 9.456, FDR = 0.0000), normal vitality (Log2FC = 10.717, FDR = 0.0000) and low sperm DFI (Log2FC = 10.063, FDR = 0.0075). A significant dominance of *Lactobacillus* in semen with normal sperm parameters reflects a positive effect on semen characteristics (**Table 15**).

Besides *Lactobacillus*, other bacterial genera including *Moryella. Sneathia, Neisseria,* and *Murdochiella* were also present in significant abundance in the semen with normal parameters. However, their presence was associated with only a few of the semen parameters and in less abundance than *Lactobacillus*. The genus *Moryella* was abundant in semen with normal viscosity (Log2FC = 6.089, FDR = 0.0033) and normal sperm concentration (Log2FC = 6.089, FDR = 0.0033). Similarly, *Sneathia* was also abundantly present in semen with normal viscosity (Log2FC = 5.090, FDR = 0.0097) and normal sperm concentration (Log2FC = 5.090, FDR = 0.0097). *Neisseria* and *Murochiella* were abundant in semen with normal morphology and vitality, though in less association than *Lactobacillus* (**Table 15**).

Table 15: Single-factor analysis, Comparison: normal vs. abnormal semen parameters

Semen parameters	Bacteria	Log2 fold change (Log2FC)	Log counts per million (logCPM)	False discovery rate (FDR)	Interpretation of association
Viscosity	*Acinetobacter*	-11.322	18.739	0.0001	Negative
	Lactobacillus	8.1123	17.101	0.0033	Positive
	Moryella	6.0892	13.846	0.0033	Positive
	Sneathia	5.2902	14.558	0.0097	Positive
Concentration	*Acinetobacter*	-11.322	18.739	0.0001	Negative
	Lactobacillus	8.1123	17.101	0.0033	Positive
	Moryella	6.0892	13.846	0.0033	Positive
	Sneathia	5.2902	14.558	0.0097	Positive
Motility	*Lactobacillus*	9.5006	17.101	0.0011	Positive
	Acinetobacter	-11.519	18.739	0.0011	Negative
Morphology	*Lactobacillus*	9.4560	17.101	0.0000	Positive
	Acinetobacter	-11.612	18.739	0.0412	Negative
	Neisseria	5.2692	15.237	0.0412	Positive
	Murdochiella	2.8084	10.511	0.0477	Positive
	Campylobacter	-6.2181	14.589	0.0477	Negative
Vitality	*Lactobacillus*	10.717	17.101	0.0000	Positive
	Murdochiella	3.3452	10.511	0.0097	Positive
Sperm DFI	*Neisseria*	5.2708	15.237	0.0157	Positive
	Lactobacillus	10.063	17.101	0.0075	Positive

Log2FC indicates the abundance of bacteria in semen with normal or abnormal parameters; a positive value indicates more abundance in semen with normal parameters; a negative value indicates more abundance in semen with abnormal parameters.

FDR= False discovery rate adjusted *p*-value; <0.05 is considered as significant

4.2 Discussion

The culture-based bacteriological analysis revealed that 25.3% of semen samples had bacteriospermia, which was found to be similar to a previous study on infertile men from Nepal, which reported that 25.8% of samples from infertile men had bacteriospermia (Pokhrel *et al.*, 2020). While another study from Nepal found bacteriospermia in 17.8% of semen samples from infertile men (Bhatt *et al.*, 2015). However, rates of bacteriospermia reported from other countries vary. The wide range in the rate of bacteriospermia (3.2 to 30%) has been reported in Germany (Volz *et al.*, 2022; Zeyad *et al.*, 2017), while higher rates were observed in Iran (35%) (Eini *et al.*, 2021) and India (35.3%) (Vilvanathan *et al.*, 2016). In contrary, the lower rates of bacteriospermia were reported in the previous studies done in Canada (15%) (Domes *et al.*, 2012) and Italy (11.1%) (Iovene *et al.*, 2017). One of the reasons for the differences in the rate of bacteriospermia by semen culture in various studies might be due to differences in the population, with respect to demographics and geographic locations. Another reason might be methodological diversity in studies, including the use of different types of culture media and techniques applied for the semen culture in different studies.

Semen culture revealed the presence of aerobic and facultatively anaerobic bacteria, with the predominance of the genus *Staphylococcus*. Within this genus, *S. aureus* was the leading species, followed by CoNS. Similar result on the predominance of *S. aureus* was also revealed in previous studies from other countries (Eneye *et al*., 2019; Enwuru *et al*., 2016; Isaiah *et al*., 2011; Tiwari & Pattanaik, 2016; Zeyad *et al*., 2017). *S. aureus* accounted for about 40% of seminal bacteria in various studies from India, Egypt, and Nigeria (Ahmad *et al*., 2016; Mu *et al*., 2021; Nasrallah *et al*., 2018). This dominance of *Staphylococcus* species, particularly *S. aureus*, is significant given their known pathogenic potential and association with various infections. The occurrence of *S. aureus* in greater abundance could be due to its ability to grow in minimal growth requirements, to colonize and establish infection within the male reproductive system (Salisu *et al*., 2018). *S. aureus* is a normal flora of the mucosal membrane of humans and animals; however, it is also a common pathogen of a variety of human diseases (Ezeamagu *et al*., 2018). *S. aureus* isolates from infertile men have higher biofilm-producing ability than those from healthy individuals (Bukharin *et al*., 2022). The biofilm-forming ability of bacteria decreases sperm motility, affecting male fertility

potential. It also produces a surface protein SIF that immobilizes sperm cells (Prabha *et al*., 2009). An *in vitro* study has shown that the coincubation of sperm cells with *S. aureus* significantly decreases the motility of sperm (Kaur & Prabha, 2012). Scanning electron microscopy of sperms after incubation with SIF showed multiple morphological alterations in the spermatozoal head, mid-piece, neck, and tail regions (Prabha *et al*., 2010). The second predominant isolate of this study was *Corynebacterium* spp., which corroborates the findings of Bukharin *et al*. Corynebacteria could reduce cytokine levels in infertile men and increase the level of biofilm formation, affecting fertility potential in infertile men (Bukharin *et al*., 2022). The presence of bacteria in semen may also increase the production of ROS, which impairs sperm function and leads to infertility (Folliero *et al*., 2022). *E. coli*, *Neisseria*, and *Micrococcus* were detected at lower frequencies (4.84% each) than *Staphylococcus* and *Corynebacterium*. Although the occurrence of *E. coli* was relatively low in the present study, its presence remains clinically important due to its detrimental effects on semen quality, such as sperm motility, sperm apoptosis, and mitochondrial dysfunction (Boguen *et al.*, 2015; Nabi *et al.*, 2022). The detection of bacteria, including *E. faecalis*, *K. pneumoniae*, *Pseudomonas*, *Streptococcus* spp., *Alcaligenes* spp., and *Sarcina* spp. at very low frequencies (1.61% each), reflects occasional or opportunistic infections of the male genital tract. *E. faecalis* had been reported as one of the commonly isolated seminal bacteria in the past studies on semen culture (Domes *et al.*, 2012; Moretti *et al.*, 2009; Ricci *et al.*, 2018; Vilvanathan *et al.*, 2016). Similar to the findings of this study, some studies have also reported a very low frequency of streptococci from semen (Fraczek *et al.*, 2016; Vilvanathan *et al.*, 2016; Zeyad *et al.*, 2018). However, *Streptococcus* spp. had also been detected as commonly isolated bacteria in the semen culture in past studies (Bhatt *et al.*, 2015; Bukharin *et al.*, 2022; Hannachi *et al.*, 2018), in contrast to the result of this study. Although these bacteria were isolated in lower frequencies in this study, these organisms are commonly associated with urinary tract infections and may ascend into the reproductive tract, particularly in individuals with poor genital hygiene, prior infections, or underlying urogenital abnormalities.

The analysis of the virulence genes *clf*A, *clf*B, and *tst i*n the isolated *S. aureus* strains reveals a pattern of virulence factor distribution with significant implications for the pathogenicity and potential clinical impact of *S. aureus* in the semen. In this study, clumping factor genes were present in *S. aureus*. The *clf*A gene and *clf*B gene were

detected in more than half of the isolates. In a similar study, both *clf*A and *clf*B genes were frequently found in urinary tract *S. aureus* isolates (Jabbar, 2024). In another study, *S. aureus* (58%) isolates from dairy farms harbored the *clf*A gene (Juwita *et al.*, 2022). Vascular abscess infection caused by *S. aureus* showed the occurrence of both *clf*A and *clf*B genes in all the isolates (Wong *et al.*, 2022). *S. aureus* can infect any part of the body due to various toxins, enzymes, and other virulence factors responsible for the pathogenicity of the bacteria. Clumping factors A and B are fibrinogen-binding proteins on the surface of *S. aureus* cells, which are virulence factors that play a role in attachment to host cells (Eldhin *et al.*, 1998; Lacey *et al.*, 2019). The *clf*A gene product, clumping factor A, is considered an important virulence factor responsible for the aggregation of protein-coated host cell parts in various infections (Herman-Bausier et al., 2018) and plays a crucial role in biofilm formation by facilitating host cell attachment (Kashi *et al.*, 2024). The presence of the *clf*B gene expression has been reported in previous research, justifying its significant contribution to the pathogenicity of *S. aureus,* which helps in bacterial colonization (Soltani *et al.*, 2019). The production of clumping factors may initiate the pathogenesis and agglutinate the sperm cells, impairing sperm motility. Another virulence gene, the *tst* gene encoding toxic shock syndrome toxin, was detected only in 9.5% of the isolates. Previous studies on *S. aureus* from different clinical samples also revealed that the *tst* gene was not a common virulence gene. The *tst* gene was not detected in *S. aureus* from seminal fluid (Esmailkhani *et al.*, 2018), abscess (Wong *et al.*, 2022), and endocervix (Akhi *et al.*, 2017). The presence of the virulence genes in bacteria indicates that their presence in semen may impair sperm function, which should not be ignored. The prevalence of the clumping factor genes, particularly *clf*A and *clf*B, is noteworthy given their crucial role in *S. aureus* adherence and pathogenesis, while the low detection rate of the *tst* gene suggests a limited presence of toxic shock syndrome-associated strains within isolated *S. aureus*. The identification of *S. aureus* harboring virulence genes in semen indicates a potential pathogenic role in male subfertility, moving beyond the view of it as a passive contaminant and arguing for its diagnostic relevance.

Bacteriospermia based on the metagenomic analysis of the 16S rRNA gene was detected in 70.81% of semen samples. The rate of bacteriospermia was found to be higher than the rate detected by the culture-based method. The difference in the rate of bacteriospermia obtained by two different techniques could be due to the inability of

the culture method to favor the growth of all the types of bacteria present in the specimen, despite being a gold-standard method for clinical specimens. Semen may contain bacteria that are difficult to culture and even uncultivable bacteria. So, culture reveals an incomplete picture of the bacterial flora. With the help of 16S rRNA gene sequencing, more than 90% of the bacteria present in the sample can be identified, which cannot be achieved by conventional identification techniques (Al-Kass *et al.*, 2020). The 16S rRNA gene sequencing reveals a complex and diverse ecosystem, predominantly populated by anaerobic and facultatively anaerobic bacteria. This composition underscores the unique microenvironment of the male reproductive tract, which is characterized by low oxygen tension. Semen microbiota in infertile men, with the application of the next-generation sequencing (NGS) techniques in Nepal, has been studied for the first time. The characterization of the diversity of bacteria in human semen communities had not been done to a greater depth in the previous studies in Nepal. However, the presence of bacteria in semen does not equate to seminal infection. Seminal infection is characterized by the presence of viable pathogenic microorganisms accompanied by an inflammatory host response, such as leukocytospermia. In contrast, the semen microbiome represents a complex community of commensal and opportunistic microorganisms that may persist in semen without inducing overt inflammation. Bacterial detection by 16S rRNA gene sequencing reflects microbial composition rather than infection status.

Based on the 16S rDNA analysis, the most common phylum of bacteria reported in this study was Firmicutes, which is comparable to other previous studies (Garcia-Segura *et al.*, 2022; Mändar *et al.*, 2017; Yao *et al.*, 2022). In this study, anaerobic bacteria were predominant in samples with abnormal semen characteristics. At the genus level, strict anaerobes such as *Prevotella* (14.2%), *Fenollaria* (12.2%), *Veillonella* (8.4%), and *Sneathia* (5.7%) collectively constitute a major portion of the community. An earlier study also reported the difficult-to-culture Gram-positive anaerobic cocci as the most abundant semen organisms (Kiessling *et al.*, 2008). In a recent study done by Kwa & Karpi (2025) on anaerobic culture of semen, *Prevotella* (49% of all isolates) was the most abundant genus, followed by *Veillonella* (10.9%), *Peptoniphilus* (10%), and *Cutibacterium* (8.7%). The presence of anaerobic organisms in semen may originate from different parts of the male genital tract, such as the urethra and prostate, or from the individual's own gut normal flora, which naturally contains anaerobic

microorganisms (Kiessling *et al*., 2008). The bacteria present in semen may be contributed from different parts of the male genital tract, such as the urethra and prostate. Anaerobe dominance in this study has been associated with impaired sperm motility and increased sperm DNA fragmentation. The past studies on metagenomic analysis of semen also revealed the presence of anaerobic bacteria in semen with abnormal sperm quality (Baud *et al.*, 2019; Weng *et al.*, 2014). Anaerobic bacteria might cause subclinical inflammation through lipopolysaccharide release, short-chain fatty acid production, and biofilm formation within accessory glands. These processes promote oxidative stress and inflammatory mediator release, leading to impaired sperm characteristics and function (Lundy *et al.*, 2020).

Prevotella, an obligately anaerobic Gram-negative bacterium, was the most abundant bacterium in this study, which was similar to the previous studies (Alqawasmeh *et al.*, 2022; Baud *et al.*, 2019; Weng *et al.*, 2014). Despite being the commensal of the human genitourinary tract, it possesses various virulence factors that may play a role in disease pathogenesis (Sharma *et al.*, 2022; Zhang *et al.*, 2017), and is present abundantly in semen with abnormal spermiogram (Baud *et al.*, 2025; Gachet *et al.*, 2022; Okwelogu *et al.*, 2021; Weng *et al.*, 2014). It is also considered to be associated with bacterial vaginosis and involved in the pathogenesis of infertility in women (Zhao *et al.*, 2020). Comparable to the present result, a study on microbiota composition of infertile couples seeking *in vitro* fertilization also detected the presence of *Prevotella, Porphyromonas, Sneathia, Gardnerella, Megasphera,* and other bacteria in oligospermic and azoospermic semen (Okwelogu *et al.*, 2021). The diversity of bacteria in semen signifies a complex seminal bacterial community with its specific implications on the reproductive health of men (Neto *et al.*, 2024). The bacterial genera identified in this study exhibit varying clinical relevance in semen. *Acinetobacter* and *Neisseria* species may represent opportunistic colonization or contamination from urethral flora; the detection of anaerobic genera such as *Sneathia* and *Moryella* suggests a dysbiotic seminal microbiome potentially associated with subclinical inflammation and impaired sperm function. *Staphylococcus*, the predominant genus isolated by semen culture in this study, showed a relative abundance of only 1% when analyzed using the 16S rRNA gene–based method. This finding suggests the presence of other bacterial taxa with higher relative abundance that could not be isolated by conventional culture techniques. In contrast, *Streptococcus* exhibited a relative abundance of 2.7% by the gene-based

method, whereas its detection rate by culture-based analysis was 1.6%, which was significantly lower than that of *Staphylococcus*. The lower frequency of *Streptococcus* isolation in culture may be attributed to the presence of non-viable bacteria in the semen samples.

Lactobacillus was the predominant genus in samples with normal semen characteristics. Various previous studies also reported the predominance of the genus *Lactobacillus* in normal semen samples (Baud *et al.*, 2019; Hou *et al.*, 2016; Weng *et al.*, 2014). A previous study based on the semen with and without leukocytes, it was found that *Lactobacillus* was more abundant in the semen of leukocytospermic males (Yao *et al.*, 2022). The presence of *Lactobacillus* in semen seems to provide benefit in the reproductive function of semen, as the number of other bacteria tends to decrease in the semen enriched with *Lactobacillus* (Yao *et al.*, 2022). The positive clinical outcome of *in vitro* fertilization (IVF) treatment was also found to be significantly higher in the male patients with the abundance of *Lactobacillus jensenii*, and also in the female patients with *Lactobacillus gasseri* colonization in the vagina (Okwelogu *et al.*, 2021), suggesting the replenishment of *Lactobacillus* before the IVF treatment for the possibility of a positive outcome.

The antibiotic susceptibility test revealed that most of the antibiotics tested were effective against *S. aureus* isolates. The most ineffective antibiotic was ampicillin (47.61%), followed by erythromycin (38.09%), doxycycline hydrochloride (19.04%), levofloxacin (14.28%), ciprofloxacin, clindamycin, and nitrofurantoin (9.52% each), gentamicin and linezolid (4.76%), whereas the least resistant was co-trimoxazole (0%). Comparable to the antibiotic resistance in this study, Esmailkhani *et al.* (2018) reported higher resistance to gentamicin (18.8%), ciprofloxacin (18.8%), and co-trimoxazole (6.5%). Closer to the current result on linezolid, a study found 100% effectiveness against *S. aureus* isolated from semen (Tiwari & Pattanaik, 2016). One previous study on seminal *S. aureus* done in Nepal reported higher resistance to co-trimoxazole (64%), followed by gentamicin (45.5%), levofloxacin (36.4%), and nitrofurantoin (18.17%) (Bhatt *et al.*, 2015). Variations in the antibiotic resistance pattern may reflect the study time frame, population, and regional variations in different studies. This study demonstrates a degree of antibiotic resistance among *Staphylococcus aureus* isolates recovered from semen samples, highlighting potential clinical and public health

concerns in the context of male reproductive health. Despite the presence of antibiotic resistance towards a few antibiotics, most isolates were sensitive to fluoroquinolones, clindamycin, gentamicin, linezolid, and co-trimoxazole, indicating the availability of effective antibiotics for clinical treatment of *S. aureus*.

Phenotypic and molecular analysis of MRSA revealed that cefoxitin resistance was detected in 9 (42.86%), whereas the *mec*A gene was present in 14 (66.67%) out of 21 isolates of *S. aureus*. There was no statistical association between the occurrence of phenotypic and genotypic methicillin resistance. A few isolates conferring the *mec*A gene did not show resistance to cefoxitin. Similar to the finding of this study, *mec*A-positive *S. aureus* were sensitive to oxacillin and cefoxitin discs (Liang *et al.*, 2022). Though the detection of *mec*A and cefoxitin susceptibility is considered highly comparable, discrepancies in the MRSA detection might occur (Adhikari *et al.*, 2017; Broekema *et al.*, 2009). This phenomenon might be possible in the isolates with low-level or absence of gene expression of an altered protein, penicillin-binding protein 2a (PBP2a) (Pardo *et al.*, 2022). Additionally, a few *S. aureus* lacking the *mec*A gene showed resistance to cefoxitin in this study. MRSA strains without the *mec*A gene were also reported in *S. aureus* isolates from milk samples in different studies (Rafif Khairullah *et al.*, 2022; Wang *et al.*, 2014) and in isolates from clinical samples (Hososaka *et al.*, 2007). This might be possible due to the presence of a *mec*C gene (a homologue of the *mec*A gene) instead of the *mec*A gene (Baum *et al.*, 2022; Larsen *et al.*, 2022) or other antibiotic resistance mechanisms. The lack of a statistically significant association between cefoxitin resistance and *mec*A gene presence underscores the complexity of methicillin resistance expression in *S. aureus* and highlights the importance of combining phenotypic and molecular methods for accurate MRSA detection. The detection of antimicrobial resistance genes in bacteria isolated from semen indicates a risk of antibiotic resistance and potential treatment failure in clinical settings.

An understanding of the co-occurrence of the virulence and antimicrobial resistance genes in *S. aureus* isolated from semen is important for assessing pathogenicity and antibiotic resistance. The co-occurrence of *mec*A and the virulence genes in *S. aureus* might further enhance the pathogenicity of bacteria. The clumping factor genes (*clf*A and *clf*B) in co-occurrence with *mec*A in MRSA increase the ability of bacteria to

adhere to spermatozoa, potentially impairing the semen quality. The correlation of the *mec*A gene with virulence genes in this study could not be established. However, a high prevalence of adhesion genes (*clf*A and *clf*B genes) among clinical *S. aureus* indicates the co-existence of adhesion and *mec*A genes (Yao *et al.*, 2022). The occurrence of virulence genes and an antibiotic resistance gene in *S. aureus* isolates indicates that the presence of a common bacterium of the human body might be a potential pathogen with antibiotic resistance properties. However, an absence of a statistically significant association between *mec*A and the virulence genes in this study indicates that antibiotic resistance and virulence may be independently acquired traits in seminal *S. aureus* isolates.

A direct effect of bacteria on seminal parameters has remained controversial (Hannachi *et al.*, 2018). The findings of the current study showed normal semen quality (45.16%) based on the semen concentration, motility, and morphology. A similar result (44%) was observed in Koju *et al.* (2021), but a higher rate (66.2%) was observed in Gautam *et al.* (2021) from two different hospitals in Nepal. The most common semen abnormality in this study was teratozoospermia, which was also common in previous studies (Pokhrel *et al.*, 2020; Vilvanathan *et al.*, 2016). However, asthenozoospermia (Koju *et al.*, 2021; Rijal & Maskey, 2020) and oligozoospermia (Pant, 2013) were also found to be the most common semen abnormal characteristics in Nepal.

There was a significant difference in the median values of sperm volume with and without culture-based bacteriospermia, and also in the median values of sperm concentration with and without 16S rRNA gene-based bacteriospermia. One previous study observed low ejaculate volume of semen with bacteriospermia (14.7%), and normal volume of sample without bacteriospermia (Shash *et al.*, 2023). Similar findings on the association between bacteriospermia and sperm concentration were also reported in the previous studies (Enwuru *et al.*, 2016; Fraczek *et al.*, 2016; Osadchiy *et al.*, 2024; Pergialiotis *et al.*, 2018). A decrease in the sperm concentration in semen and seminal volume might be due to damage to the sperm-producing cells and blockage of the reproductive ducts as a result of bacterial infection, leading to interference in the release of sperm (Farsimadan & Motamedifar, 2020). Seminal bacteria influences on ejaculate volume (Enwuru *et al.*, 2016), sperm concentration (Domes *et al.*, 2012; Eini *et al.*, 2021; Enwuru *et al.*, 2016; Hannachi *et al.*, 2018; Sergerie *et al.*, 2006) and sperm

motility (Domes *et al.*, 2012; Eini *et al.*, 2021; Enwuru *et al.*, 2016; Hannachi *et al.*, 2018) were also reported in previous studies, whereas other studies showed no effect of bacteriospermia on semen parameters including semen volume, concentration, and motility (Filipiak *et al.*, 2015; Vilvanathan *et al.*, 2016). The composition and the load of bacteria present in bovine semen also affected the quality of sperm, diminishing the fertility ability of bulls (Ďuračka *et al.*, 2021). However, the influence of bacteria on semen characteristics is controversial, as several studies did not find any significant association between bacteriospermia and semen parameters (Domes *et al.*, 2012; Hou *et al.*, 2016). Some similar studies on sperm vitality (Filipiak *et al.*, 2015; Sergerie *et al.*, 2006; Vilvanathan *et al.*, 2016) and morphology (Filipiak *et al*., 2015; Sergerie *et al*., 2006; Vilvanathan *et al*., 2016; Zeyad *et al*., 2018) were not influenced by the presence of bacteria. Sperm morphology was shown to be negatively affected by bacteriospermia in other studies (Domes *et al.*, 2012; Eini *et al.*, 2021; Enwuru *et al.*, 2016). The sensitivity of the statistical analysis on the association between seminal bacteria and semen characteristics could be increased with additional samples. The presence of bacteria in semen is considered to be an important factor that may negatively affect the process of spermatogenesis and spermatozoa functions, which may consequently increase fertility problems in men (Farsimadan & Motamedifar, 2020).

Leukocytospermia is a condition in which the number of leukocytes in semen is equal to or greater than 1 million per mL of ejaculate, which indicates seminal infection or active inflammation (Henkel, 2024). Leukocytospermia, together with bacteriospermia, is the cause of active seminal infection (Ventimiglia *et al.*, 2020). A differential leukocyte count (DLC) in addition to total leukocyte count (TLC) in semen would be more informative on the interpretation of bacterial infection or colonization in semen. The condition of leukocytospermia with an abundance of neutrophils is suggestive of active infection, whereas the presence of leukocytes less than 1 million per mL of semen with few or no neutrophils is indicative of microbial colonization in semen (Fathy *et al.*, 2014). The findings of this study on leukocyte count showed only 12.4% leukocytospermia, and the concentration of leukocytes in semen was not associated with the presence of bacteria in the samples. Similar results on leukocytospermia were also reported by Domes *et al.* (2012) and Ventimiglia *et al*. (2020). Ventimiglia *et al*. (2020) also concluded that leukocytospermia cannot be a potential indicator of positive

semen culture, as only 20% culture-positive semen had leukocytospermia. However, in a comparative study between infected and uninfected semen, leukocytospermia was significantly higher in infected samples (Eini *et al.*, 2021). Leukocytospermia may have a negative impact on male fertility; however, it may not be only due to bacteriospermia (Henkel, 2024). The higher leukocyte concentration in semen might also be due to other health conditions like varicocele (Mongioì *et al.*, 2020), and external factors like smoking (Saleh *et al.*, 2002; Zhang *et al.*, 2013). Based on this study, leukocytospermia could not be considered indicative of bacteriospermia. Bacteriospermia without leukocytospermia could be due to bacterial colonization in semen rather than infection. However, leukocytospermia in a bacteriospermic individual is an indication of infection.

Sperm DNA may be fragmented due to bacterial infection (Garcia-Segura *et al.*, 2023; Moubasher *et al.*, 2018). However, this study could not show the association between bacteriospermia and sperm DNA integrity. Aligned with the present study, a few other studies could not associate seminal bacteria with high SDF (Mowla *et al.*, 2025; Rybar *et al.*, 2012). Sperm DNA could be damaged by factors other than bacterial presence in semen. This could be due to the presence of confounding factors like lifestyle, environmental, and health factors other than bacteriospermia, and might be associated with the increased risk of sperm DNA fragmentation, and semen quality (Szabó *et al.*, 2023).

Differential abundance study based on 16SrRNA gene sequencing in semen samples revealed the bacteria of genus *Lactobacillus* and *Acinetobacter* have significant impact on the semen parameters. The abundance of genus *Acinetobacter* was high in semen with abnormal viscosity, sperm concentration, sperm motility and sperm morphology. Yang *et al.* in their study found that *Acinetobacter* was relatively abundant in semen with asthenospermia (Yang *et al.*, 2014). In an *in vitro* study with rabbit semen, *Acinetobacter baumanii* also possessed a negative influence on the motility of semen (Tvrda *et al.*, 2018). *Acinetobacter baumanii* was detected in semen of the men with oligozoospermia and teratozoospermia (Kiessling *et al.*, 2008). *Acinetobacter* can localized inflammatory responses with the production of ROS and cytokine affecting motility, membrane integrity, and DNA of sperm cells (Tvrda *et al.*, 2018).

The significantly higher abundance of *Lactobacillus* was found in the semen with normal characteristics, including sperm DFI. *Lactobacillus* has been shown to have a positive effect on sperm concentration (Weng *et al.*, 2014), sperm morphology (Baud *et al.*, 2019), and DNA integrity (Garcia-Segura *et al.*, 2022) in past studies. Several studies reported the abundance of *Lactobacillus* in semen with normal sperm characteristics (Baud *et al.*, 2019; Chen *et al.*, 2023; Farahani *et al.*, 2021). The beneficial role of *Lactobacillus* on sperm parameters has been revealed by an experimental study, which showed a significant improvement on sperm motility and sperm DNA integrity after an administration of *Lactobacillus rhamnosus* as probiotics (Valcarce *et al.*, 2017). The protective effect of *Lactobacillus* has also been seen on animal semen. *Lactobacillus* has been proven to be a potential probiotic for semen quality, as well as beneficial for restraining the negative influence of other bacteria on boar semen (Zhang *et al.*, 2020).

The present single center-based study in men suspected of contributors of couple infertility could not establish the causal relationship between bacteriospermia and impaired semen characteristics. Further multicenter-based longitudinal studies with control group of fertile men would be helpful in generalization of the study findings in larger infertile population. The limitations in the culture-based method could not detect bacteria of anaerobic and fastidious nature.

CHAPTER 5

5. CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

This study highlights the occurrence of bacteriospermia and its influence on the potency of semen related to infertility within Nepalese men. Aerobic culture revealed bacteriospermia in 25.35% of samples, with *S. aureus* being the predominant isolate. *S. aureus* exhibited antibiotic resistance and virulence factors frequently. However, its presence could not be associated with the semen quality. The 16S rRNA gene amplification revealed that 70.81% of samples had bacteriospermia. Semen culture reported a lower rate and diversity of bacteriospermia than the molecular method, which revealed a greater diversity of semen microbiome with the predominance of anaerobic bacteria. Thus, the molecular method for the detection of seminal bacteria might be useful in the context of culture-negative idiopathic male infertility cases.

Sperm DNA fragmentation analysis revealed significant loss of sperm DNA integrity in 20.21% samples. The culture-based bacteriospermia and 16S rRNA gene-based bacteriospermia did not show direct association with sperm DNA integrity. However, upon analysis of the 16S rRNA, the bacterial genus *Acinetobacter* showed a negative influence on the semen quality, whereas the genus *Lactobacillus* showed a positive influence on sperm DNA integrity and semen quality. The findings of the study shed light on the significant role of the semen microbiome in semen and its fertility potency, specifically indicating beneficial and harmful roles influencing male reproductive health.

5.2 Recommendations

- Sequencing of the 16S rRNA gene in DNA extracted from semen is more sensitive for the detection of seminal bacteria than conventional semen culture; therefore, molecular analysis may be applicable for diagnostic evaluation in cases of idiopathic male infertility.
- The dominance of particular bacteria, such as *Acinetobacter*, is significant, as these can negatively influence semen quality without indicating infection. Therefore, identification of cause and treatment of idiopathic male infertility should also focus on the specific makeup of the microbial community rather than mere culture positivity.
- *Lactobacillus* is found to show a protective effect on seminal parameters; hence, it can be considered a useful bacterium for improving the fertility potential of semen.
- Detection of virulence genes in *S. aureus* isolates indicates that their presence in semen culture should not be neglected during infertility investigation.
- Empirical antibiotic therapy for bacteriospermia should be avoided since the detection of *S. aureus* with antibiotic resistance highlights the need for a rational use of antibiotics to prevent the development of resistance.
- The study was limited to a single center. Therefore, multicenter studies across diverse populations could be performed to get comprehensive data related to the seminal microbiota in men of Nepal.
- A comparative study on fertile and infertile men would be more beneficial to establish the impact of seminal microbiota on infertility.
- Future studies exploring the mechanisms by which specific bacterial genera influence sperm function could be done, focusing on toxin production, biofilm formation, and bacterial metabolites that may affect sperm DNA integrity and other sperm characteristics.

CHAPTER 6

6. SUMMARY

The fertility center-based study for bacteriospermia and its impact on semen among infertile men in Nepal uses both culture-based and molecular methods. Among 217 male participants with infertility, 55 (25.35%) showed bacteriospermia based on semen culture, and 148 (70.81%) detected seminal bacteria by metagenomic study targeting 16S rRNA gene. The rate of bacteriospermia detected by a culture-based study was lower than the rate detected by the molecular-based study.

Sixty-two bacteria isolated from semen culture were identified as belonging to 11 genera. The identified genus of bacteria were 31 (50%) *Staphylococcus*, 15 (24.19%) *Corynebacterium*, 4 (6.45%) *Neisseria*, 3 (4.84%) *Escherichia*, and 3 (4.84%) *Micrococcus*, 1 (1.61%) *Alkaligens*, 1 (1.61%) *Enterococcus*, 1 (1.61%) *Klebsiella*, 1 (1.61%) *Pseudomonas*, 1 (1.61%) *Sarcina*, and 1 (1.61%) *Streptococcus*. *Staphylococcus aureus* was the predominant bacterium isolated in semen culture that harbored virulence genes (*clf*A, *clf*B) and also the methicillin resistance gene (*mec*A). The metagenomic study targeting the 16S rRNA gene identified a diverse seminal microbiota, dominated by the top ten genera: *Prevotella* (18.55%), *Fenollaria* (14.95%), *Ezakiella* (7.43%), *Porphyromonas* (7.10%), *Veillonella* (6.91%), *Corynebacterium* (5.35%), *Acinetobacter* (5.31%), *Peptoniphilus* (4.73%), *Sneathia* (4.44%), and *Finegoldia* (4.16%). The molecular-based method revealed a diverse seminal microbiome with the predominance of anaerobic bacterial genera.

Semen analysis showed that 188 (86.64%) out of 217 samples had normal sperm concentration, among which, 38 (20.21%) had high DNA fragmentation index (DFI ≥ 30%).

Culture-based study revealed that bacteriospermia was significantly associated with decreased ejaculate volume (p = 0.001), whereas 16S rRNA-based study revealed bacteriospermia was significantly associated with lower sperm concentration (p = 0.02). However, no significant associations were found between bacteriospermia and sperm DNA fragmentation in both culture-based and metagenomic studies. Bacteria isolated in this study could not be found to be associated with sperm DNA fragmentation.

Bacteria identified by 16S rRNA gene sequencing from DNA extracted from semen samples showed the association of bacterial genera with the semen parameters, along with sperm DNA fragmentation. Differential abundance analysis from 16S rRNA amplicon sequencing results highlighted *Acinetobacter* as negatively associated with multiple semen parameters, while the presence of *Lactobacillus* was consistent in semen with normal parameters, including lower DFI. The study indicates that *Lactobacillus* has a significant role in maintaining the sperm DNA integrity and protecting the quality of semen.

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APPENDIX - I

Reagents and Materials

Chemical reagents, culture media, biochemical media and kits used are listed in tables.

Chemicals

Chemicals	Company and origin
Absolute ethanol	Changshu Hongsheng Fine Chemical Co. Ltd/China
Catalase solution: Hydrogen peroxide 3% (H_2O_2)	Himedia/India
Glycerol	Finar Chemical/India
Oxidase reagent: [N, N, N, N-tetramethyl-p-phenylenediamine dihydrochloride]	Himedia/India
Eosin	Fisher Scientific/India
Ethylene diamine tetra-acetic acid (EDTA)	Himedia/India
Kovac's Reagent: p-dimethylamino benzaldehyde, isomylalcohol	Himedia/India
Methyl red Reagent	Himedia/India
Voges-proskour Reagent: alpha-naphthol, Potassium hydroxide	Himedia/India

Culture media

Media	Company and origin
Blood agar	Hi-media, India
Chocolate agar	
DNase agar	
Hugh and Leifson's media	
Luria-Bertani broth	
MacConkey agar	
Methyl red, Voges-Proskauer broth	
Muller Hinton agar (MHA)	
Nitrate broth	
Nutrient agar (NA)	
Nutrient broth (NB)	
Peptone water broth	
Sulphide indole motility (SIM) agar	
Simmons citrate agar	

Triple sugar iron agar
Tryptic soy broth
Urea agar

Composition of media

Blood agar

Composition of blood agar base	Gram/Litre
Peptone	10.0
Tryptone	10.0
Sodium chloride	5.0
Agar	15.0
Distilled water	1000 mL
Final pH (at 25°C)	7.3±0.2

Suspended 40.0 grams in 1000 mL distilled water, heated to boiling to dissolve the medium completely, sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes, cooled to 45-50°C, aseptically added 5% v/v sterile defibrinated blood, mixed well and poured into sterile Petri plates.

Chocolate agar (Heated blood agar)

Suspended 40.0 grams of blood agar base in 1000 mL distilled water, heated to boiling to dissolve the medium completely, sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes, cooled to about 60-70^{o}C, aseptically added 5% v/v sterile defibrinated blood, placed the media in a water bath of 75-80^{o}C, kept swirling gently until the color of media changed to brown, and poured into sterile Petri plates.

DNase agar

Composition	Gram/Litre
Tryptone	15.0
Soya peptone	5.0
Deoxyribonucleic acid (DNA)	2.0
Sodium chloride	5.0
Agar	15.0

Suspended 42 grams in 1000 mL distilled water. Heated with frequent agitation to dissolve the medium completely. Sterilized by autoclaving at 12 to 15 lbs pressure (118°C to 121°C) for 15 minutes. Cooled to 45°C and pour into sterile Petri plates. Flooded the plates with 0.1% Toluidine Blue solution after incubation.

Hugh and Leifson Agar (O/F) Medium

Composition	Gram/Litre
Peptone	2.0
Sodium Chloride	5.0
Dipotassium hydrogen phosphate	0.3
Bromothymol blue solution (2.0%)	15.0 mL
Agar	3.0
Distilled water	1000 mL
Final pH (at 25°C)	7.1±0.02

Dissolved the solids in water and sterilized by autoclaving at 121°C for 15 minutes. Adjusted the pH to 7.1 cooled the sterilized medium to 40-45°C in a water bath and added 10% aqueous sterilized solution of carbohydrates (e.g. glucose, lactose, sucrose, maltose etc.).

Luria-Bertani broth

Composition	Gram/Litre
Tryptone	10.0
Yeast extract	5.0
Sodium chloride	10.0
Final pH (at 25°C)	7.5±0.2

Suspended 25.0 grams in 1000 mL purified/distilled water. Heated if necessary to dissolve the medium completely. Mixed well. Dispensed in tubes or flasks as desired. Sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

MacConkey Agar

Composition	Gram/Litre
Peptone	20.0
Lactose10.0	
Bile salts	5.0
Sodium chloride	5.0
Neutral red	0.075
Agar 12.0	
Final pH (at 25°C)	7.4±0.2

Suspended 52 grams in 1000 mL distilled water. Boiled to dissolve completely and sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

MRVP broth

Composition	Gram/Litre
Polypeptone or a buffered peptone	7.0
Dextrose (glucose)	5.0

Dipotassium phosphate (K_2HPO_4)	5.0
Distilled water	1000 mL
Final pH (at 25°C)	6.9±0.02

Sterilized at 121°C, 15 lb, for 15 minutes.

Mueller Hinton Agar

Composition	Gram/Litre
Beef infusion from	300.0
Casein acid hydrolysate	17.5
Starch 1.5	
Agar 17.0	
Final pH (at 25°C)	7.4±0.2

Dissolved 38 gm of media in 1000 mL water and sterilized by autoclaving at 15 lbs. pressure (121°C) for 15 min.

Nitrate broth

Composition	Gram/Litre
Beef extract	3.0
Peptone	5.0
Potassium nitrate	1.0
Distilled water	1000 mL.
Final pH (at 25°C)	6.8±0.2

Sterilized by autoclaving at 121°C for 15 minutes.

Nutrient Agar

Composition	Gram/Litre
Peptone	5.0
Sodium chloride	5.0
Beef extract	1.5
Yeast extract	1.5
Agar	15.0
Final pH (at 25°C)	7.4±0.2

Added 28 grams of nutrient agar to 1000 mL distilled water. It was boiled to dissolve the medium completely, sterilized by autoclaving at 15 lbs. pressure (121°C) for 1 minutes and mixed well before pouring.

Nutrient Broth

Composition	Gram/Litre
Peptone	5.0
Sodium chloride	5.0

Beef extract	1.5
Yeast extract	1.5
Final pH (at 25°C)	7.4±0.2

Suspended 13 gm in 1000 mL of distilled water. Heated to dissolve the medium and autoclaved at 15 lbs. for 15 minutes (121°C).

SIM (Sulphide, Indole, Motility) agar

Composition	Gram/Litre
Beef extract	3.0
Peptone	30.0
Peptonized iron	0.2
Sodium thiosulphate	0.025
Agar	3.0
Final pH (at 25°C)	7.3±0.2

Suspended 36 gm in 1000 mL distilled water. Heated to dissolve the medium and autoclaved at 15 lbs. for 15 minutes (121°C).

Simmon's citrate agar

Composition	Gram/Litre
Ammonium dihydrogen phosphate	1.0
Dipotassium hydrogen phosphate	1.0
Sodium chloride	5.0
Magnesium sulphate	0.2
Agar	15.0
Bromothymol blue (1.5% alcoholic)	0.08
Distilled water	1000 mL
Final pH (at 25°C)	6.8±0.02

Dissolved all the ingredients in distilled water by heating and sterilized by autoclaving at 121°C, 15 lbs, for 15 minutes.

Triple Sugar Iron (TSI) Agar

Composition	Gram/Litre
Peptone	10.0
Tryptone	10.0
Yeast extract	3.0
Beef extract	3.0
Lactose	10.0
Saccharose (Sucrose)	10.0
Dextrose (Glucose)	1.0
Ferrous sulphate	0.2

Sodium chloride	5.0
Sodium thiosulphate	0.3
Phenol red	0.024
Agar	12.0
Final pH (at 25°C)	7.4±0.02

Dissolved 6.5 grams of TSI agar in distilled water by heating and distributed in test tubes. Then tubes were sterilized at 121°C (15 lbs pressure) for 15 minutes and made slants in test tubes.

Tryptic soya broth

Composition	Gram/Litre
Tryptone	8.50
Soya peptone	1.50
Sodium chloride	5.10
Dextrose (Glucose)	1.77
Dipotassium hydrogen phosphate	1.25
Tryptose	10.38
Yeast extract	3.0
Final pH (at 25°C)	7.2±0.2

Suspended 31.50 grams in 1000 mL distilled water. Heated to dissolve the medium completely. Dispensed 5mL portions into 16×150mm test tube. Sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

Urea Agar

Composition	Gram/Litre
Peptone	1.0
Dextrose (Glucose)	1.0
Sodium chloride	5.0
Disodium phosphate	1.2
Monopotassium phosphate	2.0
Phenol red	0.012
Agar	15.0
Final pH (at 25°C)	6.8±2

Dissolved all the ingredients in distilled water by heating and sterilized by autoclaving at 121°C (15 lbs pressure) for 15 minutes. Then added 20-gram urea in solution form and made slants in test tubes.

Kits used in the study

Name of kits	Company and origin

Morphology-D kit	Sperm Processor Pvt. Ltd., India
Leukocheck kit	Cryolab International, India
Sperm chroma kit	Cryolab International, India
QIAamp DNA Mini Kit	QIAGEN, Hilden, Germany

Materials used in molecular study

Materials	Company and origin
100 bp DNA Ladder Ready to Load	Solis Biodyne/Estonia
Agarose-electrophoresis grade	GeneDirex, Inc/Germany
FIREPol ® Master Mix Ready to Load	Solis Biodyne/Estonia
Hot Firepol Evagreen qPCR Mix Plus ROX	Solis Biodyne, Estonia

Composition of Stains and Reagents

Crystal Violet Stain

Solution A: 2.0 Gram of crystal violet was dissolved in 20 mL of 95% ethanol.

Solution B: 0.8 gm of ammonium oxalate was dissolved in 80 mL distilled water.

Then solutions A and B were mixed.

Gram's Iodine

Iodine crystals	1.0 gram
Potassium iodide	2.0 gram
Distilled water	300.0 mL.

About 2 gm of potassium iodide was dissolved in 300 mL of distilled water and then 1 gm of iodine crystal was added & dissolved.

Decolorizer

Ethanol (95%)	250 mL.
Acetone	250 mL.
Mixed these two solutions	

Safranine (Stock Solution)

Safranine	2.5 gram
Ethanol (95%)	100 mL.
Diluted stock safranine 1:5 or 1:10 with distilled water.	

Reagent for oxidase Text

1% Tetramethyl - p - phenylene diamine dihydrochloride in distilled water.

Kovac's Reagent for Indole Test

n-Amyl or Isoamyl alcohol	75 mL.
p-Dimethylamino-benzaldehyde	5 gram
Hydrochloric acid (conc)	25 mL.

Dissolved aldehyde in the alcohol and added acid

Methyl Red Indicator Solution

Methyl red	0.1 gm
Ethanol (95%)	300 mL.
Distilled water	200 mL.

Barritt's Reagents for VP Test

Solution A: 5-gram α-naphthol in 100 mL 95% ethanol

Solution B: 40% KOH or NaOH in 100 mL distilled water

Before use, these two solutions A & B were mixed in the 3:1 ratio respectively.

Nitrate Reduction Test Reagent

Solution A: α-naphthylamine, 0.5% in acetic acid (5 M/l) Solution (28.5 mL acetic acid + 71.5 mL. distilled water).

Solution B: 0.8 gram of sulphanilic acid in 100 mL acetic acid (5 M/L) i.e. 28.5 mL acetic acid + 7.15 mL distilled water.

The solution A & B were added 1 mL/l mL respectively in the culture medium while doing nitrate reduction test.

Sterile normal saline

8.5 g of NaCl was added to 1.0 L distilled water and mixed well. The mixture was autoclaved at 121°C for 15 minutes.

Sterile Phosphate-buffered saline (PBS)

A total number of 5 PBS tablets were dissolved in 500 mL of distilled water and mixed well. The mixture was autoclaved at 121°C for 15 minutes.

Preparation of 50× TAE buffer

24.2 g of Tris Base was added with 5.71 mL of glacial acetic acid and 10 mL of 0.5 M EDTA (PH 8). The mixture was topped up with distilled water to 100 mL. Finally, the solution was autoclaved at 121°C for 15 minutes.

10X TBE Buffer (Tris-Borate-EDTA)

1X TBE buffer is composed of 89 mM Tris, 89 mM boric acid, 2 mM EDTA, (pH 8.3), Stored at room temperature.

TE buffer (10 mM Tris·Cl, 1 mM EDTA, pH 8.0)

1M Tris-HCl, pH 8.0 -1mL (final concentration = 10 mM)
0.5 EDTA - 200 µL (final concentration = 1 mM)
Distilled water -100 mL
Sterilize by passing through 0.2 millipore microfilters.

Ethidium bromide solution:

10 mg/mL in water. Extreme care should be taken during handling this solution as it is highly mutagenic, store in the dark at 4°C.

Preparation of McFarland standard test

Turbidity standard was prepared by adding 0.5 mL of 0.048 mol/L $BaCl_2$ (1.175% w/v $BaCl_2.2H_2O$) to 99.5 mL of 0.18 mol/L (0.36 N) H_2SO_4 (1% v/v). The density of mixture was measured at 620 nm using spectrophotometer. The absorbance was 0.080-0.10 at 620 nm. The mixture was stored in the dark at room temperature for up to 4 months.

Preparation of 2.0% agarose gel

2.0 g of agarose powder was added to 100 mL of 1×TAE buffer and mixed gently. The mixture was boiled in a microwave for 3 min until dissolved completely. Liquid mixture was cooled and then 0.8 µL (10 mg/mL) of ethidium bromide was added and swirled gently.

APPENDIX – II

Equipment and apparatus

The equipments and apparatuses that were used in this study are shown in table.

Equipment and apparatus.

Equipment and apparatus	Company	Origin
1.5 mL Microcentrifuge tubes	Cellcare	China
0.2 mL PCR tubes	Cellcare	China
Apparatus for agarose gel electrophoresis	Narang Scientific Works Pvt. Ltd.	India
Autoclave	Equitron Medica Pvt. Ltd.	India
Centrifuge	Eppendorf	USA
Container for sample collection	Tarsons	India
Distillator	Equitron Medica Pvt. Ltd.	India
Freezer (-80^{o}C)	Arctic	
Incubator	IndoExim	India
	Memmert	Germany
Laminar air flow	Jindal Medical and Scientific Intrument company Pvt. Ltd.	India
Light Microscope	Olympus	Japan
Makler's chamber	Wondersperm	India
Micropipette	Eppendorf	USA
Microspin Centrifuge	Remi Group	India
Microwave Oven	LG Electronic Inc	Korea
Nanodrop spectrophotometer	Thermo Fischer Scientific	UK
Neubauer's chamber	Sigma Aldrich	Germany
PCR tube strips	Tarsons	India
pH meter	Hanna Intrument	India
Quan*ts*tudio™ 5 Real-Time PCR System	Thermo Fischer Scientific	UK
Refrigerator	LG	Nepal
Semen warmer (Hot block)	Sperm Processor Pvt. Ltd.	USA
Sensitive balance	Phoenix Insturment	Germany
Thermal Cycler (Bio-Rad T100)	Bio-Rad Laboratories, Inc	Singapore
Tips, different sizes; (0.1-10 µL, 0.5-300µL and 300-1000µL)	Cellcare	China
UV transilluminator, gel doc XR system	The Lab World Group	USA
Vortex	Labtical	India
Water bath	Narang Scientific Works Pvt. Ltd.	India

APPENDIX - III

Consent form

बिरामीको जानकारी र मंजुरीनामा फाराम

बिरामीको जानकारी

अनुसन्धानको शिर्षक : नेपालको नि:सन्तान केन्द्रमा आएका नि:सन्तान दम्पती मध्ये पुरुष बिरामीको विर्यमा जीवाणुको अधययन सम्बन्धी अनुसन्धान ।

अनुसन्धानकर्ताको नाम : अनिमा श्रेष्ठ

संस्थाको नाम : केन्द्रिय माइक्रोवायोलोजी विभाग, त्रि.वि., किर्तिपुर

अनुसन्धानको उद्देशय :

म अनिमा श्रेष्ठ, अनुसन्धाकर्ता पि.एच.डी.को विद्यार्थी हुँ । म नि:सन्तान पुरुषको विर्यमा जीवाणुको अनुसन्धान गरिरहेको छु । पुरुषमा नि:सन्तान हुने समस्या जीवाणुको कारणले पनि हुन सक्छ भन्ने बारेमा संसार भरी नै अध्ययन अनुसन्धान भइरहेको छ । यस्तो समस्या हाम्रो देशमा कतीको छ र कस्ता जीवाणुले यो समस्या ल्याइरहेका छन् भन्ने अनुसन्धान भएमा हाम्रो देशको नि:सन्तान पुरुषको उपचारमा पनि सघाउ पुऱ्याउने हुनाले यो अनुसन्धान गरिएको छ ।

कार्य प्रणाली :

तपाईले आफ्नो परिक्षणको क्रममा डाक्टर जाँच गरेपछि विर्य जाँच गर्न दिइने विर्यको नमूनाको थोरै अंश (१ मि.लि.) यस अनुसन्धानको लागि दिइनेछ र यस अनुसन्धानसंग सम्बन्धित तपाईको बारेमा केही प्रश्न सोध्ने छु । यो तपाईको अनुमतीमा मात्र हुनेछ । यस अनुसन्धानमा तपाईको संलग्नता स्वेच्छिक हुनेछ । सबै जानकारीहरु गोप्य राखिने छ र त्यसमा अनुसन्धानकर्ता बाहेक अरुको पहुँच हुने छैन । अन्तर्वार्ताको अवधि बढीमा १० मिनेट हुनेछ र तपाईले वीर्यको नमूना दिनु भए पश्चात जान सक्नु हुनेछ । तपाईलाई फेरी यस अनुसन्धानको लागि बोलाइने छैन ।

हानी र नोक्सानी :

तपाईलाई नमूना संकलन गर्दा वा अन्तर्वार्ताको क्रममा कुनै असजिलोपन महसुस हुन गयो भने तपाई यस अनुसन्धानमा संलग्न हुन अस्विकार गर्न सक्नु हुन्छ । तर तपाईलाई यस क्रममा कुनै हानी वा नोक्सानी हुने छैन ।

फाईदा :

यो अनुसन्धानले भविष्यमा नि:सन्तान दम्पतीको उपचारमा केही सहयोग पुऱ्याउने छ भन्ने आशा छ । यदी वीर्यमा जीवाणु पाइएमा उपचारको निम्ती उपयुक्त औषधी पत्ता लाईनेछ । यस अनुसन्धानबाट प्राप्त जानकारीको बारेमा सम्बन्धित चिकित्सकसंग छलफल गर्न सक्नु हुनेछ ।

रकम :
यस अनुसन्धानमा भाग लिए वापत कुनै किसिमको रकम लिईने/दिईने छैन ।

गोपनियता :
यस अनुसन्धानबाट प्राप्त जानकारीहरु गोप्य राखिने छन् । यो अध्ययनमा तपाईले दिनु भएको जानकारी फाइलमा राखिनेछ । जसमा तपाइको नाम उल्लेख गरिने छैन् । पहिचानको लागि सांकेतिक अंक प्रयोग गरिनेछ । कुनै पनि जानकारी माथि बताइएको बाहेक अन्य अनुसन्धानको लागि प्रयोग हुने छैन ।

अनुसन्धानबाट प्राप्त नतिजा प्रस्तुती :
यो अनुसधान पुरा भएपछी यस्को प्रतिफल थेसिसको रुपमा माईक्रोवायोलोजी विभाग, विज्ञान तथा प्रविधि अध्ययन संस्थान, त्रि.वि. र केन्द्रिय पुस्तकालयमा राखिने छ । साथै विभिन्न जर्नलहरुमा प्रकाशित गरिनेछ । त्यो रिपोर्टमा तपाईको नाम र व्यक्तिगत विवरण राखिने छैन ।
भाग लिने वा नलिने अधिकार :
तपाईले चाहेको खण्डमा यस अनुसन्धानमा भाग लिन सक्नुहुन्छ । यदी तपाई त्यसमा संलग्न हुन चाहनु हुन्न भने कुनै पनि बेला अस्विकार गर्न सक्नु हुन्छ ।

कसलाई सम्पर्क गर्ने ?
नेपाल स्वास्थ्य परिष्द, रामशाह पथ काठमाडौंमा रहेको भ्तजष्अर्बा च्भखष्भध द्वयबचम को काम अनुसन्धानमा सहभागी जनाउनेहरु नोक्सान हुनबाट सुरक्षित छन् भन्ने विश्वास दिलाउनु हो । संस्थाको बारेमा सिस्तृत जानकारी चाहनु हुन्छ भने नेपाल स्वास्थ्य अनुसन्धान परिष्दको फोन नं.०१४२५४२२० मा सम्पर्क गर्न सक्नु हुनेछ ।
यदी तपाईको यस अनुसन्धान बारे केही प्रश्न छ भने तत्कालै वा पछी सोध्न सक्नु हुनेछ । यदी पछी प्रश्न सोध्न चाहनु हुन्छ भने मलाई फोन नं.९८४१२४८३४३ मा कुनै पनि बेला सम्पर्क गर्न सक्नु हुन्छ ।

मंजुरीनामा

अगाडी दिइएको जानकारी मैले आफैले पढेको हुँ /मलाई पढेर सुनाईएको हो । मलाई सबै किसिमको प्रश्न सोध्ने अवसर प्रदान गरिएको थियो र मैले सोधेको सबै प्रश्नको जवाफ पाए । म यस अनुसन्धानमा आफ्नो ईच्छाले स्वयंसेवीको रुपमा सहभागि हुन मंजुर छ । मलाई जुनसुकै बेला यसबाट वाहिरिने अधिकार छ र यसबाट म र मेरो परिवारलाई कुनै किसिमको हानी नहुने कुरामा म विस्वस्त छु ।

सहभागिको नाम :
हस्ताक्षर :
मिति :

APPENDIX - IV

Questionnaire form

Patient Information

1. S. no.
2. Patient ID:
3. Date:
4. Address:
5. Age (years):
6. Height: Weight (kg):
7. Blood group:
8. Married since (in years):
9. Type of infertility:
   a. Primary b. Secondary
10. Trying to conceive since (in years):
11. Occupation:
12. Risk factors at work:
    a. None b. Chemicals c. Radiation d. Humidity
    e. Physical overload f. Mental overload
13. Exposure to heat: a. Yes b. No
14. Smoking:
    a. Never b. in the past c. periodically d. currently
15. Alcohol consumption:
    a. never b. 1-4 times/yr c. 1x/month d. 2x/month
    f. 1-2x/week g. daily b. in the past
16. Circumcision status:
    a. Yes b. No
17. Physical activity:
    a. No b. Walking/Jogging c. cycling d. Swimming e. aerobics
    f. yoga g. dancing h. gym i. games
    j. others

Clinical history

18. Diabetes: a. Yes b. No
19. Genital tract infection:
    i. Prostatitis: a. Yes b. No
    ii. Epididymitis: a. Yes b. No
    iii. Urethritis: a. Yes b. No
20. Hydrocoele: a. Yes b. No
21. Varicose vein: a. Yes b. No
22. Genital trauma: a. Yes b. No
23. Surgery in genital tract region: a. Yes b. No
24. Cardiovascular disease: a. Yes b. No
25. Cancer: a. Yes b. No
26. Chemotherapy: a. Yes b. No
27. Radiotherapy: a. Yes b. No
28. Antibiotic therapy at present: a. Yes b. No
29. Mumps: a. Yes b. No
    If yes, at which age:
30. UTI: a. Yes b. No

Semen Analysis

Sample No.:

Date of sample collection:

Period of abstinence (days):

Liquefaction time: a. within 30 min b. 30-60 min c. not liquefied till 60 min

Viscosity: a. Normal b. Abnormal

Appearance: a. Greyish white, opalescent b. Transluscent

Volume (mL):

Sperm concentration (million/mL):

Sperm count (million/ejaculate):

Agglutination: a. Present b. Absent

Total motility%:

Progressive motility%:

Rapid progressive motility%:

Slow/moderate progressive motility%:

Non-progressive motility%:

Vitality%:

Morphology%:

Leukocyte count:

DFI%:

APPENDIX – V

Ethical clearance

Government of Nepal

Nepal Health Research Council (NHRC)

Estd. 1991

Ref. No.: 1933

23 February 2020

Ms. Anima Shrestha
Principal Investigator
Tribhuvan University
Kathmandu

Ref: Approval of thesis proposal

Dear Ms. Shrestha,

This is to certify that the following protocol and related documents have been granted approval by the Ethical Review Board, NHRC for implementation.

If the researcher requires transfer of the bio-samples to other countries, the investigator should apply to the NHRC for the permission. The researchers will not be allowed to ship any raw/crude human biomaterial outside the country, only extracted and amplified samples can be taken to laboratories outside of Nepal for specific study, as per the protocol submitted and approved by the NHRC. The remaining samples of the lab should be destroyed as per standard operating procedure and the process should be documented and informed to the NHRC timely.

ERB Protocol No	874/2019 PhD	**Sponsor Protocol No**	NA
Principal Investigator/s	Ms. Anima Shrestha	**Sponsor**	NA
Title	Bacteriospermia in men among infertile couples in Nepalese community and its relation to sperm DNA integrity		
Protocol Version No	Version 6.0	**Version Date**	10 February 2020
ICF Version No. (V.N,)	Version 6.0	**Version Date**	10 February 2020
Other Documents 1. Data Collection Tools 2. Acceptance letter from study site			
Members of research team			
Study Site	1. Creator's IVF Nepal Pvt. Ltd		

Tel: +977 1 4254220, Fax: +977 1 4262469, Ramshah Path, PO Box: 7626, Kathmandu, Nepal
Website: http://www.nhrc.gov.np, E-mail: nhrc@nhrc.gov.np

Government of Nepal

Nepal Health Research Council (NHRC)

Estd. 1991

Ref. No.: 1933

Type of Review	√	**Expedited**	**Duration of Approval** 23 February 2020 to 23 February 2021	**Frequency of continuing review** At least once in a year
		Full Board		
	Meeting Date: 10 February 2020			
Total budget of research	**NRs 4,65,000.00**			
Ethical review processing fee	**NRs 1,000.00**			
Executive Chief of NHRC	**Name**		**Date**	
	Dr. Pradip Gyanwali		23 February 2020	

Investigator Responsibilities

- Any amendments shall be approved from the ERB before implementing them
- Submit Serious Adverse Events (SAE) and Suspected Unexpected Serious Adverse Reaction (SUSAR) reports to the ERB within 48hours
- Submit progress report every 3 months
- Submit final report after completion of protocol procedures at the study site
- Report protocol deviation / violation within 7 days
- Comply with all relevant international and NHRC guidelines
- Abide by the principles of Good Clinical Practice and ethical conduct of the research

If you have any questions, please contact the Ethical Review M & E Section at NHRC.

Thanking you,

Dr. Pradip Gyanwali
Executive Chief
(Member- Secretary)

Tel: +977 1 4254220, Fax: +977 1 4262469, Ramshah Path, PO Box: 7626, Kathmandu, Nepal
Website: http://www.nhrc.gov.np, E-mail: nhrc@nhrc.gov.np

Government of Nepal

Nepal Health Research Council (NHRC)

Estd. 1991

Ref. No.: 237

28 July 2021

Ms. Anima Shrestha
Principal Investigator
Tribhuvan University
Kathmandu

Subject: **Approval of requested Amendment for a research** study entitled **Bacteriospermia in men among infertile couples in Nepalese community and its relation to sperm DNA integrity (Reg. no: 874/2019, Approved on 10 February 2020)**

Dear Ms. Shrestha,

The meeting of the Expedite Review Sub-Committee of Nepal Health Research Council held on 26 July 2021 discussed the amendment requested on 7 July 2021. The meeting has approved the amendment to extend timeline till 10 February 2022 as sample collection for the study could not be done due to COVID-19 pandemic and country lockdown.

If you have any queries, please feel free to contact the Ethical Review M & E section of NHRC.

Thanking You,

Dr. Pradip Gyanwali
Member Secretary
(Executive Chief)

Tel: +977 1 4254220, Fax: +977 1 4262469, Ramshah Path, PO Box: 7626, Kathmandu, Nepal
Website: http://www.nhrc.gov.np, E-mail: nhrc@nhrc.gov.np

Government of Nepal

Nepal Health Research Council (NHRC)

Estd. 1991

Ref. No.: 678

20 September 2022

Ms. Anima Shrestha
Principal Investigator
Tribhuvan University, Kirtipur

Subject: **Approval of the requested amendment** for the study entitled **Bacteriospermia in men among infertile couples in Nepalese community and its relation to sperm DNA integrity (Reg. no. 874/2019, Approved on 10 February 2020)**

Dear Ms. Shrestha,

The meeting of the Expedited Review Sub-Committee of Nepal Health Research Council held on 26 August 2022 discussed the amendment requested on 7 July 2022. The meeting has approved the amendment to extend timeline till July 2023 as the targeted samples could not be collected as planned due to COVID-19 pandemic.

If you have any queries, please feel free to contact the Ethical Review M & E Section of NHRC.
Thanking you!

Namita.

Ms. Namita Ghimire
Acting Administrative Chief

Tel: +977 1 4254220, Fax: +977 1 4262469, Ramshah Path, PO Box: 7626, Kathmandu, Nepal
Website: http://www.nhrc.gov.np, E-mail: nhrc@nhrc.gov.np

APPENDIX - VI

Assessment of morphology

(Manufacturer's instructions)

Procedure

Step 1: Allow smear to air dry

Step 2: Lay smear horizontally and cover the entire smear with 1 mL of Fixative solution. Keep it for 15 seconds.

Step 3: Drain off the fixative solution and allow smear to air-dry.

Step 4: Lay smear horizontally and cover the entire smear with 1 mL of Stain - I solution. Keep it for 12 seconds.

Step 5: Drain off Stain - I solution

Step 6: Lay smear horizontally and cover the entire smear with 1 mL of Stain – II solution. Keep it for 8 seconds.

Step 7: Drain off Stain - II solution

Step 8: Rinse smear in distilled water

Step 9: Drain off distilled water and clean the back of the slide with a filter paper.

Step 10: Allow smear to air-dry.

Examination

• Put a drop of immersion oil on dry smear.

• Examine the smear under microscope with the help of 100x lens.

• Examine at least 200 sperm and evaluate as Normal Sperm or Abnormal Sperm

• Calculate the percentage of normal & abnormal sperm.

Morphology of Normal Sperm

• Head: Smooth oval configuration

Dimensions: Length: 5.0µm - 6.0µm

Width: 2.5µm - 3.5µm

• Acrosome: Acrosome should comprise of 40 - 70% of the anterior sperm head.

• Borderline Forms: Consider all borderline forms as abnormal.

• Neck / mid-piece: No abaxial implantations - Slender approximately 1µm in width and 6-7µm in length. Cytoplasmic droplets >30% of head size are considered abnormal.
• Tail: Uniform, slightly thinner than the mid-piece, uncoiled with principal piece 45-50µm and terminal segment of 4-6µm.

Normal Sperm

• If sperm dimensions for Head, Neck & Tail are within normal limits, then it is called as 'Normal Sperm'.

Abnormal Sperm

• When any one of the parameters of sperm for Head, Neck or Tail is not within normal limits, then it is called as 'Abnormal Sperm' (Strict Criteria).

(Source: Morphology-D kit, Sperm Processor Pvt. Ltd., India)

APPENDIX - VII

Leukocyte count

(Manufacturer's instructions)

Leukocytes, predominantly polymorphonuclear leukocytes (PMN, neutrophils), are present in most human ejaculates. Elevated concentrations of leukocytes in semen indicated male accessory gland infections and associated with poor semen quality and reduced fertility. Also, leukocytospermia is known to induce oxidative stress which may lead to DNA damage in sperms. Leukocheck kit, Cryolab International is simple, quick and inexpensive for screening of leukocytes. The test identify leukocytes in semen based on the cyto-chemical stain for peroxidase activity. Cells (leukocytes) that contain peroxidase activity stain brown and other cells counterstain pink.

Kit contents: Solution A and Solution B

Procedure

1. Prepare the working solution by adding 50µL of Solution B to 1mL of Solution A in microcentrifuge tube. The working solution is stable for 24 h at ambient temperature.

2. Mix 10µL of liquefied semen and 10µL of working solution on a glass slide and mix well. Wait for 1-2 min.

3. Place 10µL on Neubauer's chamber, put cover glass and count the number of brown colored cells in four corner squares under 40X objective, and calculate the average of one square (Volume of sample in corner square is 10^{-4} mL).

4. Express the concentration of leukocytes as no. of leukocytes in millions per mL of sample.

(Source: Leukocheck kit, Cryolab International)

APPENDIX - VIII

Sperm DNA fragmentation test

(Manufacturer's instructions)

Sperm chromatin dispersion test for assessing human sperm DNA fragmentation. The integrity of sperm DNA is being recognized as an important factor for successful reproductive outcomes. Sperm DNA fragmentation testing has gained importance as it offers a better diagnostic and prognostic potential than routine semen analysis. Several techniques exist to detect sperm DNA fragmentation, but sperm chromatin dispersion (SCD) test is a simple, reliable, and reproducible technique. Using SCD test, DNA fragmentation can be accurately measured using conventional bright-field microscopy.

Kit contents:

Pre-coated slides, Agarose, Solution A, Solution B, Solution C, Solution D

Procedure

1. Assess the semen sample for its concentration. Dilute the sample with culture medium to 5-10 million/mL.

2. Melt agarose at 90 ^{o}C for 1 to 5 min.

3. Transfer the agarose to maintain temperature at 37 ^{o}C for 5 min.

4. Add 25µL of the semen sample to agarose and mix well.

5. Place the sperm cell suspension immediately onto the pre-treated slides and place the cover slip. Avoid formation of air bubbles.

6. Leave the slide at 4 ^{o}C for 5 min.

7. After 5 min, remove the slide. Carefully recover the cover slip by sliding it off gently.

8. Take care to maintain the slide in a horizontal position throughout the procedure.

9. Incubate the slides horizontally in Solution A for 7 min.

10. After 7 min, incubate the slide horizontally in lysis solution for 25 min. (Mix the lysis solution before use)

11. Leave the slide in distilled water for 5 min.

12. Place the slide in 70% ethanol (2 min), followed by 90% ethanol (2 min) and finally in 100% ethanol (2 min). Leave to dry at room temperature.

13. Mix the Solution C and Solution D (1:1), and deposit the layer of stain, horizontally.
14. Leave to stain for 15-20 min.

15. Decant the stain and gently wash with distilled water and dry at room temperature.

16. Visualize the slide under bright field microscope using 20X or 40X objective and grade the spermatozoa. The slide can be re-stained if the staining is weak. If the staining

is too strong, the slide can be de-stained by rinsing gently in distilled water or 10% ethanol.

17. Classify at least 300-500 sperm cells as follows.

Sperm without DNA fragmentation: Big or medium halo around the sperm head

Sperm with DNA fragmentation: Small halo or no halo around the sperm head or degraded

Calculate the percentage of sperm DNA fragmentation (SDF) as follows:

SDF%= 100 X No. of sperms with fragmented DNA/ No. of sperms counted

(Source: Sperm Chroma kit, CryoLab International, India)

APPENDIX - IX

Zone size Interpretative chart of antibiotics used for *S. aureus*

Antimicrobial Agent	Disk Content	Interpretive Categories and Zone of inhibition (Diameter in mm)		
		Sensitive mm or more	Intermediate mm	Resistant mm or less
Ampicillin	10 mcg	29	-	28
Cefoxitin	30 mcg	18	15 - 17	14
Ciprofloxacin	5 mcg	21	16 - 20	15
Clindamycin	2 mcg	21	15 - 20	14
Co-trimoxazole	25 mcg	16	11 - 15	10
Doxycycline hydrochloride	30 mcg	16	13 - 15	12
Erythromycin	15 mcg	23	14 - 22	13
Gentamicin	10 mcg	15	13 - 14	12
Levofloxacin	5 mcg	19	16 - 18	15
Linezolid	30 mcg	21	-	20
Nitrofurantoin	300 mcg	17	15 - 16	14

APPENDIX - X

Protocol of DNA extraction from semen

(Manufacturer's instructions)

Protocol: DNA Purification from Blood or Body Fluids (Spin Protocol)

This protocol is for purification of total (genomic, mitochondrial, and viral) DNA from whole blood, plasma, serum, buffy coat, lymphocytes, and body fluids using a microcentrifuge.

Things to do before starting

■ Equilibrate samples to room temperature (15–25 °C).

■ Heat a water bath or heating block to 56°C for use in step 4.

■ Equilibrate Buffer AE or distilled water to room temperature for elution in step 11.

■ Ensure that Buffer AW1, Buffer AW2, and QIAGEN Protease have been prepared according to the instructions.

■ If a precipitate has formed in Buffer AL, dissolve by incubating at 56 °C.

Procedure

1. Pipette 20 µL QIAGEN Protease (or proteinase K) into the bottom of a 1.5 mL microcentrifuge tube.

2. Add 200 µL sample to the microcentrifuge tube. Blood or Body Fluid

3. Add 200 µL Buffer AL to the sample. Mix by pulse-vortexing for 15 s.

To ensure efficient lysis, it is essential that the sample and Buffer AL are mixed thoroughly to yield a homogeneous solution.

4. Incubate at 56 °C for 10 min.

DNA yield reaches a maximum after lysis for 10 min at 56 °C. Longer incubation times have no effect on yield or quality of the purified DNA.

5. Briefly centrifuge the 1.5 mL microcentrifuge tube to remove drops from the inside of the lid.

6. Add 200 µL ethanol (96–100%) to the sample, and mix again by pulse-vortexing for 15 s. After mixing, briefly centrifuge the 1.5 mL microcentrifuge tube to remove drops from the inside of the lid.

7. Carefully apply the mixture from step 6 to the QIAamp Mini spin column (in a 2 mL collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 x *g* (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 mL collection tube

(provided), and discard the tube containing the filtrate. Close each spin column to avoid aerosol formation during centrifugation. Centrifugation is performed at 6000 x *g* (8000 rpm) to reduce noise. Centrifugation at full speed will not affect the yield or purity of the DNA. If the lysate has not completely passed through the column after centrifugation, centrifuge again at higher speed until the QIAamp Mini spin column is empty.

8. Carefully open the QIAamp Mini spin column and add 500 µL Buffer AW1 without wetting the rim. Close the cap and centrifuge at 6000 x *g* (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 mL collection tube (provided), and discard the collection tube containing the filtrate. Protocol

9. Carefully open the QIAamp Mini spin column and add 500 µL Buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed (20,000 x *g*; 14,000 rpm) for 3 min.

10. Recommended: Place the QIAamp Mini spin column in a new 2 mL collection tube (Not provided) and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min. This step helps to eliminate the chance of possible Buffer AW2 carryover.

11. Place the QIAamp Mini spin column in a clean 1.5 mL microcentrifuge tube (not provided), and discard the collection tube containing the filtrate. Carefully open the QIAamp Mini spin column and add 200 µL Buffer AE or distilled water. Incubate at room temperature (15–25°C) for 1 min, and then centrifuge at 6000 x *g* (8000 rpm) for 1 min.

Incubating the QIAamp Mini spin column loaded with Buffer AE or water for 5 min at room temperature before centrifugation generally increases DNA yield. A second elution step with a further 200 µL Buffer AE will increase yields by upto 15%.

For samples containing less than 1 µg of DNA, elution in 50 µL Buffer AE or water is recommended. For long-term storage of DNA, eluting in Buffer AE and storing at –30 to

–15 °C is recommended, since DNA stored in water is subject to acid hydrolysis.

(Source: QIAamp® DNA Mini and Blood Mini Handbook)

APPENDIX - XI

Calculation of gene copy number in the positive control of 16S rRNA gene

Length of pUC 57 plasmid vector = 2710 bp

Length of positive control = 148 bp

Total length of plasmid (L) = 2710 bp + 148 bp = 2858 bp

Formula for the calculation of gene copies in standard (positive control)

$N = 10^{-9} x\ C\ x\ NA\ /\ L\ x\ M$ (copies/mL) where N = gene copy number concentration

NA = Avogadro constant (6.02×10^{23}/mol)

C = nucleic acid concentration (ng/µL)

= 4 µg/100µL = 40 ng/µL

L = total length of plasmid (bp)

M = 660 dalton/bp (1 dalton = 1 gm/mol)

Using the formula,

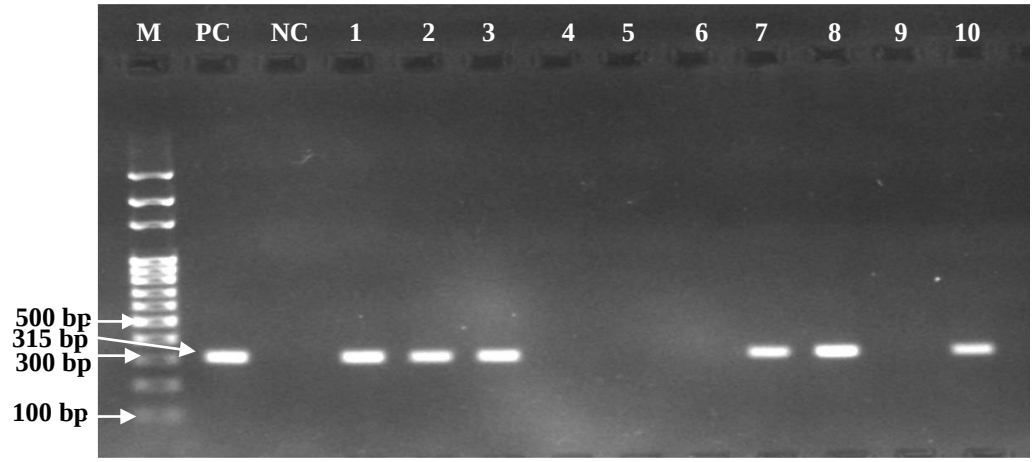
$N\ (copies/\mu L) = 10^{-9} \times 40 \times 6.02 \times 10^{23} / 2858 \times 660$

$= 1.276 \times 10^{-4} \times 10^{14}$

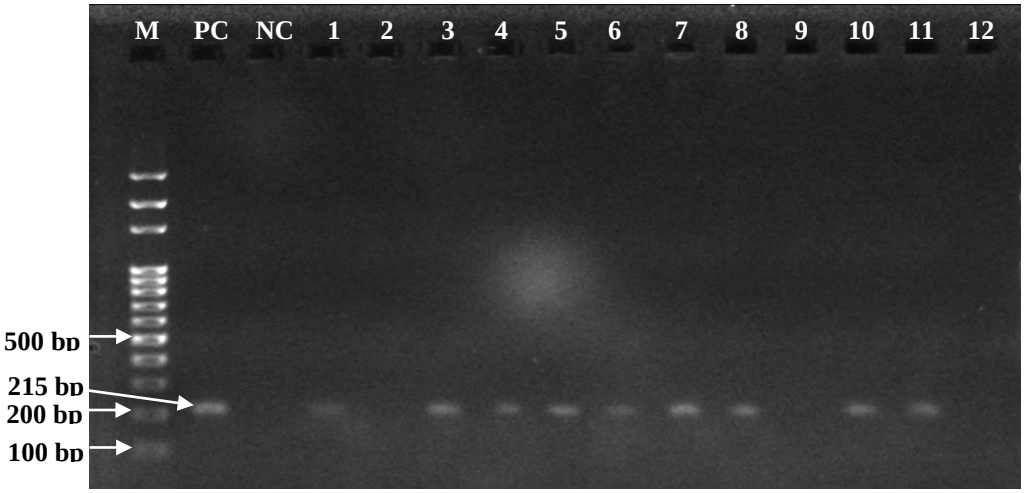
$= 1.276 \times 10^{10}$ = concentration of standard positive control

APPENDIX – XII

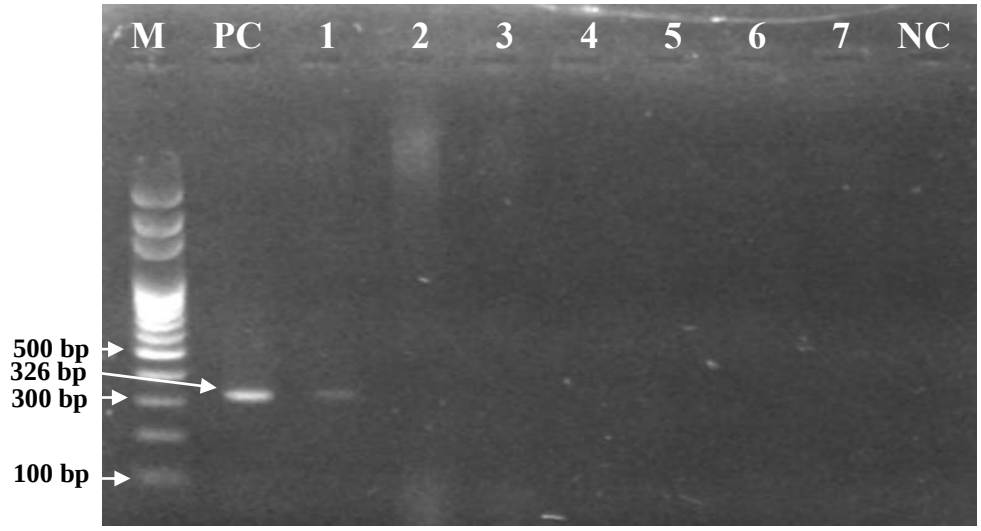
Photographs



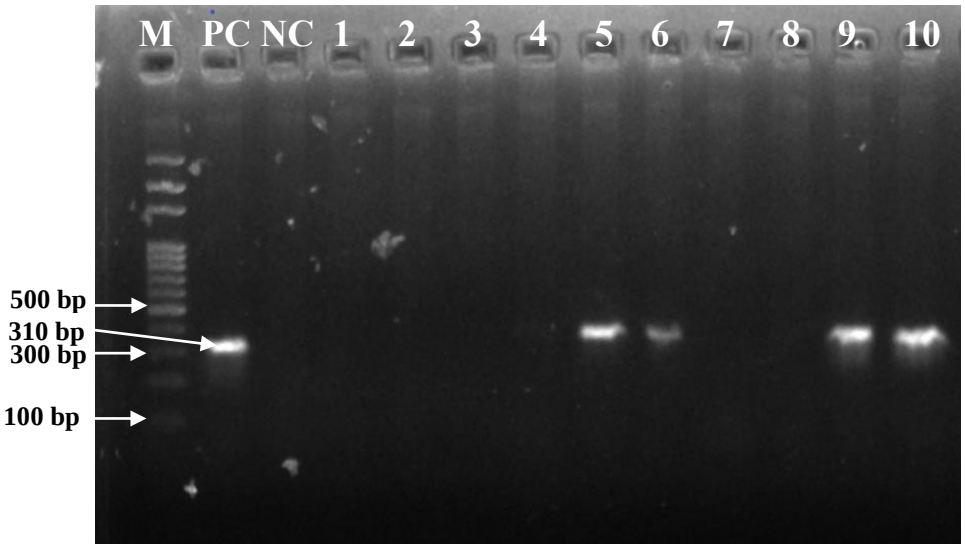
Photograph 1: PCR products of *clf*A gene in *S.aureus* (From left to right: M-100bp DNA marker PC- positive control with approx. 315 bp, NC- negative control, 1-3,7,8,10-positive for *clf*A gene and 4-6, 9-negative for *clf*A gene)



Photograph 2: PCR products of *clf*B gene in *S. aureus* (From left to right: M-100bp DNA marker, PC- positive control with approx. 215 bp, NC- negative control, Lane 1,3-8,10,11-positive for *clf*B gene and 2, 9,12 -negative for *clf*B gene)



Photograph 3:PCR products of *tst* gene in *S. aureus* (From left to right: M- 100bp DNA marker, PC- positive control with approx. 326 bp, 1-positive for *tst* gene and 2-7 -negative for *tst* gene, NC- negative control for *tst* gene)



Photograph 4: PCR products of *mec*A gene in *S. aureus* (From left to right: M- 100 bp marker DNA, PC- positive control of approx. 310 bp, NC- negative control, Lane 1-4,7, 8- negative for *mec*A gene, Lane 5, 6, 9,10- positive for *mec*A gene)

APPENDIX XIII
List of Publications

1. Bacteriospermia in men among infertile couples in the Nepalese population

a. Anima Shrestha, Dev Raj Joshi, Dijan Vaidya, Sanu Maiya Shrestha, Anjana Singh
b. Systems Biology in Reproductive Medicine, Volume 70, No. 1, August 2024.
c. https://doi.org/10.1080/19396368.2024.2391052

2. Influence of Bacteriospermia, Host and Lifestyle Factors on Sperm DNA Integrity: A Cross-Sectional Study Based on a Fertility Center of Nepal

a. Anima Shrestha, Dev Raj Joshi, Dijan Vaidya, Sanu Maiya Shrestha, Anjana Singh
b. Journal of Family and Reproductive Health (2025), Volume 19, No. 1, March 2025
c. https://doi.org/10.18502/jfrh.v19i1.18438

APPENDIX-XIV

Seminar/Conference presentations

1. Oral presentation on “Bacteriospermia in men among couples undergoing fertility investigation” at Ninth National Conference on Science and Technology, organized by Nepal Academy of Science and Technology (NAST), Kathmandu, Nepal, 26-18 June 2022.

2. Poster presentation on “Bacteriological profile and semen quality in Nepalese men with potential infertility” at Ninth National Summit of Health and Population Scientists in Nepal – 2023, Organized by Government of Nepal, Nepal Health Research Council (NHRC), Kathmandu, Nepal, 11-12 April 2023.

3. Poster presentation on “Bacterial diversity in semen of infertile men in Nepal” at Ph.D. festival 2023, organized by Institute of Science and Technology (IoST), Tribhuvan University, 9-10 October 2023.

4. Poster presentation on “Bacterial community composition of human semen from suspected infertile Nepalese males” at Second South Asian Symposium on Microbial Ecology 2023 (SASME 2023), organized by Microbial Ecology Network Nepal, Dhulikhel, Nepal, 1-3 November 2023.

5. Oral presentation on “Influence of Bacteriospermia, host and lifestyle factors on sperm DNA fragmentation” at Fourth Conference on Recent Trends in Science, Technology, and Innovation (R*TST*I) 2025, organized by Pokhara University, Pokhara, Nepal, 19-21 March 2025.

Nepal Academy of Science and Technology (NAST)

CERTIFICATE OF PARTICIPATION

Awarded to

ANIMA SHRESTHA

for Presentation in Oral / Poster / Participation in the

9th National Conference on Science and Technology

June 26-28,2022 (Asar 12-14, 2079)

Khumaltar, Lalitpur, Nepal

Ms. Luna Vajra
Chief, Promotion Division

Prof. Dr. Mahesh K. Adhikari
Secretary

Dr. Sunil Babu Shrestha
Vice Chancellor

"Research for Health: Translating evidence and innovation into Action"

Ninth National Summit of Health and Population Scientists in Nepal - 2023

Certificate of Poster Presentation

This is to certify that

Anima Shrestha

has made a poster presentation in the Ninth national summit of health and population scientists in Nepal

11-12 April 2023, Kathmandu, Nepal.

Organized by Nepal Health Research Council (NHRC)

Dr Pradip Gyanwali
Member-Secretary

Prof Dr Gehanath Baral
Chairman

TRIBHUVAN UNIVERSITY
INSTITUTE OF SCIENCE & TECHNOLOGY
Kirtipur, Kathmandu

PARTICIPATION CERTIFICATE

AWARDED TO

ANIMA SHRESTHA

With appreciation and good wishes for participation with a poster presentation in the

Ph.D. Festival 2023

Held on 9-10 October, 2023 (22-23 Asoj 2080) in University Campus, Kirtipur, Nepal.

Prof. Dr. Binil Aryal

Organized by

Prof. Dr. Shiva Lal Bhusal

SECOND SASME 2023

CERTIFICATE

OF PARTICIPATION

Microbial Ecology Network Nepal | Central Department of Microbiology | Central Department of Biotechnology

Awarded to

Anima Shrestha

from *Tribhuvan University, Nepal*

for participating as **Poster Presenter** in

"Second South Asian Symposium on Microbial Ecology (SASME 2023)"

held on 1-3 November, 2023 in Dhulikhel, Nepal.

Prof. Colin Murrell
Member, International Advisory Board
International Society for Microbial Ecology

Prof. Thulani Makhalanyane
Member, International Board
International Society for Microbial Ecology

Dr. Dev Raj Joshi
President
Microbial Ecology Network Nepal

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Science, Technology, and Innovation to build the nation

4th Conference on Recent Trends in Science, Technology and Innovation-2025

RTSTI-2025 | Pokhara, Nepal

CERTIFICATE OF PARTICIPATION

This certificate is proudly presented to

Anima Shrestha

19-21 March 2025

for his/her participation as a **Oral Presenter** in the 4th Conference on Recent Trends in Science, Technology, and Innovation-2025 organized by Pokhara University.

Organizing Chair
Prof. Dr. Bed Raj KC
Vice-Chancellor

Convener
Assoc. Prof. Dr. Namraj Dhami
Executive Director
Pokhara University Research Centre