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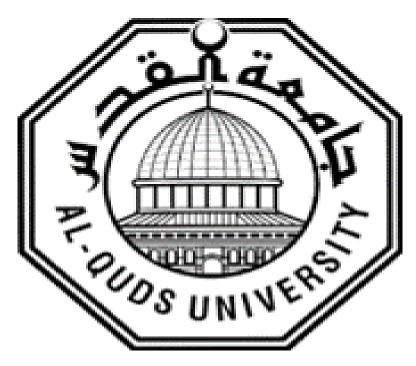

COMPARSION BETWEEN CULTIVATION AND 16s RNA SEQUENCING BASED – APPROACHES ANALYSIS FOR BACTERIA IN URINE AND CERBROSPINAL FLUID.

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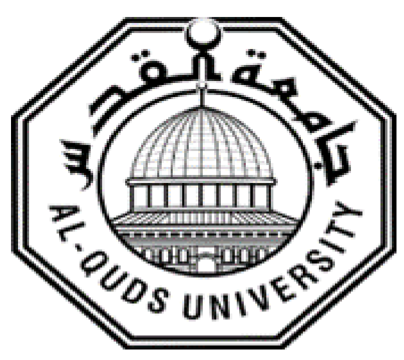

Comparison between cultivation and 16s RNA sequencing based- approaches analysis for bacteria in urine and cerebrospinal fluid.

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1443/2022

Dedication

I dedicate this work to my Parents

I dedicate this work to my dear Mamuon Jaffal

I dedicate this work to my Lovely Kids; Sama and Lama,

I dedicate this work to my brothers,

I dedicate my work to all teachers who teach me,

I dedicate my work to my friends.

Declaration

I certify that this thesis submitted for the degree of Master in microbiology and immunology is the result of my own research, except where otherwise acknowledged, and that this thesis (or any part of the same) has not been submitted for a higher degree to any other university or institution.

Signature: …………….

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Abstract:

Bacterial identification remains a challenge in the microbiology laboratories, specifically in hospitals, to choose the more sensitive and specific methods to identify the causative agents of diseases, especially in emergency cases and outbreak diseases.

Hospital laboratories collected many of samples like blood and body fluids, in this study focused on the urine and cerebrospinal fluids samples, which are the important samples, taken in hospitals; especially when the symptoms do not appear to physicians, particularly in babies and the elderly, many bacterial identities fail to detect the bacteria present in these samples.

Bacterial meningitis is still a serious condition with a significant risk of complications that might result in death or harmful complications. The determining the clinical cause of diseases is essential to the evaluation of novel treatment strategies.

The culture method, also called the classical identification method, has many challenges in sample collection, adequate sample volume, timing consumption during the bacterial incubation, antibacterial resistance and an uncultivable bacteria.

The main aim of the current study is to choose specific diagnostic methods to identify bacterial infection in the clinical samples and compare these methods.

The central methodology used in this study is the next-generation sequencing (NGS) compared with the classical culture method, for evaluation and diagnosis of most abundant bacteria present in the urine and CSF.

NGS technique is a relatively new platform that helps for mass sequencing and the simultaneous creation of a huge array of genomic information from numerous species, as well as a different quantitative counts measurements for every sequenced DNA segment type.

In this study, we collected 50 urine samples (30 positive bacterial growth, 15 no significant bacterial growth and 5 no bacterial growth) and 14 samples of CSF (4 positive bacterial growth and 10 no bacterial growth) from Al-Makassed hospital microbiology laboratory -Jerusalem-Palestine. These samples were cultured at the same lab and collected the results from it. The collected sample DNA extraction was done at Al-Quds university laboratory, followed by bacterial DNA fragment amplification using specific primers adapted to be used later in Illumina MiSeq DNA sequence analysis. A total of 64 collected samples were NGS done.

The data were obtained as FASTQ files to FASTA and then analyzed according to genus and species bacterial classification.

The specific bacterial species that were identified by NGS in this study were considered from urine pathogenic bacteria, the most important identified species are *Escherichia coli*, *Pseudomonas, Klebsiella, Lactobacillus, Enterobacter, streptococcus Group B, Acinetobacter, Staphylococcus, Rickettsia and Gardnerella.*

These species are present as a pathogenic bacteria in the urinary tract and cause urinary tract infections, most abundant and important are the *Escherichia coli* species that cause UTIs. The result is the same for the two methods.

In the CSF samples, the most abundant bacteria are *Pseudomonas, Enterobacter, Escherichia, Staphylococcus and Lactobacillus.*

And in the CSF the most abundant bacterial species present as a pathogenic bacteria in CNS and cause meningitis, is *Staphylococcus haemolyticus*. And the result is the same in both methods.

In conclusion, the NGS method is a very sensitive and specific method that can detect slow-growing, fastidious bacteria, and low-frequency bacteria in the no significant and negative growth results samples at the culture method.

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List of Abbreviations

Abbreviation	Full Word
CSF	Cerebrospinal fluid
DNA	Deoxyribonucleic acid
NGS	Next Generation Sequencing
RNA	Ribonucleic acid
PCR	Polymerase Chain Reaction
UTI	Urinary Tract Infection
MRSA	Methicillin-resistant Staphylococcus aureus
SBA	Sheep blood agar
Mac	MacConkey agar

Chapter One

Introduction

The goal of this study is to find the difference between the standard culture dependent and culture independent methods by using two types of samples, which are cerebrospinal fluids and urine, to describe the optimum strategy to identify the bacterial pathogen.

This chapter demonstrates the background of this study, the primary research problem. In addition, justification of the study, aims and objectives and the hypotheses are listed here.

1.1 Background

In nature, the microbes exist in a complex community shared with other species rather than pure culture occupying a specific ecological niche. The microbiota in healthy humans and various human disease states, such as chronic diseases, autoimmune disorders and metabolic disease, are no exception, often cohabiting organ systems or functioning in concert as polymicrobial biofilms.(Salipante et al., 2013)

Early and accurate detection of disease etiology is critical for developing effective therapies for pathogenic infection, but it is often impossible due to the determinations of current diagnostic microbiologic tests in terms of speed, sensitive, and assay target spectrum(Langelier et al., 2018), and rapid diagnosis and treatment are extremely important in reducing mortality and disability of patients. Such as *Tuberculous meningitis* (TBM), remains difficult due to nonspecific clinical features and the inefficiency of diagnostic technique.(Li et al., 2020)

Advances in genome sequencing hold promise for overcoming these diagnostic challenges by allowing for culture-independent analysis of microbial genomes from microliter volumes of clinical samples. The utility of next-generation sequencing (NGS) for rapid and actionable diagnosis of complicated infections has been highlighted in recent research. (Langelier et al., 2018)

1.2 problem statement :

Emerging infections are infections that has recently emerged in inhabitants or previously existed but are rapidly expanding in happening or geographic range, that have influenced the course of human past and caused huge suffering and death. (Morens et al., 2004)

Infectious diseases will continue to arise and resurface, posing unpredictable epidemics as well as challenges to public health, microbiology, and associated sciences.

Rapid clinical diagnosis, detection, and containment in populations and the environment are critical elements in controlling them, whether they are naturally occurring or deliberately engineered. This study aims to assess and compare between this methods:

Culture- dependent and culture -independent methods.

1.3 Study justification :

The end of the 19th century saw the birth of the well-known and long-accepted culture-dependent methodology. Bacterial populations in a variety of habitats this despite its limitations, the technique drove improvements in microbiology .Well-known, significant limits, primarily related to selectivity of the nutritional media and cultural circumstances.(Al-Awadhi et al., 2013)

Molecular methods were developed to address this problem. Molecular methods offer speed, and specificity and sensitivity. Both DNA and RNA have been analyzed by using molecular amplification methods such as polymerase chain reaction (PCR) , next generation sequencing (NGS) .

1.4 Study hypothesis :

In this study, we hypothesized that found the other types of bacteria in the samples by used the culture-independent methods another than by culture-dependent method.

1.5 study goals :

This study aimed to compare the efficiency of using advanced methods of bacterial identification based on DNA analysis with the classical culture methods currently used for bacterial identification.

1.6 Study objectives :

1. To identify bacterial species found in urine and CSF samples of body fluid isolated from suspected individuals.
2. To extract total DNA material directly from the isolated clinical samples and apply 16s rRNA PCR test.
3. To do NGS sequence analysis of the amplified DNA from the tested samples (using 16s RNA PCR). The NGS analysis will be done based on Illumina MiSeq sequence analysis system for bacterial analysis.
4. To analyze the obtained data (Fastq files of each sample) using advanced bioinformatics methods adapted on Galaxy online NGS analysis program.
5. To perform the statistical analysis of the obtained bacteria data.

Chapter Two

Literature Review

This chapter provides an overview of on the pathogenic identification and the technique types and their history. The main focus of this overview is the comparison between culture -dependent and culture- independent techniques.

2.1 Microbiome identification:

In the past decade we have seen an immense increasing of investigations numbers that aim to characterize the structure, function and dynamic of the host-associated with a microbial communities (microbiota) within the context of host development, diet, and pathophysiology, and environment perturbations. Microbial communities are defined as groups of microorganisms that live together. Microbial communities are more specifically defined as many-species gathering in which (micro) organisms interact with one another in a joining environment.(A., 2009)

Whipps and colleagues in 1988, worked on the ecology of rhizosphere microorganisms and provided the first definition of a term microbiome, they described the “microbiome” as a combination of the words “micro” and “biome”, naming a “characteristic microbial community” in a “reasonably well-defined habitat which has distinct physiochemical properties” as their “theatre of activity”. (Whipps J, 1988)

However, many other definitions of microbiome have been present in the last decades. Like the definition by Lederberg describes microbiomes within an ecological context, as a community of symbiotic ,commensal , and pathogenic microorganisms within a body space or other environment(Lederberg J, 2001).

The collecting of present living microorganisms in a defined concept is referred to as the microbiota. Viruses, phages, prions, plasmids, viroids and DNA (free) are not considered living microorganisms, so they are not included in the microbiota. Whipps and coworkers proposed the word "microbiome," which includes not only the community of microorganisms but also their "theatre of activity."

The latter refers to a complete range of molecules produced by microorganisms, including structural elements (nucleic acids, proteins, lipids, polysaccharides), metabolites (signaling molecules, toxins, organic, and inorganic molecules), and molecules produced by living to hosts and structured through environmental conditions.

As a result, the term "microbiome" should include all mobile genetic elements such as phages, viruses, "relic" and extracellular DNA. (Berg et al., 2020)

The microbiota is a community of microorganisms under to various kingdoms, the bacteria were classifieds under prokaryotic kingdom. (Berg et al., 2020).

These studies have shown a wide array of important parts that the pathogenic microbes play in human and animal health.

2.2 Methods for detecting the microbes:

Many methods have been developed for detecting the bacterial pathogens. Culture dependent method, a pure culture, as proposed by Robert Koch, is the foundation of all infectious disease research. The first isolation of a bacterium allows for the development of experimental Models to study virulence and fulfil Koch's criteria, establishing a link between microorganisms and infectious diseases. Bacterial culture also allows researchers to investigate bacteria's antibiotic susceptibility, which is the first step in developing treatment recommendations(Lagier et al., 2015)

Bacterial culture is more difficult than molecular techniques and often necessitates more training. As a result, the number of specialists has been steadily declining for the past 30 years, and there are now few specialists compared to the 1970s. Clinical microbiologists who specialize in intracellular bacteria sparked a renewed interest in bacterial culture in large part.(Bollmann et al., 2007)

Increases fungal infections, including increasing diversity in the species infecting immunocompromised patients, and difficulties in rapidly diagnosing fungal infections, necessitate increased vigilance and the use of more sensitive methods to achieve rapid and accurate diagnoses and targeted treatments, especially critical when the fungus might spread to numerous tissues, making effective therapy more difficult and harmful. Fungal infections often mimic bacterial illnesses at first. (Sidiq et al., 2016)

The first culture media were developed empirically, using environmental components. Overall, the choice of nutrients, .atmosphere, temperature, and time of incubation are the four primary elements that determine the growth of bacteria.(Kaeberlein et al., 2002)

Due to the drastic reduction in costs and its high-throughput capacity, next-generation sequencing has become the preferred method to study the collective genetic contents of microbial communities (microbiome). The application of next-generation sequencing technologies has

revolutionized microbiome research by allowing high-throughput profiling of the genetics contents of microbial communities.(Dhariwal et al., 2017)

In microbiology, there are two main strategies for isolating pure cultures. Enrichment culture had used as the first step in both strategies. The first strategy aims to isolate colonies by streaking them on a solid medium repeatedly (or using pour plates or agar shake tubes), while the second strategy aims to isolate cells by dilutions in a liquid medium repeatedly.

These traditional methods have resulted in the isolation of a huge number of strains from types with few or no pure culture representatives.(Alain and Querellou, 2009)

Traditional cultivation is laborious of microorganisms, time-consuming, and, most significant, selective and limited growth for the specific bacteria. The majority of bacterial cells are obtained from the environment and can be visualized under microscopy, but they can't be visualized typically from the visible colonies on the plates. This phenomenon may meditate the artificial conditions inherent in most culture media (for example, extremely high substrate concentrations or the absence of specific nutrients required for growth). In addition, studies using changed media demonstrated the recovery of organisms not previously identified in culture traditional cultivation methods.(Zengler et al., 2002) .

An organisms that were difficult to culture or grow slowly on laboratory media, as well as those that were relatively inert biochemically or produce variable reactions in phenotypic identification schemes, advantage from DNA sequence identification.

New culture-independent identification methods were needed to enhance the etiological detecting for infectious diseases, guiding to an improvement in clinical outcomes for patients, best antimicrobial, improved detection and controlling of disease outbreaks, the detection of viable but non-cultivable (VBNC) or other difficult-to-culture microorganisms, and investigations of unknown pathogens.(Caliendo et al., 2013)

DNA sequencing technology was first developed in 1975 and has based on the selective incorporation of labelling chain-terminating ddNTPs by DNA polymerase during in vitro DNA replication by Frederick Sanger. It was historically expensive, time-consuming and laborious for high-throughput studies.(Sanger and Coulson, 1975)

Polymerase chain reaction (PCR) provides an alternative, more rapid and accurate diagnostic tool for the identification of bacterial infection, PCR was created by Kary Mullins in 1983 and its benefits were immediately clear. When compared to culture, PCR is faster, less expensive, and more accurate at detecting organisms in a sample.

PCR is a three-step process:

1. Hot denaturation of a double-stranded DNA template initially.
2. Targeted annealing of particular primers.
3. Making the annealed primers longer.

These DNA templates were then amplified to a sufficient degree to identify the pathogens. PCR's high sensitivity and specificity have allowed it to detect rare microbes, which has fueled its diagnostic clinical uses, particularly in the diagnosis of bodily fluid infections. And it's can identifying more bacteria and discriminating more fastidious bacteria than traditional culture in patients.(Dixon et al., 2020)

Multiplex polymerase chain reaction (PCR) is a type of PCR in which two or more target sequences can be amplified by using more than one pair of primers in the same process. Multiplex PCR has the potential to deliver significant time and effort savings in the laboratory. Since it has initially described in 1988. (Markoulatos et al., 2002)

Polymerase chain reaction techniques are a powerful tool for the amplification of small amounts of starting target sequences. The great majority of PCR procedures involve reactions that amplify a single target.

The analysis of polymerase chain reaction products, and gel electrophoresis sequencing was chosen for its large separation scale, and extraordinary resolution.(Xu et al., 2012)

The term 'electrophoresis' had derived from the Greek electron and the Latin phore. The Greek term for amber is an electron, which has a material that may be charged to a high voltage of static electricity by rubbing it with a cloth. The Latin word forbearer is phore. The electrical charges 'carried' by the molecules are referred to as electrophoresis. The phrase goes back to 1909 when it was first used to describe the movement of colloidal particles in an electric field. Negatively charged molecules are nucleic acids. They move towards the positive electrode when exposed to an electric field. They move quickly as the voltage Increases.(Martin, 1996)

Their development is influenced by the material they pass through as well as their overall form. As a result, different sizes are required.

Leroy Hood and their workers have developed the automated Sanger sequencing.(Smith, 1986)The dideoxynucleotydes has labelled by fluorescent dyes that permit sequencing in a single reaction. The DNA fragments pass through capillary electrophoresis in a single reaction, and the fluorescence had detected by four different color plots (four nucleotides (A, C, T and G)). The Sanger sequencer has been considered as the first sequencing generation technology, the only sequencing

method from(1977-2004) .(Schuster, 2008) And the capacity of read is up ton1200bp.(Zhang et al., 2011)

By combining new sequencing methods with computational processing, the Shotgun sequencing methodology has accelerated the creation of sequencing data.(Pop, 2009).

Shotgun sequencing breaks very large DNA or RNA fragment or perhaps an organism's whole genome into several small overlapping parts at random. Using computer tools known as genome assemblers, these smaller parts are then sequenced and reassembled to recover the entire sequence of the original genome. Between 1995 and 2005, shotgun sequencing was the most advanced technique for genome sequencing. Even though the shotgun sequencing process accelerated the completion of the human genome sequencing project, it has taken more than ten years to complete a human genome sequence (using the Sanger sequencer). In 2001, the first consensus of the human genome were obtained by Sanger sequencing technique, and the first individual human's diploid.(MacLean et al., 2009)

A revolution in sequencing technology began in 2004 with the release of Roche's 454 GS20 platforms in 2005. Using the platform, James D.Waston second complete genome was sequenced(Zhang et al., 2011).Other platforms were introduced after the invention of the 454 platforms, including Solexa/Illumina in 2006 and the SOLiD technology in 2007.(Metzker, 2010; Pop, 2009)

To increase speed and rapidly decreasing costs, it is now possible to conduct sequencing projects with relative case. These sequencing technologies are called the next-generation sequencing (NGS) platforms and are now also available as benchtop instruments, including the Roche 454 GS Junior, Illumina MiSeq, and Life Technology Ion Proton and Ion Torrent Personal Genome Machines. (Mardis, 2008; Metzker, 2010; Zhang et al., 2011)

Non-Sanger-based sequencing techniques it is a new generation techniques, has delivered on its promise of sequencing DNA at unheard-of speeds, enabling remarkable scientific breakthroughs and innovative biological applications. However, NGS had to overcome the inertia of a field that had relied on Sanger sequencing for 30 years before breaking through.

This technique involved arraying hundreds of thousands of sequencing templates in either Pico titer plates or agarose thin layers so that these sequences could be examined in parallel - a massive increase above the maximum of 96 sequencing templates on a modern Sanger capillary sequencer. (Schuster, 2008)

NGS is one of the culture-independent technology that can be used in routine clinical microbiology but not in microbiology research only. The application of NGS technology and its different methodological variants now makes it possible to detect various types of microorganisms present within a microbial sample simultaneously, using a culture-independent approach and in a single sequencing run(Goodwin et al., 2016). The technical advancements that make high-throughput construction of next-generation sequencing libraries possible from Pico gram quantities of RNA enable studies of whole-transcriptome expression profiles in single cells.(Trombetta et al., 2014)

Next-generation sequencing (NGS) technologies read DNA templates at random over the entire genome. It was performed by first dividing the complete genome into small parts and then ligating those little pieces of DNA to specific adapters for random reading during DNA synthesis (sequencing-by-synthesis). As a result, NGS technology has frequently been referred to as massively parallel sequencing.

NGS read length (the number of continuously sequenced bases) is substantially shorter than Sanger sequencing. Because NGS currently only provides 50–500 continuous base pair reads, sequencing findings were classified as short reads. These short reads are a significant restriction of the present technology; however, emerging NGS methods, such as single-molecule sequencing, may outperform Sanger procedures and have the ability to read several continuous kilo base pairs (Kbps). Coverage is a critical issue because NGS can produce short readings. These short reads that overlap each other inside a certain genomic region has been described as coverage.(Zhang et al., 2011)

This method may lead to improved detection of difficult-to-culture bacteria, for example, obligate anaerobic bacteria, in clinical samples. Further, obligate anaerobes are known to cause serious infections, their detection may be sub-optimal within routine clinical microbiological diagnostic laboratories using traditional specimen collection and detection techniques, as special precautions are required to help preserve the anaerobic environment during specimen collection and transport, and the laboratory needs to provide growth components for culture-based detection methods,(Brook, 2002) the collection, transport, and culture-independent NGS methods could play a major role in the identification and detection of any other infection caused by fastidious or viable but non-cultivable (VBNC) microorganisms—examples of VBNCs include antibiotic 'damaged' microorganisms that may be present within patients during antimicrobial therapy.(Pasquaroli et al., 2013).

Targeted amplicon sequencing was used approach for identification microbial communities. From a clinical sample the DNA was extracted then subjected to PCR amplification using a PCR primer, was putted according the targets a taxonomically informative gene of the bacteria. After

amplification, the output results amplicons were sequenced from the NGS and then identified and characterized by using bioinformatics tools to analysis the data was obtained, which can search for specific sequence from databases to determine which bacteria or other microbes were present in the clinical sample and relative abundance. NGS technology advances at now that the latest amplicon-based NGS protocols enable extensive multiplexing of clinical samples, allowing scientist to uses hundreds of clinical samples and analyzing a millions of PCR amplicons in a single NGS run.(Kozich et al., 2013).

The 16S rRNA gene sequencing allows the microbiologists to infer phylogenetic relationships as the 16S rRNA gene encodes for the RNA component of the small subunit (SSU) of prokaryotic ribosomes and possesses a slow rate of evolution, and the16S rRNA gene size is approximately 1500 base pairs, structure comprises 9 highly conserved and 9 hypervariable regions (V1–V9). The conserved regions can use as universal primer binding sites for the PCR amplification of gene fragments, whereas the hypervariable regions contain considerable sequence diversity, useful for prokaryotic identification purposes.(Van de Peer et al., 1996)

The Illumina Genome Analyzer, introduced in 2006, was based on the expression of 'sequencing by synthesis (SBS)' to synthesize sequence reads of (32–40 bp) from tens to millions of surface amplified DNA fragments at the same time.(Mardis, 2008)

The oligonucleotides on the slide were spaced so that the DNA, which was then subjected to multiple rounds of amplification, forms clonal "clusters" of about 1000 copies of each oligonucleotide fragment. Each glass slide can support millions of parallel cluster reactions. During the synthesis reactions, proprietary modified nucleotides, one for each of the four bases, were used.(Chen et al., 2017)

The choice for a particular hypervariable region also depends on the technological limitations of the NGS platforms were used. For example, in the 16S rRNA gene, the short length of V4 region (~ 250 bp) allows for the full overlap of DNA sequences that were obtained from both ends of the PCR amplicon using Illumina's MiSeq NGS-platform, which was currently the most commonly used NGS platform. This technique generates lower error rates, which have resulted in additional accurate diversity estimates, compared to the results obtained from the not completely overlapping V3–V4 and V4–V5 regions, though the accompanying cost was a decrease of discriminatory power due to the shorter amplicon size.(Kozich et al., 2013)

The bioinformatics algorithms, it was the successes of the NGS and to transform obtained data into actionable information.(Oliver et al., 2015)

The bioinformatician was a very important part of genetic and microbiologic testing laboratories, and sending these information from research laboratories to clinical settings was required to provide high-quality testing frame work.

Bioinformatics has become a very important tools in the clinical and search laboratories for (generated, analyzed, maintained, and interpreted) these data from genetics testing.(Oliver et al., 2015) Commercial bioinformatics software licenses can be expensive and restrictive in terms of flexibility.

Table (1) : comparison between cultivation and 16s sequencing based approaches:
(Leddy, 2018)

Methods	**Description**	**Advantages**	**Limitation**
Culture	Cultivation of selected bacteria on selective media	Inexpensive, quantitative method, greater expertise required, viability confirmed, phenotype characterization.	Appropriate growth conditions required, only identification organisms that can be grown in lab, only individual microorganisms identified and detected, low sensitivity.
Polymerase Chain Reaction (PCR)	Amplification of DNA /RNA/Genes to identify targeted organisms, quantify genes via quantitative polymerase chain reaction (qPCR).	Rapid; high sensitivity, usually bacteria identified independent of growth; can also target resistant or virulent genes, quantitative method.	PCR bias, individual microorganisms targeted and identified, viability not confirmed; greater expertise and relativity sophisticated instrumentation needed.
Next Generation Sequencing (NGS)	Uses high – throughput sequencing to sequence the whole genomes from all members of microbial community, combined with data analysis (bioinformatics) DNA and RNA sequenced.	Rapid ; high specificity , identifies in each sample the pathogens to species and strain-level ,antibiotic resistant, pathogenicity and functional genes identified greater accuracy and utility , quantitative method.	Need greater expertise and instrumentation required, more complex database and advanced computations.

2.3 Identification of microbiome in urine and cerebrospinal fluids:

In urine, urine is the fluid (sterile) produced by the two kidneys that carry water and wastes through the urinary tract and go out body. The urine culture is a test that detects and identifies bacteria and yeast in the urine, which causes a urinary tract infection (UTI).

UTIs are one of the most frequent bacterial infections in the world. Their treatment is becoming increasingly insufficient as standard antibiotic resistance rates increase. The rise in antibiotic resistance and multi-drug resistance (MDR) bacteria in urinary tract infections have linked to be a greater rate of ineffective empirical therapy caused by poor antibiotic coverage. (Bischoff et al., 2018)

Urinary tract infections are generated by Gram-negative, Gram-positive bacteria, certain fungi and parasites. For both complicated and uncomplicated UTIs is *Escherichia coli* which is the most common bacterial causative agent, the most common bacteria causes UTIs: *Staphylococcus saprophyticus, Klebsiella pneumoniae, Staphylococcus aureus ,Enterococcus facials, , Proteus mirabilis, Group B Streptococcus, Pseudomonas aeruginosa and Candida species* .(Flores-Mireles et al., 2015)

The urinary microbiome presents a unique opportunity to improve our understanding of these situations, and potentially optimize treatment. In the standard culture method was adopted to include UTIs, in the different studies that we showed that the $\geq 10^5$ CFU/mL threshold where as other is the ≥1000 CFU/mL, insufficient to detect infections.(Hooton et al., 2013) The use of 16S rRNA rapid (NGS) has become demonstrated the bladder is not a sterile zone, but it is home to its unique microbiome. NGS was developed to diagnosis these microbiomes. (Hilt et al., 2014)

The most commonly antimicrobial drugs used to treat UTIs are: a combination of trimethoprim and sulfamethoxazole, β-Lactams, trimethoprim, fluoroquinolones, fosfomycin tromethamine, nitrofurantoin, Ampicillin and ciprofloxacin. (Jancel et al., 2002)

In CSF, cerebrospinal fluid is the secretion product of the central nervous system that fills the ventricles and the subarachnoid space of the brain and spinal column.(Wishart et al., 2008)

Acute bacterial meningitis is a disease with a quick onset, the potential for outbreak and epidemic, and high rates of mortality and morbidity. (Mace et al., 2008)

For certain infectious clinical conditions, such as septic shock and bacterial meningitis, early antibiotic therapy is crucial. However, unnecessary broad-spectrum antibiotic treatment has adverse effects and has an ecological cost due to the selection of resistant microorganisms. Many

patients, particularly those who report to the emergency department with common community-acquired diseases, are infected with bacteria resistant to narrow-spectrum antibiotics. Furthermore, many suspected bacterial illnesses are non-bacterial (e.g. fungal or viral) or non-infectious diseases. (Nauclér et al., 2021)

Next-generation sequencing (NGS) is a promising approach for the detected of infectious disease, causes of bacterial, viral, parasitic, and fungi can be detected by a single run.

The clinical has been used NGS for diagnosing neurologic infections in a series of patients with acute meningitis, encephalitis, or myelitis at the time of enrollment, in parallel with conventional microbiologic testing. Thus, the performance of NGS testing is difficult to identify by current issues of accessibility, cost, and turnaround time. The highest diagnostic yield resulted from a combination of NGS of CSF and conventional testing, including serologic testing and testing of sample types other than CSF. The NGS assay identified more potential pathogens than direct-detection testing of CSF.(Wilson et al., 2019)

Latex agglutination tests, the most rapid CSF tests available, are less sensitive, giving accurate results only for samples containing more than 10^5 CFU/ ml.

Immunological tests are insufficiently sensitive since bacterial loads of less than 10^5 CFU/ml are present in up to 45 % of meningitis cases.(Greisen et al.,1994)

The examination and culture of CSF collected from a lumbar puncture are used to confirm the diagnosis of bacterial meningitis. Typically, the CSF shows an increased neutrophil count, increased protein, decreased glucose, a positive Gram stain, and bacteria growth on proper culture media. The antibiotics are determined by the bacteria's antibiotic sensitivity. The most suspected empirical antibiotic treatment for acute bacterial meningitis is the third generation of cephalosporin, ceftriaxone. The ceftriaxone lysis the bacterial cell. (Mace, 2008).

The most common pathogens infected the CNS are: *Group B streptococci, Haemophilus influenzae, Neisseria meningitidis, Escherichia coli and Streptococcus pneumonia*. (Overturf, 2005).

Chapter Three

Materials and Methods:

3.1 Sample collection:

In this study, the clinical samples (urine and cerebrospinal fluids) were collected from Al-Makassed Hospital in Jerusalem -Palestine, according to standard clinical procedures. About 50 samples were collected from urine (30 positive bacterial growth, 15 no significant growth and 5 no bacterial growth) .Table (2-a), 14 samples from CSF (4 positive bacterial growth and 10 no bacterial growth) .Table (2-b).

All samples were collected by standard sterile methods, 3ml of urine was collected in a urine transport tube, sterile wide mouth container or sterile screw–cap tube. (Fresh or refrigerate to 24 hours).

CSF was collected in a sterile tube, 1 ml for adults and children and 0.5 ml for the neonate, samples were processed as soon as possible, and they were not refrigerated. Part of the CSF specimen was used for cytological and chemical examination and the remainder for the microbiological examination. (If the chemical test was positive results, the staff reported them to the microbiology staff in the lab.).

Table 2:

a- Urine Samples

Number of sample	Sample type	Result
1	Urine	Positive
2	Urine	Positive
3	Urine	Positive
4	Urine	Positive
5	Urine	Positive
6	Urine	Positive
7	Urine	Positive
8	Urine	Positive
9	Urine	Positive
10	Urine	Positive
11	Urine	Positive
12	Urine	Positive
13	Urine	Positive
14	Urine	Positive
15	Urine	Positive
16	Urine	Positive
17	Urine	Positive
18	Urine	Positive
19	Urine	Positive
20	Urine	Positive
21	Urine	Positive
22	Urine	Positive
23	Urine	Positive
24	Urine	Positive
25	Urine	Positive
26	Urine	Positive
27	Urine	Positive
28	Urine	Positive
29	Urine	Positive
30	Urine	Positive
31	Urine	Negative
32	Urine	Negative
33	Urine	Negative

34	Urine	Negative
35	Urine	Negative
36	Urine	Negative
37	Urine	Negative
38	Urine	Negative
39	Urine	Negative
40	Urine	Negative
41	Urine	Negative
42	Urine	Negative
43	Urine	Negative
44	Urine	Negative
45	Urine	Negative
46	Urine	Negative
47	Urine	Negative
48	Urine	Negative
49	Urine	Negative
50	Urine	Negative

b- CERBROSPINAL FLUIDS :

Number of sample	Sample type :	Result
51	CSF	Positive
52	CSF	Positive
53	CSF	Positive
54	CSF	Positive
55	CSF	Negative
56	CSF	Negative
57	CSF	Negative
58	CSF	Negative
59	CSF	Negative
60	CSF	Negative
61	CSF	Negative
62	CSF	Negative
63	CSF	Negative
64	CSF	Negative

3.2- Classical culture analysis:

The collected samples were identified for presence of different bacterial infections using the classical culture methods. All samples were identified as positive or negative and identifying the bacterial species were done in Al-Makassed hospital laboratory applying the standard detection methods used in the microbiology lab.

Urine culture:

1μL of urine sample was inoculated into divided SBA / Mac plate media by the loop, the straight line down the center of the plate by using the loop on each half separately and streak the urine by making a series of passes at 90 angles through the inoculum (Figure 1), then incubate at 35 to 37 ˚C for 24 hours.

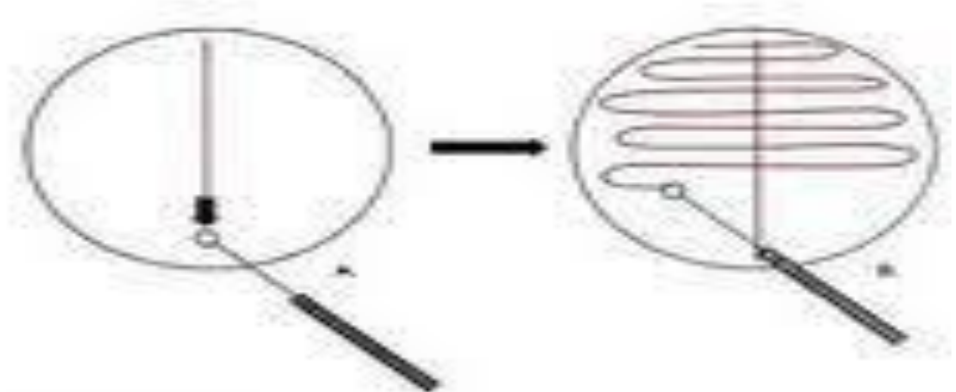

Figure (1): Streak plate technique.

If the plate does not have growth of any colony the result was negative. If the plate has a growth of a colony (count 10^5 colony or more) per millimeter (ml) of urine, the result is positive. Then doing the full of identification and sensitivity test manual.

CSF culture: were processed under a biosafety cabinet, to avoid contamination of the culture or specimen and personal safety. The processing of specimens was done as follow:

a) Preparation of CSF specimens, the volume and gross appearance of CSF was recorded, if the specimens were cloudy, a gram stain should be done before the centrifugation.

b) The specimens were centrifuged, and the sedimentation was cultured onto enrichment agar (Blood Agar Plate (BAP), Chocolate agar (CHOC)) to encourage the growth of any bacteria. From the remained sediment a smear was done and then Gram stain.

3.3- DNA extraction:

DNA extraction from the collected total samples were performed following standard method of phenol extraction and ethanol precipitation. All DNA samples were treated in standard way in order to enable results comparison.

Phenol DNA extraction protocol:

DNA was extracted from a total of 64 samples, after using phenol DNA extraction protocol.

1. DNA was extracted from these clinical samples directly by adding 200 µl of DNA lysis buffer (50 mM NaCl, 10 mM EDTA, 50 mM Tris-HCl pH 7.4, 1% Triton X-100) to sample tubes, then vortex it.
2. Then 20 µl of 10 mg/ml proteinase K was added to each tube.
3. The solution was kept at 60C for 2 hours followed by added 20 µl of phenol solution (pH =8.0) for extraction of proteins. After these steps, the tubes were vortexed for 1 minute then centrifugation for 30 minutes at high speed (14.000 rpm), the top of the aqueous solution layer was transferred 100 µl from it and labelled in Eppendorf tubes.
4. Then, ethanol precipitation using a final concentration of 0.2 M NaCl and the addition of 3 volumes of cold absolute ethanol. After incubation at -20 C overnight to precipitation the DNA.
5. DNA was recovered by centrifugation at high speed (14,000 rpm) for 10 minutes, a small pellet was seen at these steps. And then the precipitated DNA was washed using 70% cold ethanol.
6. The extracted DNA was suspended in 100 µl of sterilized double distilled water and kept for PCR reaction.

   (Abbasi et al.; 2013)

3.4- PCR and DNA amplification using universal 16s RNA microbiome primers:

The extracted DNA was used to detect possible bacterial species found in the tested clinical samples. This was done using standard 16s RNA primers(Illumina, 2013), (Table 3) that have the potential to amplify the V3/V4 region from the total 1400 bp complete bacterial rRNA gene.

Polymerase Chain Reaction (PCR):

All PCR were prepared in a total volume of 25µl(containing ready mix Taq DNA polymerase mixture)that included 5 pmol of each primer (about 0.5 µl of primer mixture) (Table 3), 5µl of DNA sample, and up to 25 µl pure sterile distilled water. All amplification reactions were hot started at 95°C for 3 min. The PCR thermo-cycling profile included 35 cycles of 95°C for 30 sec, 60°C for 30 sec, and 72°C for 60 sec, followed by a final step at 72°C for 10 minutes.
So, a total of 68 reactions were performed the 16s PCR system. It is very important to note that, each of the shown direct or reverse primers has addition of oligonucleotide sequence (known as Illumina adaptors) that is needed for next step of index addition and later used as a site for NGS sequence analysis (Table 3). A similar quantity (15µl) from all the obtained PCRs were used in preparing the NGS (Miseq) library.
The bellow table indicated the sequence of the primers that were used in this study:

Table (3): Direct and reverse primer sequences that were used for bacterial DNA amplification.

PCR system	Purpose	Primers*	Tm (°C)
16S rRNA	Microbiome	**Direct:** CCTACGGGNGGCWGCAG **Reverse:** AGGACTACHVGGGTATCTAATCC	**60**

Primers were linked to forward and reverse adaptor as indicated below:

- **Forward adaptor**: TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG
- **Reverse adaptor:** GTCTCGTGGGCTCGGAGATGTGTATAAGAGAC

3.5- Agarose Gel Electrophoreses:

At al-Quds university laboratory, the PCR products were run on a 1.5% agarose gel (1.5g agarose, 100ml 1X TAE and 10µlEthidium bromide). The 50X TAE electrophoresis running buffer (242g Tris base, 57.1ml glacial acetic acid and 100ml 0.5M EDTA (pH 8.0)). The Gene Ruler 100bp DNA ladder (**Thermo Scientific™**, #SM0371) was used as size marker to analyze the PCR amplified products.

3.6- NGS sequence analysis:

High throughput DNA deep sequencing using Illumina MiSeq platform:

For NGS sequence analysis the MiSeq DNA sequencing protocol from (Illumina, USA) was used as it is indicated in 16s Miseq microbiome sequence analysis. The exact protocol is called Nextera microbiome MiSeq DNA sequence protocol. (Abbasi et al., 2019).

For this purpose, the first PCR for 16s rRNA gene was applied for all samples, and it is worth to indicate that the used primers include two regions: the gene specific target primers (forward and reverse) which is about 20bp, that is linked to Illumina Nextera MiSeq adaptor sequences. The linked adaptors are used to link for each individual PCR product 8bp specific sequence index from both, that is later will be treated as dual indices and they will be utilized for specific sample identification.

Preparation of DNA Template

In order to enable identification of individual sequences of different DNAs in pooled samples i.e., urine, CSF dual barcode sequences were added to the ends of all DNA amplicons. These sequences, known as indices, allow for the identification of individual amplicons and their respective sequences for each sample. A unique multiple indices are used for each different urine and CSF. Sample barcodes (indices) were added by a second PCR system (the thermo-cycling profile was only for 8 cycles and this only to add the indices to both 16sRNA amplicons). Figure 2, shows the primers' regions used in this MiSeq analysis. For the addition of these primers and indices two PCRs were carried out: the first one as indicated above to

amplify bacteria, and the second PCR as it is indicated below was used for the addition of the dual bar code indices.

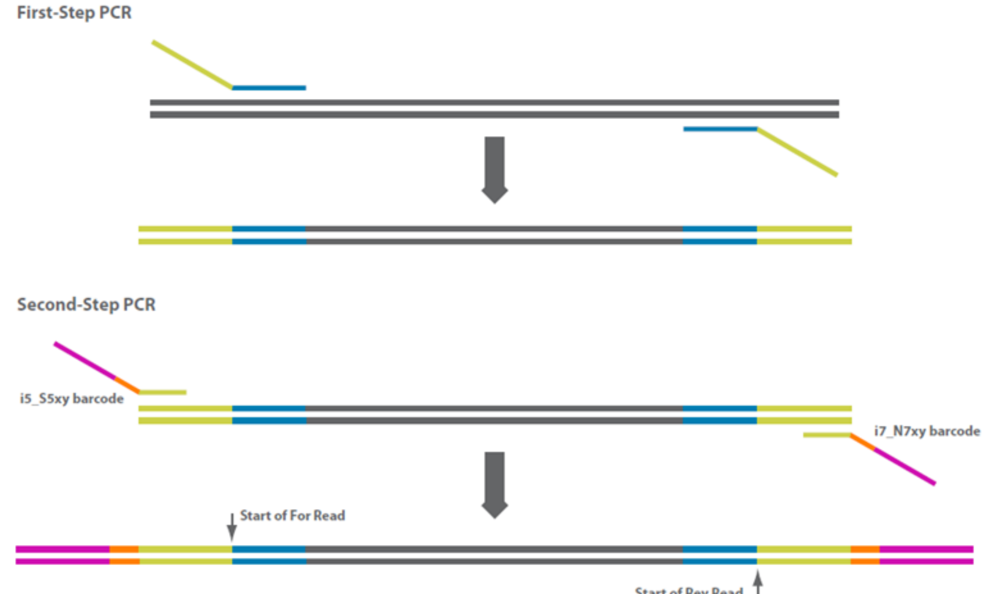

Figure (2): Overview of the double index strategy used in Illumina two-step protocol.

After samples labeling with dual indices, all samples will be collected in one tube and to be sequences by Illumina NGS MiSeq sequencing machine. Sequencing will be of paired-end in which we will sequence 250bp from each side.

Preparation of MiSeq DNA amplicon library:

The library consists of a total of 68 PCR products of clinical samples. The amplified amplicons (50 urine and the 14 CSF samples, and a 4 pools of negative controls) were purified using magnetic beads method as indicated below and were pooled in one tube named (NGS MiSeq library). The NGS sequence analysis was done as outsourcing service via one of the local biotechnology companies that use Miseq machine using 500 cycle kit from Illumina Co.).

1. **PCR products pooling and cleanup using AMPure XP beads (magnetic beads):**

   The importance of this step is to remove un-incorporated nucleotides, primers and salts using highly efficient magnetic beads purification kit (AMPure XP beads kit / Beckman coulter, USA). The following purification protocol was used:

- To each tube, containing the 25 µl of the of PCR products, add 15 µl of AMPure XP magnetic beads and mix well.

- Keep the mixed components in the strips at room temperature for 5 minutes.

- Transfer the strips to 96 well magnetic plate stand, and leave for another 5 minutes until the beads attach to tube side. (DNA is supposed to bind to the magnetic beads).

- Remove solution by gentile pipetting making sure not to disturb the attached beads (if possible use the multichannel pipette).

- Add 200 µl of freshly prepared 70% ethanol and leave for 1 minute while strips are not in the magnetic plate.

- Return the strips to magnetic plate and let beads to attached to tube side (about 1 minute), and then remove the ethanol by pipetting.

- Repeat washing with alcohol one more time, and at the end of the second wash leave strips containing the beads without any ethanol on the magnetic plates for about 5 minutes to dry.

- Transfer strips to PCR tube strips holder, and then add 30 µl of double distilled water to elute bound DNA. Leave the beads in DDW for about 2-3 minutes.

- Transfer strips to magnetic plate tube and then pipette the eluted DNA (20 µl) into fresh tubes.

2. **<u>Final preparation of MiSeq pooled and barcoded sequencing library:</u>**

After addition of the indexes by the second PCR, all the reactions were purified using the AMPure XP magnetic beads protocol as indicated above. Then all eluted DNA was pooled in one tube and sent for NGS DNA sequence analysis.

7- **Bioinformatics and data analysis:**

Normally the recovered NGS data using the MiSeq machine, give two kind of files: a- the Fastq R1 and R2 data of all NGS samples and b- a summary file for all microbiome analysis given as Excel or Notepad data file. In this study we used the summary Excel classification file that was given for each samples and shows all possible found bacterial species in these samples. For microbiome identification the Illumina machine is using BLAST bioinformatics analysis tools, which is identifies homologous sequences by locating short matches between the two sequences being compared.(Donkor, 2014)

Chapter Four:

RESULT:

4.1- Results of classical identification culture method:

Table (4) depicting the bacterial species identified by classical culture methods for both urine and CSF clinical samples. In table (4.A), we showed the results of classical urine samples culture, 30 positive growth result samples, 15 no significant bacterial growth samples and 4 negative bacterial growth samples.

The most abundant bacterial species found by this method was shown to be Escherichia *coli*. Other species were also found (*Klebsiella, Enterococcus species, Strep Group B, Acinetobacter,* and *Proteus*). Although bacterial species was identified in some cases, but this is not absolute and it is assumption as only this specific species from that pathogenic genus is mainly found in human urine samples.

Similarly it was possible to identify some bacterial species in the examined 4 positive CSF while no bacterial species were identified in culture negative CSF samples. The bacterial species that was found in positive culture CSF samples were (*Coagulase negative staphylococcus, and Enterobacter spp*). The other samples were categorized as negative samples as there was no bacterial growth in the used culturing method.

Table (4): Bacteria species identified in collected urine and CSF samples by classical culture methods.

A) Urine sample result :

Number of sample	Sample type	Patients gender	Result	Evolution
1	Urine	Male	*E.coli -ESBL*	Positive
2	Urine	Female	*Enterococcus species*	Positive
3	Urine	Female	*Klebsiella spp*	Positive
4	Urine	Female	*E.coli –ESBL*	Positive
5	Urine	Female	*Enterococcus species*	Positive
6	Urine	Male	*E.coli*	Positive
7	Urine	Female	*E.coli*	Positive
8	Urine	Female	*E.coli*	Positive
9	Urine	Male	*Strep Group B*	Positive
10	Urine	Female	*Strep Group B*	Positive
11	Urine	Female	*Lactobacillus spp*	Positive
12	Urine	Male	*E.coli*	Positive
13	Urine	Female	*Klebsiella spp*	Positive
14	Urine	Female	*E.coli*	Positive
15	Urine	Female	*E.coli*	Positive
16	Urine	Female	*Strep Group B*	Positive
17	Urine	Female	*Acinetobacter baumanii*	Positive
18	Urine	Male	*E.coli*	Positive
19	Urine	Male	*Enterococcus species- ESBL*	Positive
20	Urine	Male	*E.coli*	Positive
21	Urine	Female	*Lactobacillus spp and Strep Group B*	Positive
22	Urine	Male	*Enterococcus species*	Positive
23	Urine	Male	*Enterococcus species*	Positive
24	Urine	Female	*Enterococcus species*	Positive
25	Urine	Male	*Enterococcus species*	Positive
26	Urine	Male	*E.coli*	Positive

27	Urine	Male	*Klebsiella oxytoca*	Positive
28	Urine	Female	*E.coli*	Positive
29	Urine	Female	*Proteus mirabilis*	Positive
30	Urine	Male	*E.coli*	Positive
31	Urine	ND	No significant growth	Negative
32	Urine	ND	No significant growth	Negative
33	Urine	ND	No significant growth	Negative
34	Urine	ND	No significant growth	Negative
35	Urine	ND	No significant growth	Negative
36	Urine	ND	No significant growth	Negative
37	Urine	ND	No significant growth	Negative
38	Urine	ND	No significant growth	Negative
39	Urine	ND	No significant growth	Negative
40	Urine	ND	No significant growth	Negative
41	Urine	ND	No significant growth	Negative
42	Urine	ND	No significant growth	Negative
43	Urine	ND	No significant growth	Negative
44	Urine	ND	No significant growth	Negative
45	Urine	ND	No significant growth	Negative
46	Urine	ND	No growth	Negative
47	Urine	ND	No growth	Negative
48	Urine	ND	No growth	Negative
49	Urine	ND	No growth	Negative
50	Urine	ND	No growth	Negative

B) Results of CSF culture method.

Number of sample	Sample type	Patients Gender	Result	Evaluation
51	CSF	*Female*	*Enterobacter spp-CRE*	Positive
52	CSF	*Female*	*Coagulase Neg.Stap*	Positive
53	CSF	*Female*	*MRSA*	Positive
54	CSF	*Female*	*Coagulase Neg.Stap*	Positive
55	CSF	ND	No growth	Negative
56	CSF	ND	No growth	Negative
57	CSF	ND	No growth	Negative
58	CSF	ND	No growth	Negative
59	CSF	ND	No growth	Negative
60	CSF	ND	No growth	Negative
61	CSF	ND	No growth	Negative
62	CSF	ND	No growth	Negative
63	CSF	ND	No growth	Negative
64	CSF	ND	No growth	Negative

4.2- PCR amplification:

a. 16s **rRNA Microbiome PCR:** Successful amplification was tested each time on 1.5% agarose gel electrophoresis for each individual PCR reaction. It is worth to mention that the 16s rRNA microbiome analysis is a well-established and optimized from many other previous studies. Figure 3 showed a represented agarose gel electrophoresis results of the bacterial 16s rRNA PCR system for the 2 different clinical samples after extracted DNA. A successful DNA amplification was obtained from all examined samples.

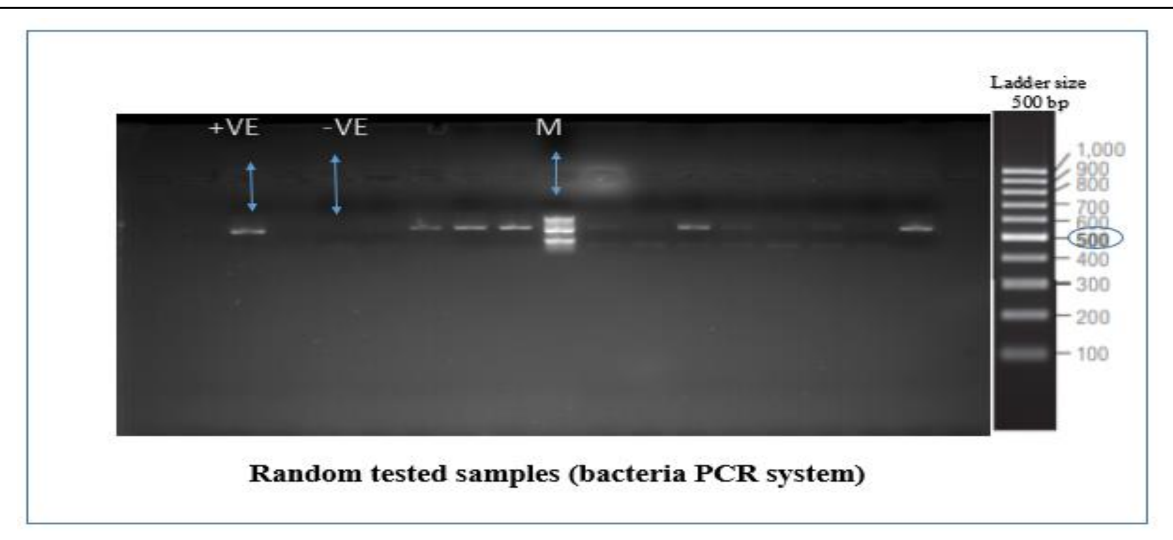

Figure (3): Agarose gel electrophoresis analysis of amplified microbiome 16s rRNA From random clinical samples collected over the study period.

Similar results of agarose gel electrophoresis for larger number of samples also could be seen in figure 4. In fact most of the samples showed successful amplification of the bacterial DNA using the 16s rRNA PCR system. The samples that showed negative results were of the negative controls or the samples that clinically were classified as no growth following classical identification methods and growing on agar plates.

Since there will be further sequencing analysis to be done after this first PCR, then the PCR results are not necessarily needed to be analyzed on agarose gel electrophoresis. Also, later and once similar quantities are used from each samples for further NGS sequence analysis this will show a quantitative results that is needed for comparison purposes and also could clinical determine the significant of this current bacterial infections. All of this taking into account that the NGS is very sensitive method and only about 1 μl of the PCR products is needed for further sequence analysis.

The bands that are seen in this gel are mainly of different bacterial species, and the main bacterial species that cause the current infection will the most representative and this only can be revealed after DNA sequence analysis of all the amplified DNA amplicons. This sequencing problem only could be resolved by NGS DNA sequence analysis.

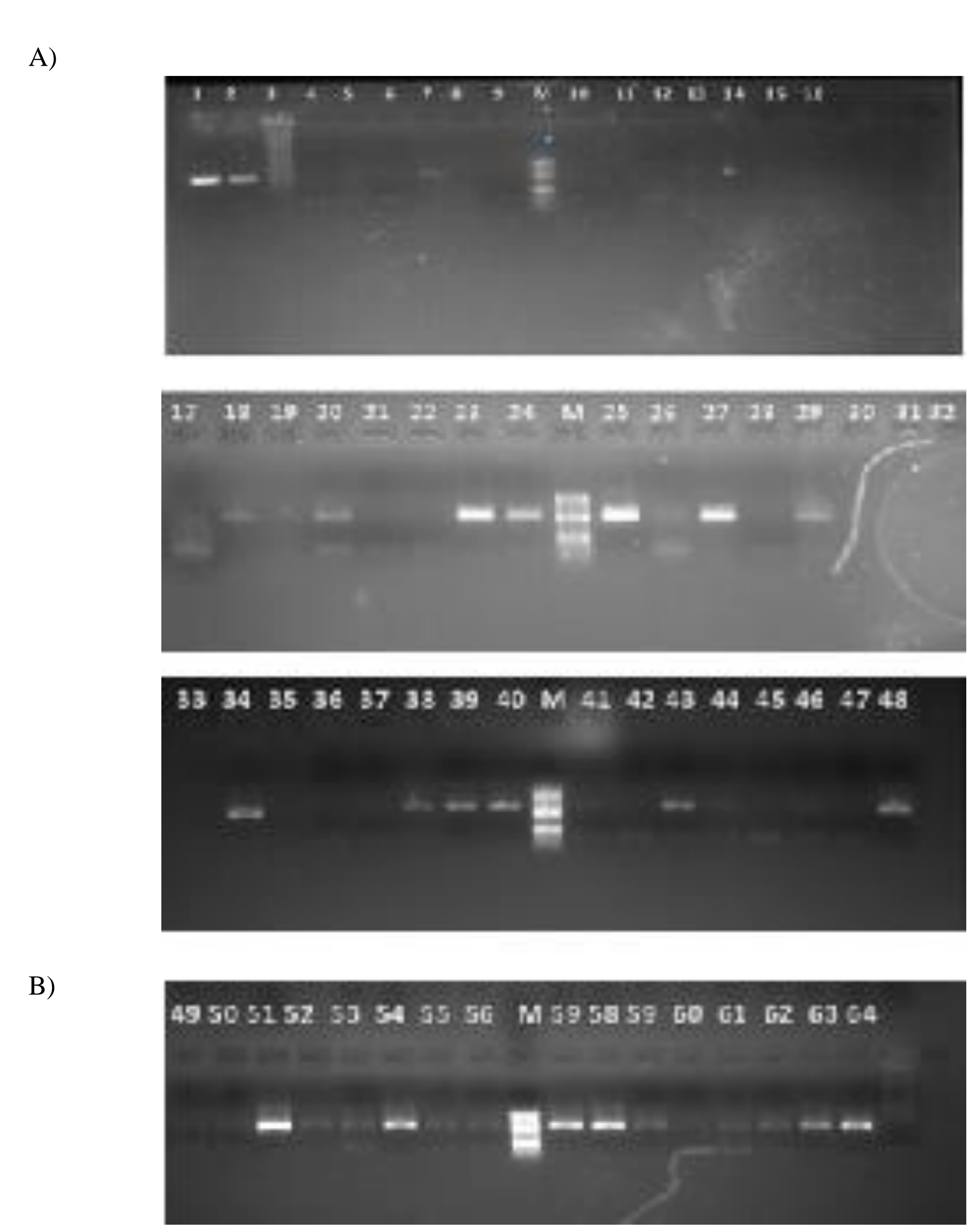

Figure (4): Agarose gel electrophoresis analysis of amplified microbiome 16s rRNA , From urine samples. A) Urine samples from (1-30 positive growth,31-45 no significant growth and 46-50 negative growth) and B)Cerebrospinal fluids from (51 -54positive growth and 55-64 negative growth), M 100bp size marker.

3.3 NGS microbiome analysis: results from urine and CSF samples (species and genus specific observations):

As it was indicated in material and methods, the NGS results could be obtained as an excel-sheet that include all the taxonomic classification of the found microbiome. The sheet includes classification at all the taxonomic levels. In this study we chose to look at the genus and species taxonomic classification data for each of the analyzed sample.

To make the comparison easier between classical and NGS analysis we grouped the obtained NGS data into five different groups: 1- Urine positive samples, 2- Urine samples with no significant growth, 3- Urine samples that showed negative growth in agar plates, 4- Positive CSF (growth on agar plates), 5- CSF negative samples (no growth on agar plates). The below histograms and the tables in the appendix show that genus and species microbiome data in these five groups.

We here demonstrated the NGS results obtained from the machine (as an excel-sheet) by transforming the raw data of FASTQ files into FASTA files, and this to obtain the exact number of total amplicons reads for each separate analyzed samples and representing them as the table, first is a bacterium genus, second is a bacterium species, then ordered by the number of maximum obtained reads and the frequency in the clinical samples as indicated in the above urine and CSF groups. Tables of most frequent bacterial species and those with most abundant were presented as histogram figures, while the exact tables used for construction of these figures are present in the appendices (Table 5- 14).

Figure (5): shows a histogram draw for positive infected urine amplicon reads representing bacteria amplified DNA fragments from it. The first histogram shows the distribution of bacterial DNA amplicons reads per sample, the vertical line represents two colors, the blue line showing the most abundant reads of bacterial genus per sample, and the orange line showing of the number of these bacteria in the samples. The highest numbers of reads were shown for the *Enterococcus* genus near 2455 reads in 13 samples from 30 samples (table (5)), which are among the most common nosocomial pathogens and causes important infections such as urinary tract and intra-abdominal infections, followed by the *Escherichia* genus, which is a facultative anaerobic genus and the most common pathogen causes UTI, which the number of pathogens present in the samples above the *Enterococcus* genus, about 15 from 30 samples. The second histogram shows the most frequently found bacterial genus, such as *Escherichia, Enterococcus* and *Klebsiella. Klebsiella* genus the most common uropathogenic bacteria causing UTI, the most abundant read was 30 amplicons from low frequent about 6 from 30 samples.

Figure (6): The highest number of reads (1732) was recorded for the most pathogenic bacteria (*Escherichia coli*), which is the most abundant bacterial species that can be detected in the urine culture samples and causes the UTI, especially in female, and it present in 15 samples from 30 positive infected urine samples. The *Enterococcus faecials* frequency in the positive urine samples were 8 amplicons per 30 samples. Then, the *Gardnerella vaginalis* caused vaginitis, although UTI and vaginitis are different, it's can present both at the same time. And it is a lowest abundant reads about 111 amplicons in 6 samples, it is present in the urine sample and maybe contaminated or poor hygiene.

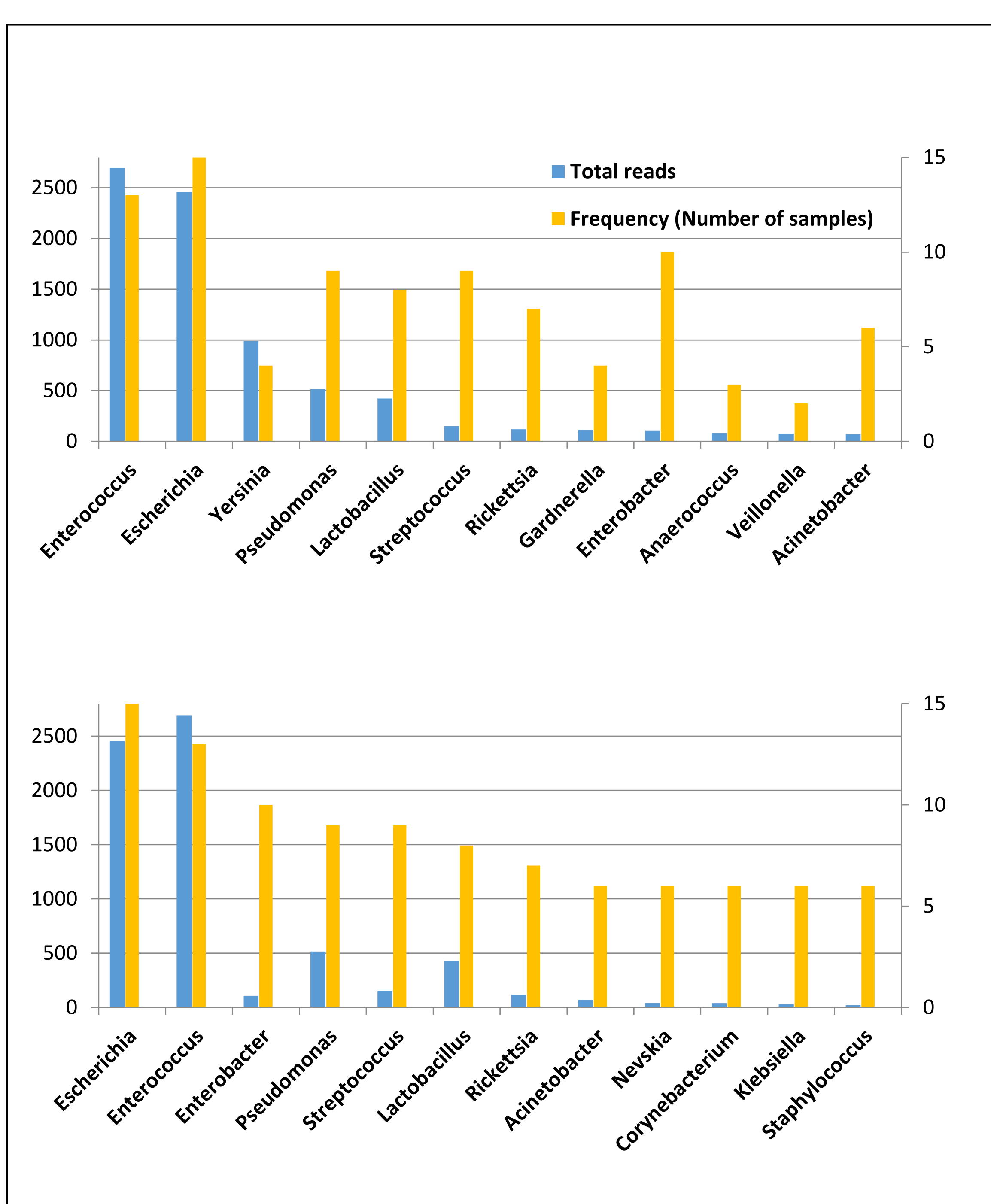

Figure 5: Histogram that represents the most occurred bacterial genus as they were obtained from positive infected urine samples. The above histogram shows bacterial genus arranged as the most abundant genus, and the lower represents the same results but according to the most frequently found bacterial genus.

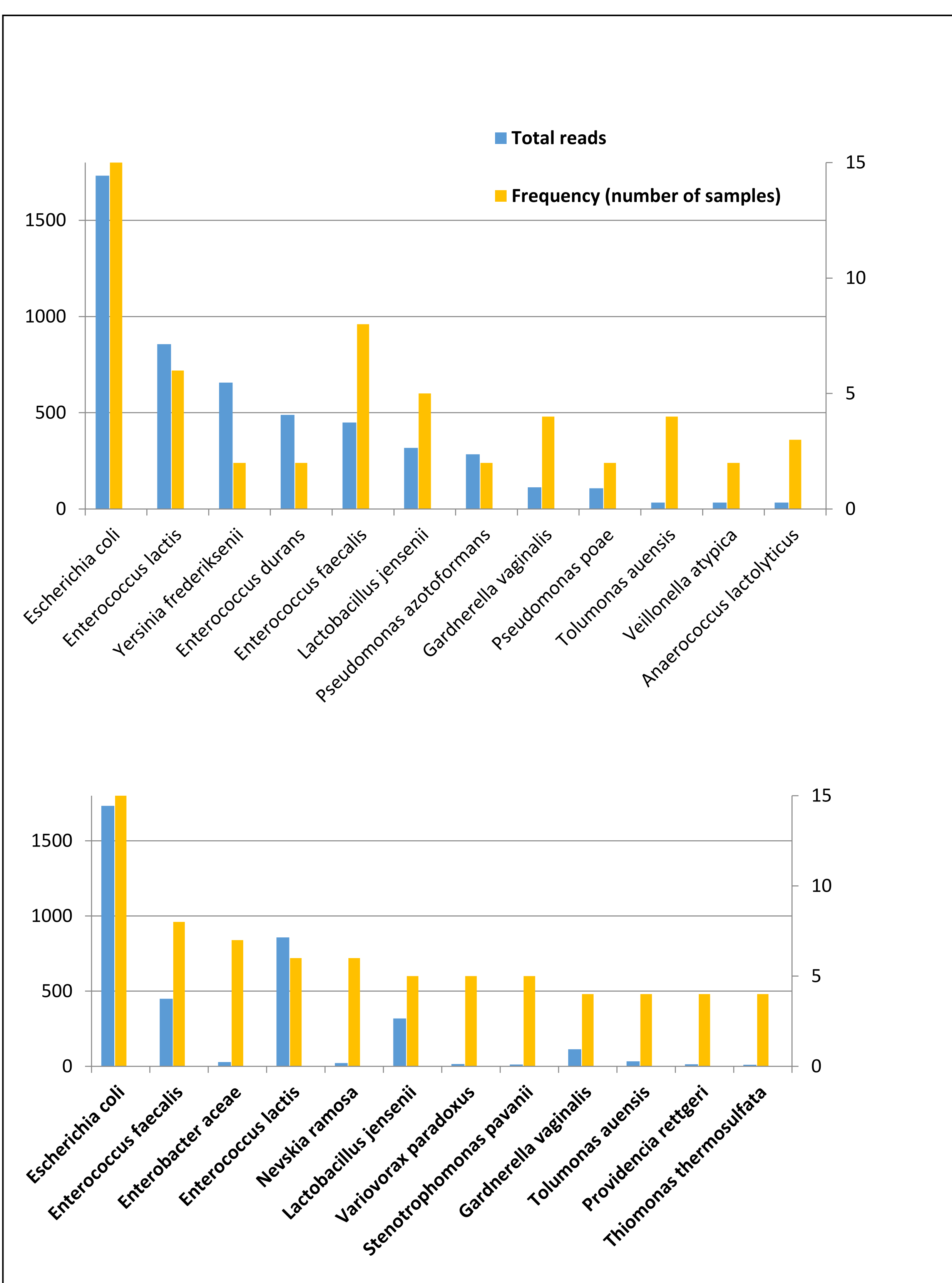

Figure 6: Histogram that represents the most occurred bacterial species as they were obtained from positive infected urine samples. The above histogram shows bacterial species arranged as the most abundant species and the lower represents the same results but according to the most frequently found bacterial species.

Figure (7): We here show the histogram from NGS results of the no significant growth urine samples. it is possible to see that the most abundant bacteria genus and that have the highest reads of amplicons in these samples were in this arrange: *Escherichia* (2626), *Enterococcus* (2150) then Pseudomonas(1178), and they are the pathogenic bacteria caused UTI, the no significant growth in the classical methods mean the count of bacterial cell less than 103 CFUs per ml in the urine culture (Giuliano et al., 2019)is associated with no infection , the NGS methods help to precaution stages of infected or a partially treated UTI.

The most frequent pathogenic bacteria genus are: *Escherichia, Pseudomonas, Enterococcus and Lactobacillus.* (Table (7)).

Figure (8): the highest recorded most abundant bacterial species in the histogram of no significant growth have classified as species it is an *Escherichia coli*, with the frequently (9 from 15) samples and its high frequency, NGS is more sensitive than the classical methods and can detect in one cell per sample. And it is the most frequent species, maybe the sample collected at a very early stage, with no signs and symptoms, therefore in culture cannot be detected.

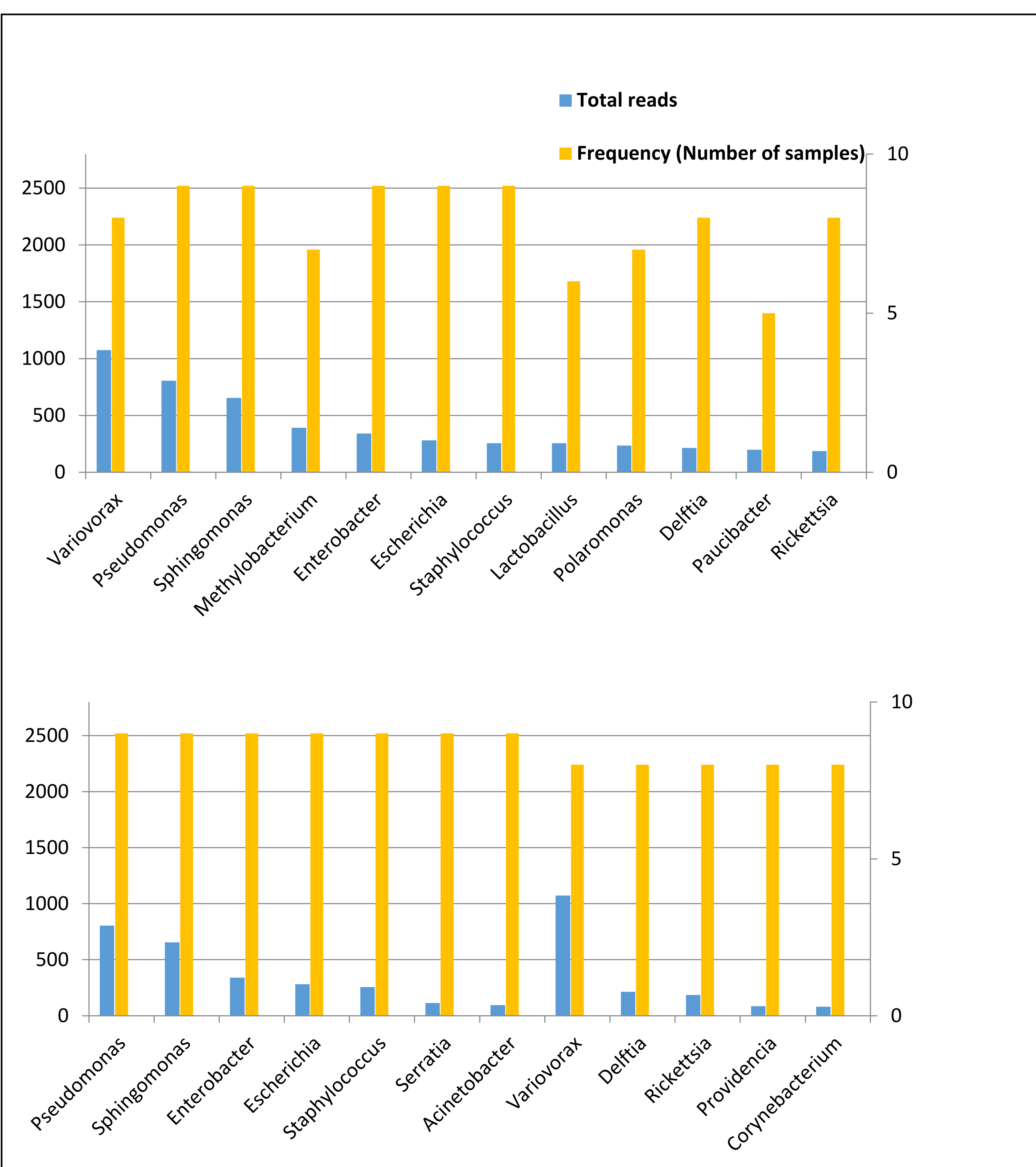

Figure 7: Histogram that represents the most occurred bacterial genus as they were obtained from urine samples that showed no significant bacterial growth in classical method. The above histogram shows bacterial genus arranged as the most abundant genus, and the lower represents the same results but according to the most frequently found bacterial genus.

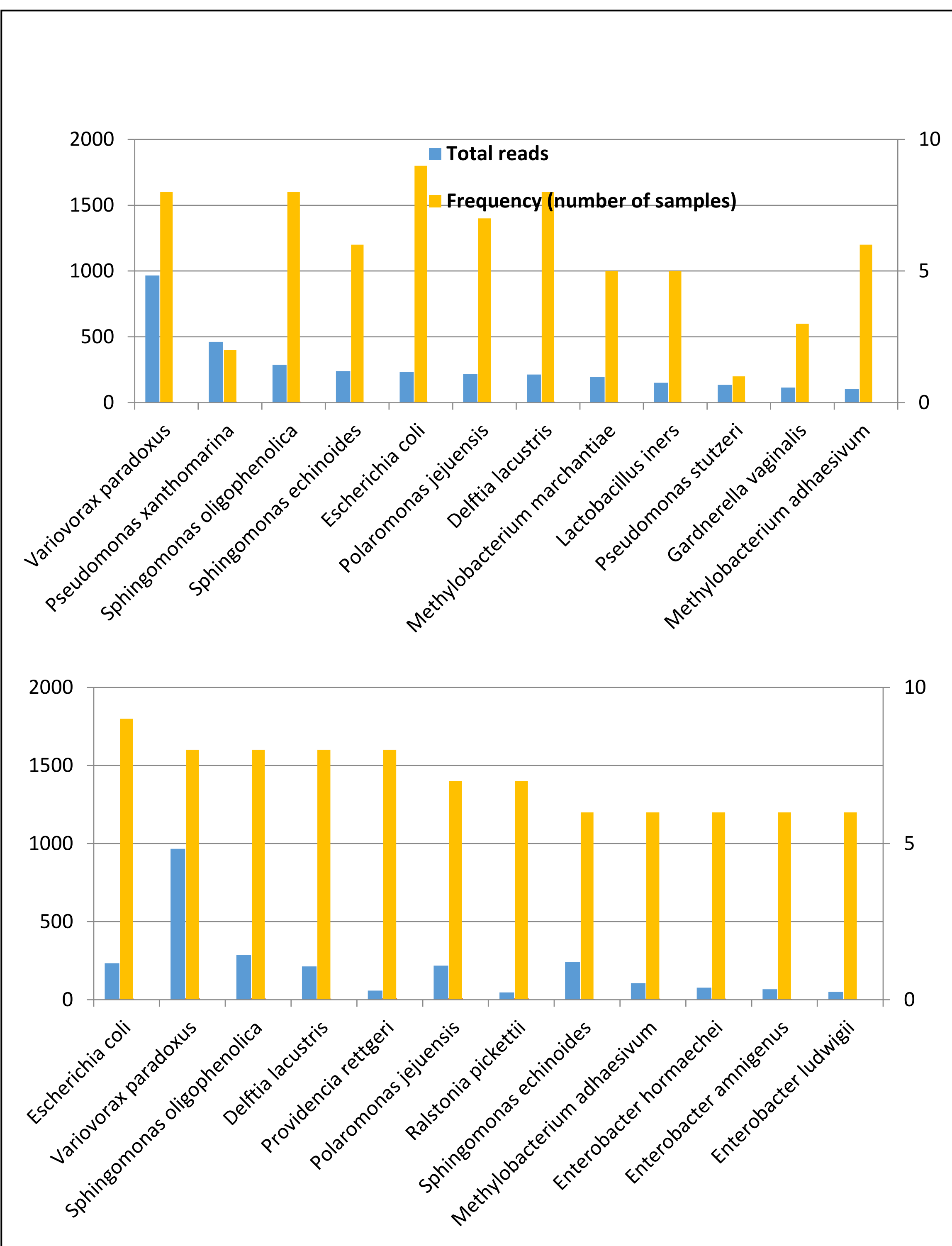

Figure 8: Histogram that represents the most occurred bacterial species as they were obtained from urine samples that showed no significant bacterial growth in classical method. The above histogram shows bacterial genus arranged as the most abundant species, and the lower represents the same results but according to the most frequently found bacterial species.

Figure (9): The most common bacteria genus obtained by NGS DNA sequence analysis from urine samples that showed no bacterial growth.

The *staphylococcus* is the most abundant read at 578 with the frequency is 2 from 5 negative samples.

The *Klebsiella* read was 381 , the frequency was 4 from 5 negative samples, and *Lactobacillus* read 194 and the frequency 2 from 5 negative samples. Table(9)

These read it is a very low comparing with positive result reads in the NGS, and these sample cannot gives positive growth in the plate on the classical methods (culture method) , some of these bacteria are facultative anaerobic or fastidious, it is need appropriate condition like the human body to grow up .

Figure (10): The most common bacterial species was obtained by NGS DNA sequence analysis from urine samples that showed no bacterial growth in classical method. Bacterial classification based on species.

Klebsiella variicola is a gram negative bacillus, facultative anaerobic, a total read is 244, the frequency is 3 from 5 negative samples. An *Escherichia coli* is the most frequency species, is 4 from 5 negative samples. Table (10)

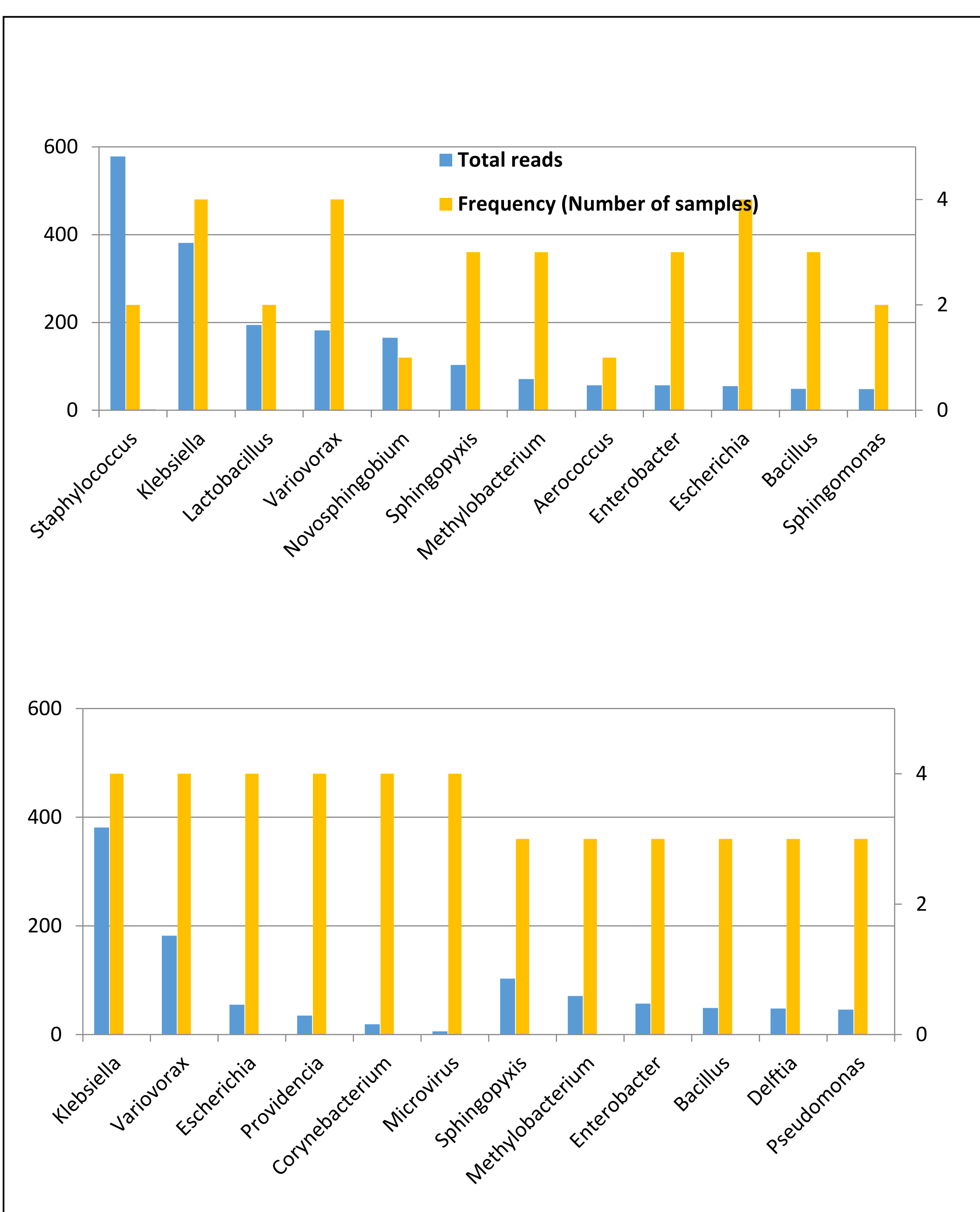

Figure 9: Histogram that represents the most occurred bacterial genus as they were obtained from urine samples that showed no bacterial growth in classical method. The above histogram shows bacterial genus arranged as the most abundant genus, and the lower represents the same results but according to the most frequently found bacterial genus.

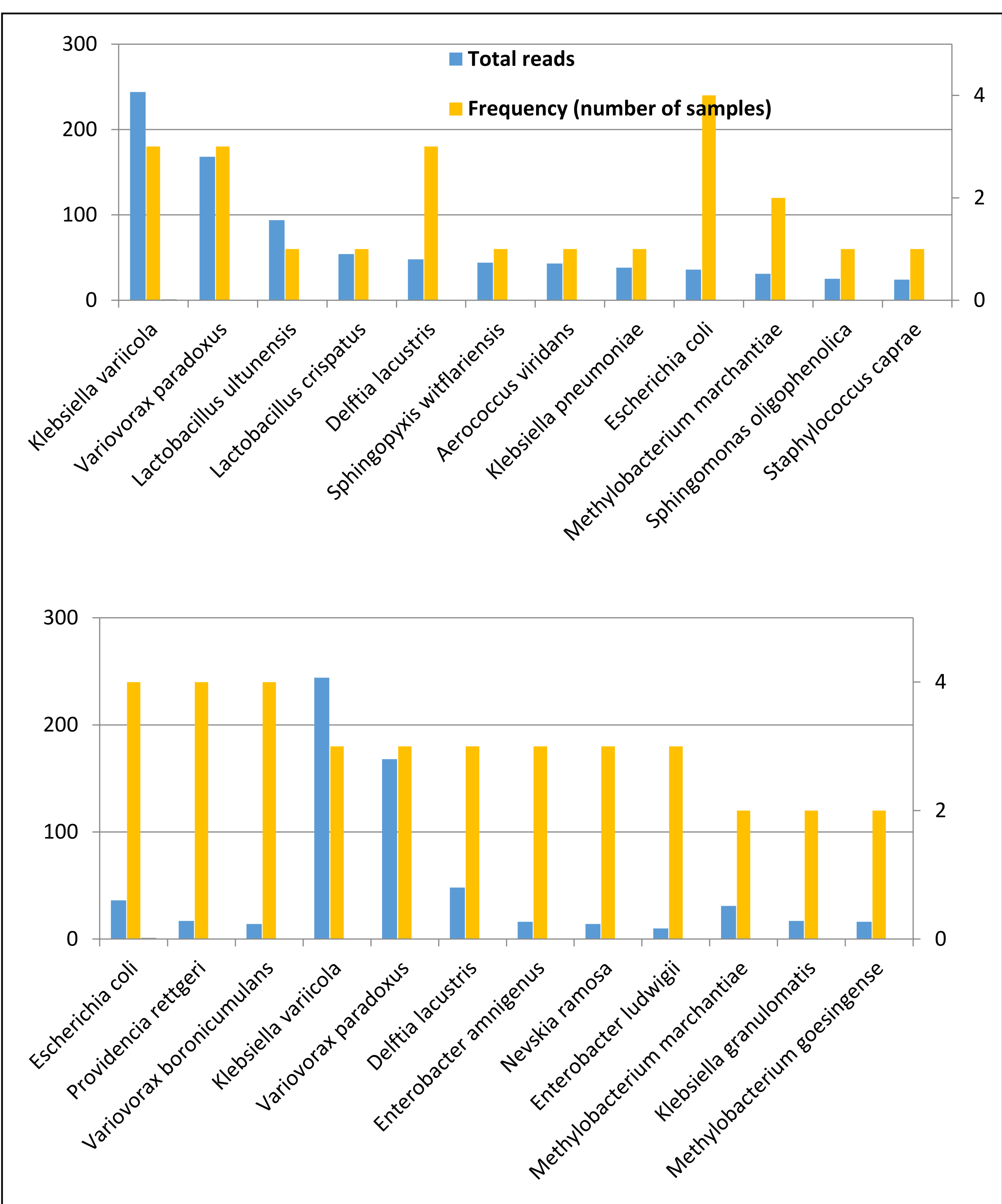

Figure 10: Histogram that represents the most occurred bacterial species as they were obtained from urine samples that showed no bacterial growth in classical method. The above histogram shows bacterial species arranged as the most abundant genus, and the lower represents the same results but according to the most frequently found bacterial species.

Figure (11): We show the most abundant bacterial genus obtained by NGS DNA sequence analysis from CSF samples with positive bacterial growth. *Staphylococcus* genus, is a gram-positive bacteria, facultative anaerobic and causes meningitis after a complication of surgery or as infected from the bloodstream. The frequency of these bacteria is 3 from 4 positive samples. Table (11), the NGS can detected them, and in culture method was detected.

Figure (12): In this histogram, we showed the most abundant bacterial species obtained by NGS DNA sequence analysis from CSF samples of positive bacterial growth. The most abundant bacteria is a *Staphylococcus haemolyticus* with a read of 664 and frequency 2 from 4 with positive growth sample. Table (12) these bacteria cause severe infection in many organs and causes meningitis.

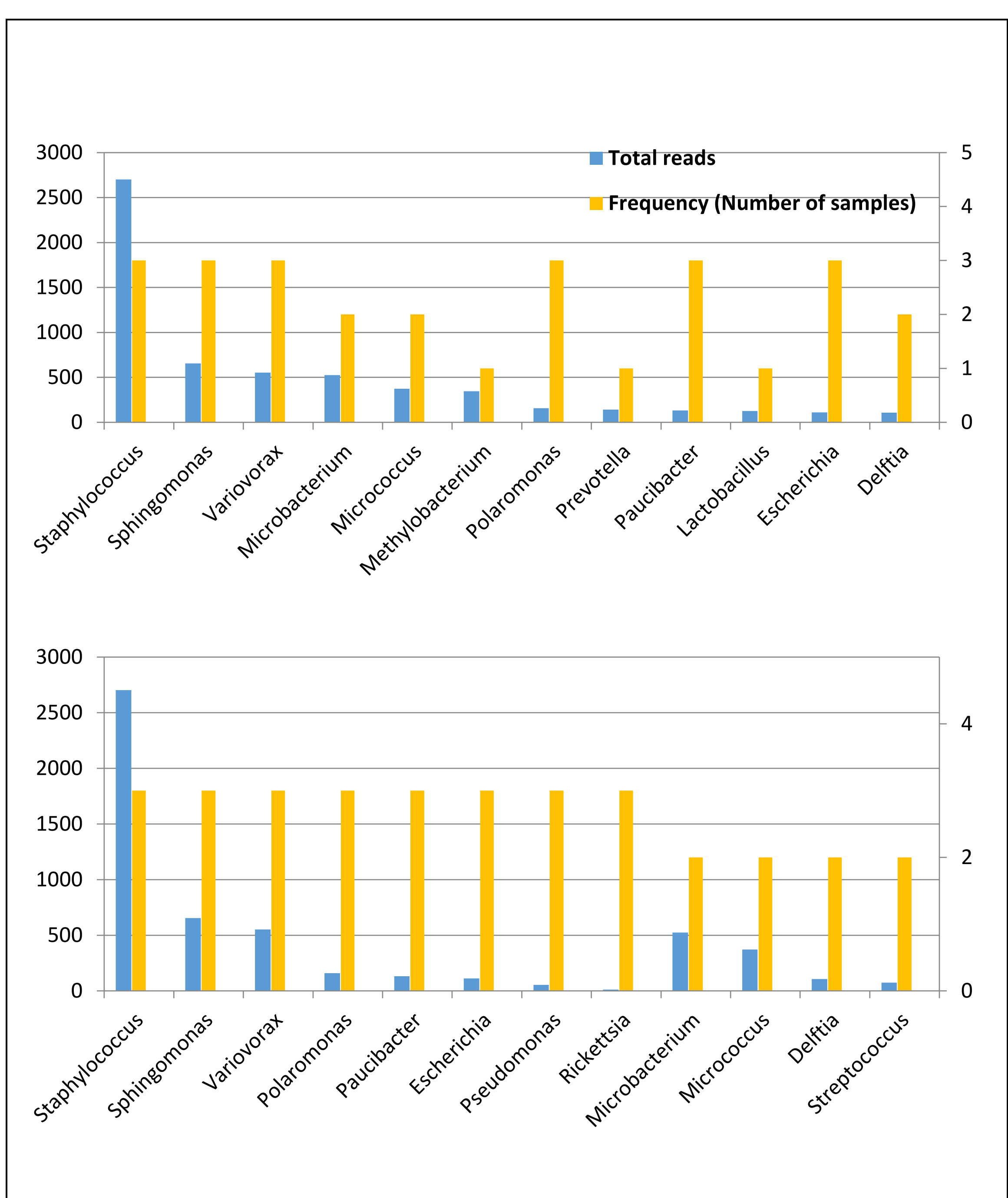

Figure 11: Histogram that represents the most occurred bacterial genus as they were obtained from CSF samples positive bacterial growth in classical method. The above histogram shows bacterial genus arranged as the most abundant genus, and the lower represents the same results but according to the most frequently found bacterial genus.

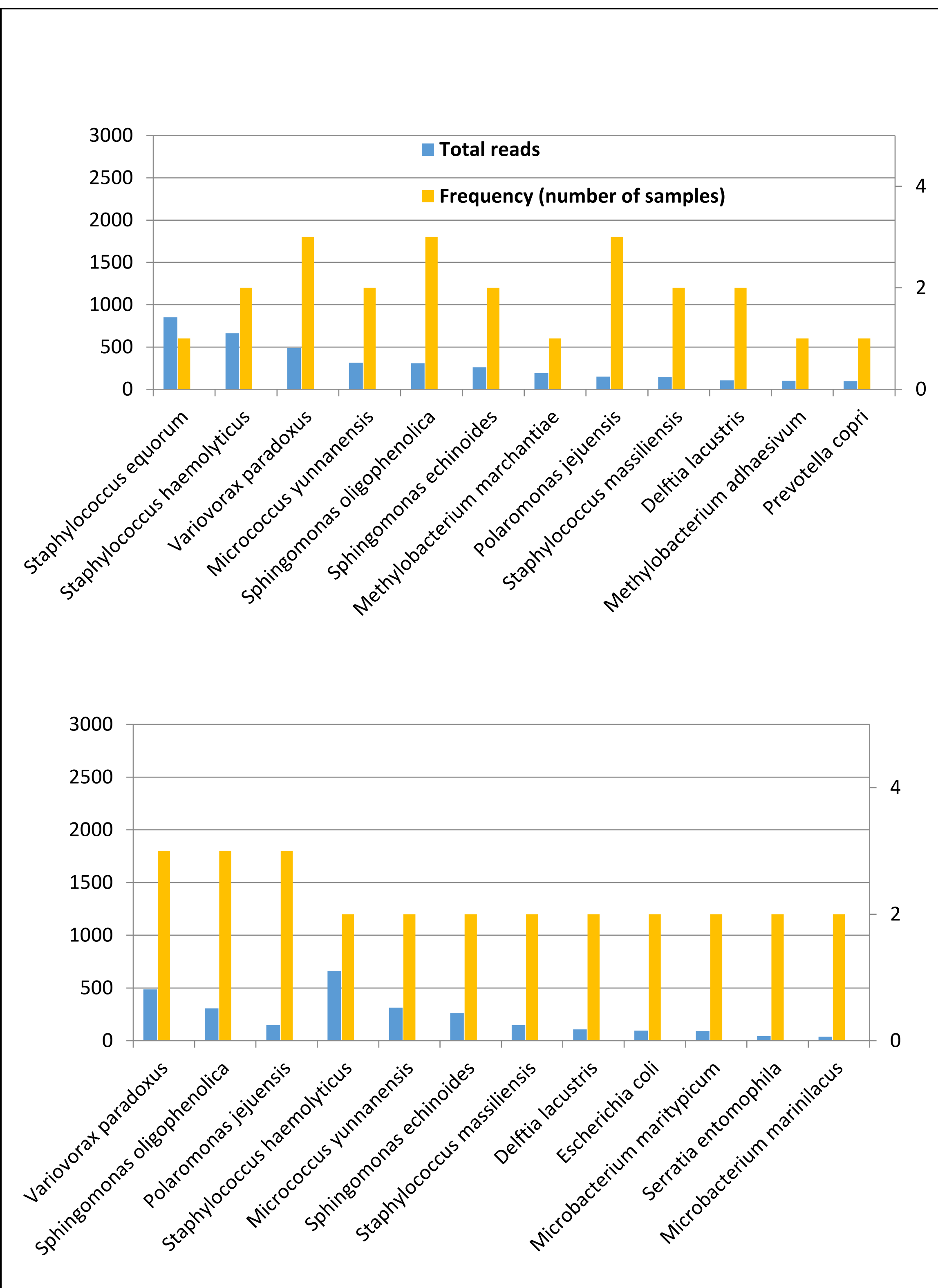

Figure 12: Histogram that represents the most occurred bacterial species as they were obtained from CSF samples positive bacterial growth in classical method. The above histogram shows bacterial genus arranged as the most abundant species, and the lower represents the same results but according to the most frequently found bacterial species.

Figure (13): in this histogram, we showed the most abundant bacteria obtained by NGS DNA sequence analysis from CSF samples with no bacterial growth. Bacterial classification has based on a genus.

The bacterial genus is: *Pseudomonas* 805 read, *Enterobacter* 340 read, *Escherichia* 282, *Staphylococcus* 255 read and *Rickettsia* 187 read (Table (13)).

And the second histogram, we were showed the most frequent pathogenic bacterial genus as *Enterobacter, Pseudomonas, Escherichia and Staphylococcus*. All of these bacteria causes meningitis.

Figure (14): The most common micro-organisms obtained by NGS DNA sequence analysis from CSF samples, showed negative bacterial growth. Bacterial classification based on species.

Escherichia coli only the species was 234 read, and most frequent *E.coli* is one of the most common neonatal meningitis causes, and it's rare to cause meningitis in an adult. Table (14)

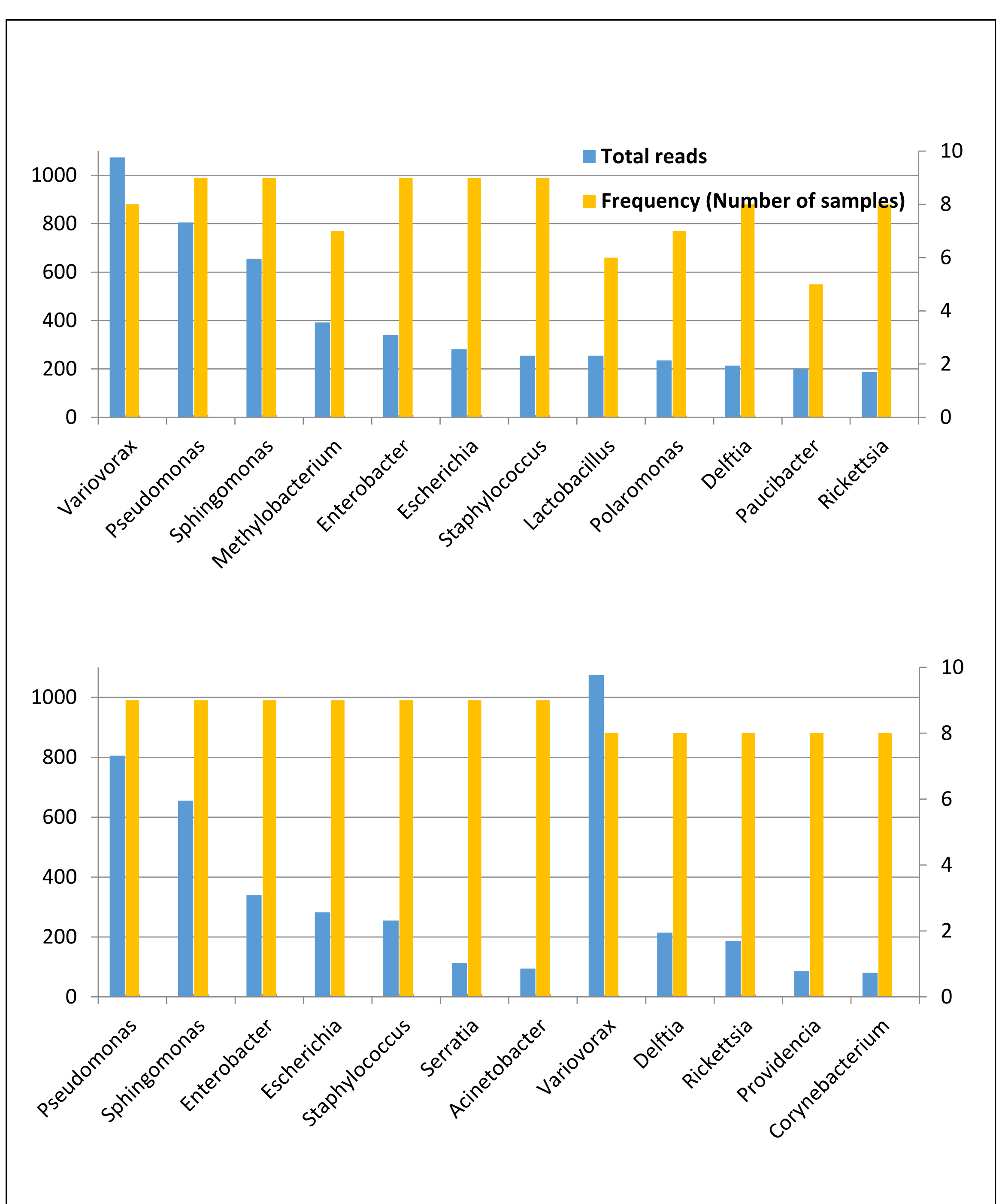

Figure 13: Histogram that represents the most occurred bacterial genus as they were obtained from CSF samples negative bacterial growth in classical method. The above histogram shows bacterial genus arranged as the most abundant genus, and the lower represents the same results but according to the most frequently found bacterial genus.

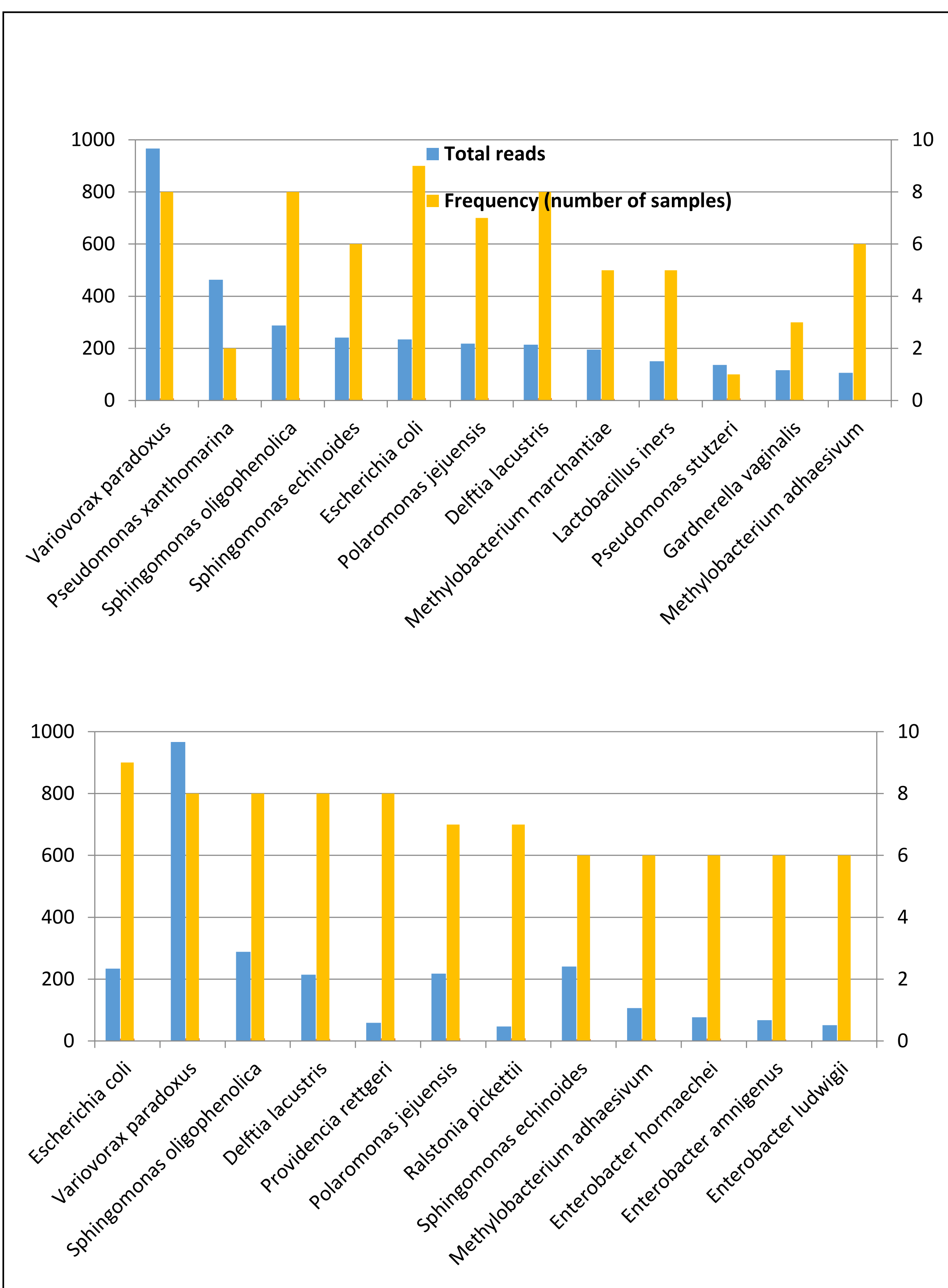

Figure14: Histogram that represents the most occurred bacterial species as they were obtained from CSF samples negative bacterial growth in classical method. The above histogram shows bacterial species arranged as the most abundant genus, and the lower represents the same results but according to the most frequently found bacterial species.

Chapter Five: Discussion

Microbes affect all life in our plants and are determinants of human health, a microbiology science is central to studying life as other science because it is study microorganisms, their structure, shape and other properties.

Clinical microbiology (diagnostic microbiology), focuses on the association with the diagnosis of human infectious diseases, by isolation the microorganisms from human clinical specimens, choosing the specific tests to identify the pathogens and determining the sensitivity or resistance of it's to the drugs. The results of these tests to assist the physicians in the diagnosis, treatment and controlled the infectious diseases.

The gold standard for diagnosing bacteria is a culture method.(Baspinar et al., 2017) Cultural methods are essential for both the study and diagnosis of microbial-mediated bio medication, yet the proportion and identity of the microbial community in the clinical samples that are cultivable have remained difficult and unclear for some pathogens (especially bacteria causes the disease). This is time-consuming for the detection of slow-growing or fastidious pathogens, leading to increase patient morbidity and mortality, usage a broad-spectrum antibiotic and the ability of transmission the pathogen to new patients, especially in the hospital (nosocomial infection).(Srinivasan et al., 2015)

The development of a methodology that allows for the thorough examination of clinical samples containing a microbiome, we suggest that both culture-dependent and culture-independent methods complement one another in diagnosing the microbiome.

Nowadays, bacteria can be identified via a sequence of nucleotides from the 16S rRNA gene, which is a short, conserved gene-specific to bacterial genus (96%) and some species (87.5%), PCR is a method for bacterial identification, culture-independent and only requires the DNA of the tested bacteria. In addition, it's used to determine bacterial pathogens from clinical samples directly were obtained from the patients. The 16S rRNA gene sequencing-based approach is helpful to avoid potential culture-related biases in pathogen identification in cases of culture test results.(Muhamad Rizal et al., 2020)

There are several critical limits to PCR. Our abilities to design oligonucleotide primers are limited to our understanding of a microorganism's genome and the ability of publicly - available

sequence databases to represent all variations of that bacterium. It is typical for microbial genomes to have unforeseen mutations that decrease or eliminate the function of a PCR. False-positives related to carrying contamination have traditionally created significant problems in the routine use of PCR in clinical laboratories, leading to numerous requirements for the design of laboratories specialized in performing PCR.(Mackay, 2004)

Next-generation sequencing (NGS), and it called a high-throughput or massively parallel sequencing, it is a type of technology that allows for the simultaneous and independent sequencing of hundreds to billions of DNA fragments. The uses of NGS in clinical microbiological testing are numerous, allowing for an impartial method of pathogen identification. NGS analysis in the microbiology laboratory includes series 1-clinical sample processing, 2-library preparation, and 3-sequencing stages. This is followed by bioinformatics analysis and NGS data interpretation in the computational lab.(Gu et al., 2019)

MiSeq NGS may also be used in metagenomics research, which analyzes a wide range of genetic information from multiple species originating from a single sample. Additionally, NGS gives all quantitative and qualitative data of every amplicon species while removing the time-consuming cloning processes necessary for classical sequencing. The outcomes include a profusion of sequencing for each amplicon, indicating its abundance in the original sample.(Abbasi et al., 2019).

Using NGS technology instead of Sanger's traditional approach minimizes the cost of DNA sequencing by carefully considering the massive number of samples that can be analyzed at once and avoids time consuming and difficult traditional cloning operations for sequencing every single amplified DNA fragment. The major advantage of NGS technology is its highly sensitive and precision in sequencing.(de Paz et al., 2018; Zhu et al., 2017) Because it provides sequence information for multiple DNA molecules that use the same, it may efficiently provide a large amount of information on short sequence repetitions or single nucleotide polymorphism (SNP). Using the NGS sequencing technique, universal primers that amplify a group of microorganisms, such as the 16S rRNA genes for bacterial species, may be used.(Salipante et al., 2013).

The results obtained will include a thousand numbers of sequences from each sample, classified as genus and species.

NGS has helped the resolution of this constraint and has dramatically improved our understanding of entire microbial communities, enhancing our understanding of microbial ecology

and microbiology in general. The restrictions and boundaries associated with traditional culture-based techniques have been overcome by next-generation sequencing (NGS). NGS technology has allowed researchers to explore a wide range of microbiomes in their natural habitats, including the human skin microbiome, human microbiome, and other microbiomes. The identification of endosymbiotic bacterial phyla, human disease pathogens associated with epidemics, bacteria and viruses related with inflammatory bowel illnesses, and the identification of commensal gut bacteria are just a few of the novel discoveries made possible by NGS.(Malla et al., 2018)

NGS analysis has a significant advantage over sensitivity, and it is a quantitative method. The significance of these parameters was established in the identification of microbiome genus and species in urine and CSF samples.

The benefit of using NGS technology is related to the method's ability to sequence every single amplicon; on the one hand, it can identify any mixed pathogenic organisms, such as mixed bacterial infections, by sequencing the unique 16s rDNA gene; on the other hand, it will provide the number of amplified amplicons.

The main disadvantage of NGS in the healthcare situation is establishing the basic infrastructure, such as computer capacity and storage, as well as the personal knowledge that are required to thoroughly analyze and interpret the resulting data. Furthermore, the volume of data must be managed expertly in order to retrieve therapeutically essential information in a clear and robust interface, the price is still high for routine diagnostics purposes, and for bacterial pathogens, information in these databases is rich, whereas data for fungal pathogens are insufficient, and absence of high-quality reference information can produce misleading results. (Gabaldón et al.; 2019)

In this study, we concentrated to identifying the bacterial species found in the clinical samples of the body fluids, these fluids were isolated from suspected patients, which is an important sample for detecting the infectious disease, and these samples could contain a pathogenic bacteria, viruses or other parasites, especially in the emergency units in the hospitals.

We focused on the bacterial 16S DNA isolated from the samples, the most abundant bacterial species present in the infected urine samples: *Escherichia coli, Staphylococcus saprophyticus, Klebsiella pneumoniae, Staphylococcus aureus, Enterococcus faecalis, Proteus mirabilis, Group*

B Streptococcus, Pseudomonas aeruginosa.(Flores-Mireles et al., 2015)**.** This pathogens affecting urinary tract and causing urinary tract infection.

The most common bacteria present in the cerebrospinal fluids: *Group B streptococci, Haemophilus influenzae, Neisseria meningitidis, Escherichia coli and Streptococcus pneumonia.* These infected the central nervous system and causes meningitis and other disease.

Meningitis is still a medical emergency that requires fast diagnosis and treatment to avoid death or significant neurological consequences. Meningitis is almost always caused by microbial organisms, primarily bacteria.

In this study, the results from two methods were interpreted and analyzed to compare them. The first clinical samples of bacterial urine infections were tested by culture-dependent method and culture-independent methods (PCR and NGS). The detailed results of 30 patients who were identified with bacterial infections by culture method were demonstrated in the table (4), PCR and gel electrophoresis were demonstrated in the figure (4), finally the NGS was done and the results were showed in the figure (5, 6) containing a histogram, the results have the same bacterial pathogens in culture method results and NGS, but the NGS present a new bacterial genus not present in culture method. Hence Table (5, 6) was lists the reads of bacterial pathogens and frequency of this bacteria in the samples. We showed in table (4) bacteria identified by the traditional culture method.

In gel electrophoresis present 12 bands from 30 positive growth urine samples, number of band it was less than the results from culture , maybe it the primer mismatch with the extracted DNA , then the PCR not amplify the segments of bacterial DNA or the concentration of sequence not enough to consist the bands and can appeared.

By comparison, *Enterococcus, Escherichia, Pseudomonas, Lactobacillus, Streptococcus, Rickettsia and Gardnerella,* pathogenic bacteria were detected through NGS technology. In addition, by the culture method *Escherichia, Klebsiella, Enterococcus, strep Group B, Lactobacillus, Acinetobacter and Proteus* were identified. Overall, were detected by *Enterococcus, Escherichia, Lactobacillus,* and *Streptococcus* in both classical culture and NGS.

The infections of *Gardnerella* were detected by NGS only. The *Klebsiella* genus was found in the infected Sample urine of the culture method, but not by NGS. The NGS method had a great advantage in the detection of bacteria, and the classical culture method cannot detect some bacterial pathogens currently. For example, *Gardnerella* has a specific cell wall, which is Gram-

positive and has Gram-negative properties, and it is difficult to detect through the culture method.(Sadhu et al., 1989).

According to (Table (5, 6) we showed the highest reads of most abundant bacterial genus and species. For example: the *Enterococcus* genus near 2455 reads in 13 samples from 30 samples from NGS. And present in 7 urine sample from 30 positive growth through the culture method.

The most abundant bacterial species is *Escherichia coli* (1732) was recorded for the most pathogenic bacterial in positive urine sample.

The second clinical samples of bacterial urine infections were tested by culture-dependent method and culture-independent methods (PCR and NGS). The detailed results of 15 patients who were identified with no significant growth by culture method were demonstrated in the table (4), PCR and gel electrophoresis were demonstrated in the figure (4), finally the NGS was done and the results were showed in the figure (7, 8) containing a histogram, the results were reported as no significant growth in culture method results, no pathogenic bacteria identity, but the NGS present a bacterial genus not present in culture method. Hence Table (7, 8) was lists the reads of bacterial pathogens and the frequency of these bacteria in the samples. With the no significant growth the colony count was less than 100,000 CFU/ml.

In-gel electrophoresis present 6bands from 15 no significant growth urine samples, several bands it was reported to detect a bacterial DNA, maybe it was due to the presence of the bacteria (dead or alive)the patient takes an antibiotic, the concentration of bacterial cell in the sample or it is could an early infection or recurrent infection.

The NGS result: *Enterobacter, Escherichia, Pseudomonas, Lactobacillus, Staphylococcus* and pathogenic bacteria were detected through NGS technology. The NGS method had a great advantage in the detection of bacteria with low concentration, and the classical culture method cannot detect it. The most abundant bacterial genus is Pseudomonas and has a read of 750 and the most frequent 10 from 15 samples. In the species classification, the *Pseudomonas* read near 400 and the most frequent is *Escherichia coli*. Both PCR and NGS helped in the detection of bacterial pathogens present in urine samples, in the classical method it is considered as no significant growth (no pathogenic growth), and the patient not treated and gives the treatment in some cases.

The third urine samples of negative growth were tested by culture-dependent method and culture-independent methods (PCR and NGS). The detailed results of 5 patients who were identified with no growth by culture method were demonstrated in the table (4), PCR and gel

electrophoresis were demonstrated in the figure (4), finally the NGS was done and the results were showed in the figure (9, 10) containing a histogram, the results were reported as no growth in culture method results, no pathogenic bacteria present, but the NGS present a bacterial genus not present in culture method. Hence Table (9, 10) was lists the reads of bacterial pathogens and the frequency of these bacteria in the samples.

In-gel electrophoresis present 4 bands from 5 no growth urine samples, several bands it was reported to detect a bacterial DNA, maybe it was due to the presence of the bacteria (dead or alive)the patient takes an antibiotic, low concentration of bacterial cell in the sample or it is could an early infection or recurrent infection.

The NGS result: *Streptococcus, Enterobacter, Escherichia, Lactobacillus, Klebsiella* and these pathogenic bacteria were detected through NGS technology. The NGS method had a great advantage in the detection of bacteria with low concentration or present the one cell of the bacteria, and the classical culture method cannot detect it. The most abundant bacterial genus is *staphylococcus* which has a read of 578 and the most frequent 2 from 5 samples. In the species classification, the *Klebsiella* read near 381 and the frequency is 4 from 5 negative samples.

Both PCR and NGS helped in the detection of bacterial pathogens present in urine samples, in the classical method it is considered as no growth, and the PCR, NGS have the same result.

It is the series bacterial identification in the cerebrospinal fluids, CSF is a sterile body fluid such as the blood, and many bacteria can infect the central nervous system and caused meningitis and septicemia.(Greisen et al., 1994)

The specific bacteria species were of most importance in causes the meningitis, which are: *Group B streptococci (agalactia), Haemophilus influenzae, Neisseria meningitidis, Escherichia coli* and *Streptococcus pneumonia, Mycobacterium tuberculosis* and *Listeria monocytogenes.*(Greisen et al., 1994; Overturf, 2005)

The CSF clinical samples were tested by culture-dependent methods and culture-independent methods (PCR and NGS). The detailed results of 4 patients who were identified with positive growth by culture method were demonstrated in the table (4), PCR and gel electrophoresis were demonstrated in the figure (4), finally the NGS was done and the results were showed in the figure (11, 12) containing a histogram, the results were reported as positive growth in culture method results, positive bacteria identity, but the NGS present a bacterial genus present in culture method.

Hence Table (11, 12) was lists the reads of bacterial pathogens and the frequency of these bacteria in the samples.

The *staphylococcus* genus was reported in the NGS and culture methods as *MRSA* and *Coagulase Neg.Stap.*

In-gel electrophoresis presented 4 bands from 14 CSF samples (positive and negative), a several bands it was reported to detect a bacterial DNA, maybe it was due to the presence of the bacteria (dead or alive)the patient takes an antibiotic, the concentration of bacterial cell in the sample or it is could an early infection or recurrent infection.

The culture method results: *Enterobacter, Methicillin-resistant Staphylococcus aureus (MRSA), coagulase-negative staphylococcus.*

The NGS results: *Staphylococcus haemolyticus.*

In two methods we can show the same bacterial genus from the positive CSF samples.

The most abundant bacterial genus is *Staphylococcus* with 2702 read, which a high read is and the frequent is 3 from 4 positive CSF samples. The *Staphylococcus haemolyticus* is the most abundant species and it is a member of the *Coagulase-Negative Staphylococci*, is present on the human skin and can cause meningitis after nosocomial neurosurgical infection.(Bryce et al., 2021)

The CSF clinical samples were tested by culture-dependent methods and culture-independent methods (PCR and NGS). The detailed results of 10 patients who were identified with negative growth by culture method were demonstrated in the table (4), PCR and gel electrophoresis were demonstrated in the figure (4), finally the NGS was done and the results were showed in the figure (13,14) containing a histogram, the results were reported as no growth in culture method results, negative bacteria identity, but the NGS present a bacterial genus not present in culture method. Hence Table (13,14) was lists the reads of bacterial pathogens and the frequency of these bacteria in the samples.

In-gel electrophoresis presented 13 bands from 14 CSF samples (positive and negative),7 light bands and 6 thick bands, these bands it were reported to detect a bacterial DNA, maybe it was due to the presence of the bacteria (dead or alive)the patient takes an antibiotic, the concentration of bacterial cell in the sample or it is could an early infection or recurrent infection.

The NGS results: *Pseudomonas, Escherichia, Staphylococcus, Enterobacter and Staphylococcus.*

The most abundant bacterial genus is *Pseudomonas* with 805 read and frequency 9 from 10 negative CSF samples. The *Escherichia* genus frequency is 9 from 10 negative CSF samples and the most abundant species is *Escherichia coli* with 234 reads and frequency 9 from 10 negative growth samples.

Our study proposed using NGS analysis with specified indices to establish a complete diagnosis technique for infectious pathogens.

In comparison to the classic culture technique, PCR and NGS technologies have substantial benefits in bacterial identification, demonstrating their exceptional therapeutic efficacy.

To detect bacterial pathogens, the detectability of PCR and NGS was higher than that of classic culture methods. PCR can detect the existence of bacteria without saying what type it is, the PCR limitation, including sequence length, primer binding capacity and GC nucleotide-content. The NGS approach is proposed to combine with the standard culture method in the diagnosis of infected patients due to the detective rate of the parallel diagnostic test. As a result of its ability to accurately and efficiently screen for many gene targets closely associated to infections, NGS technology can be of tremendous aid to the process of precision medicine in infectious diseases.(Chen et al., 2020)

NGS is a culture-independent technique, that used 16s rRNA which is a specific and conserved sequence in the bacterial DNA. This technique can impact the DNA extraction from the fastidious, slow-growing, dead or live bacterial cells present in the clinical samples.

It has a huge benefit for patients by discovering the most appropriate therapy, antibiotic resistance and the development of new diagnostic methods.

The NGS data need bioinformaticians with advanced skills in bioinformatics, to translate the results.

NGS technology has a wide application in diagnostic microbiology, starting from diagnostic the infectious disease to finding the therapeutic procedures. The future of NGS can detect rather than a single organism that causes the disease but can detect different types of organisms like bacterial, viral and fungal infections in the same run. (Gabaldón et al.; 2019)

The NGS can resolve all defects or biases and find the solutions for diagnostic the microbiome, and understanding the gap between two other techniques (culture (gold standard) and PCR).

We hope to develop microbiology labs in our country and used this technique.(Boers et al., 2019).

Appendices A:

Escherichia coli (E. coli); is a Gram-negative, rod-shaped, facultative anaerobic bacterium.

Enterococcus species; are a gram-positive cocci, catalase negative, usually facultative, anaerobic bacteria.

Group B Streptococcus (GBS) is a gram-positive bacterium, cocci in pairs and short chains on Gram stain.

Klebsiella species**:** The genus *Klebsiella* consists of non-motile, aerobic and facultative anaerobic, Gram negative rods**.**

Klebsiella pneumoniae is responsible for a variety of diseases in humans and animals and it is a prominent nosocomial pathogen mainly responsible for urinary tract, respiratory tract or blood infections.

Klebsiella oxytoca (K. oxytoca) is a rod-shaped, non-motile, Gram-negative bacterium with
A prominent polysaccharide capsule, which provides a resistance against host defense mechanisms.

Acinetobacter baumannii is a Gram-negative bacillus that is aerobic, pleomorphic and non-motile.

Lactobacillus species are non-spore-forming Gram-positive rods that are microaerophilic and catalase negative.

Proteus mirabilis is a Gram-negative bacterium and is well known for its ability to robustly swarm across surfaces in a striking bulls'-eye pattern.

Enterobacter species are a Gram-negative, non-spore-forming bacteria of the family *Enterobacteriaceae* .A few *Enterobacter species*, e.g., *Enterobacter cloacae, Enterobacter asburiae, and Enterobacter hormaechei,* are common pathogens of human infections, particularly hospital-acquired infections . (Nhung, 2007)

Staphylococcus aureus, a Gram-positive, coagulase-positive pathogen belonging to the family *Staphylococcaceae*, is a spherical bacterium of approximately 1 μm in diameter forming grape-like clusters.

Penicillin-resistant epidemic S. aureus strains; called "archaic" MRSA strains, a major issue associated with *S. aureus* is the remarkable level of acquisition of resistance against multiple antibiotic classes and complicating treatment, infections due to methicillin-resistant strains of *S. aureus* are associated with higher mortality rates than infections caused by methicillin-susceptible strains.

Coagulase-negative staphylococci are gram-positive, aerobic organisms distinguished from the closely related *Staphylococcus aureus.*

S. epidermidis, S. haemolyticus and S. hominis, S. capitis and *S. lugdunensis* are example of *coagulase negative staphylococci*.

Haemophilus influenzae is a bacteria characterized as a small, facultative anaerobic, pleomorphic, and capnophilic gram-negative coccobacillus.

Haemophilus parainfluenzae : are fastidious Gram-negative, non-motile coccobaccili and it is opportunistic pathogens.

Serratia species are opportunistic gram-negative bacteria.

Appendices B:

Table 5: The most common micro-organisms obtained by NGS DNA sequence analysis from urine positive samples. Bacterial classification based on genus.

Bacterial genus	**Total reads**	**Frequency (Sample Number)**
Enterococcus	2693	13
Escherichia	2455	15
Yersinia	987	4
Pseudomonas	515	9
Lactobacillus	423	8
Streptococcus	150	9
Rickettsia	118	7
Gardnerella	113	4
Enterobacter	107	10
Anaerococcus	83	3
Veillonella	76	2
Acinetobacter	69	6
Peptoniphilus	56	2
Ralstonia	52	4
Varibaculum	45	1
Nevskia	41	6
Prevotella	41	5
Corynebacterium	40	6
Tolumonas	34	4
Klebsiella	30	6
Gemella	28	1
Rothia	27	1
Providencia	25	5
Agrobacterium	24	4
Staphylococcus	21	6
Stenotrophomonas	21	5
Alkaliphilus	19	4
Micrococcus	19	2
Porphyromonas	19	1
Limnobacter	18	6
Variovorax	18	6
Bacillus	18	5
Actinomyces	18	4
Methylobacterium	17	3
Negativicoccus	15	1
Peptostreptococcus	14	2
Leptotrichia	13	1

Thiomonas	11	4
Swaminathania	11	2
Novosphingobium	11	2
Mobiluncus	11	2

Table 6: The most common micro-organisms obtained by NGS DNA sequence analysis from urine positive samples. Bacterial classification based on species.

Bacterial species	Total reads	Frequency (Sample Number)
Escherichia coli	1732	15
Enterococcus lactis	857	6
Yersinia frederiksenii	657	2
Enterococcus durans	489	2
Enterococcus faecalis	450	8
Lactobacillus jensenii	318	5
Pseudomonas azotoformans	284	2
Gardnerella vaginalis	113	4
Pseudomonas poae	108	2
Tolumonas auensis	34	4
Veillonella atypica	34	2
Anaerococcus lactolyticus	33	3
Enterobacter aceae	29	7
Acinetobacter tjernbergiae	28	2
Lactobacillus iners	28	1
Rothia mucilaginosa	24	1
Nevskia ramosa	22	6
Enterobacter nickellidurans	22	2
Streptococcus parasanguinis	22	1
Lactobacillus gigeriorum	21	1
Veillonella dispar	20	1
Streptococcus anginosus	20	1
Lactobacillus taiwanensis	17	3
Micrococcus yunnanensis	17	2
Klebsiella oxytoca	17	1
Variovorax paradoxus	15	5
Providencia rettgeri	14	4
Streptococcus tigurinus	14	3
Peptoniphilus gorbachii	14	1
Peptostreptococcus anaerobius	13	2
Stenotrophomonas pavanii	12	5
Streptococcus pseudopneumoniae	12	3

Ralstonia detusculanense	12	3
Prevotella melaninogenica	12	1
Streptococcus bovis	12	1
Anaerococcus tetradius	12	1
Pseudomonas chloritidismutans	12	1
Thiomonas thermosulfata	11	4
Enterobacter ludwigii	11	3
Yersinia massiliensis	11	1

Table 7: The most common micro-organisms obtained by NGS DNA sequence analysis from urine samples that showed no significant bacterial growth. Bacterial classification based on genus.

Bacterial genus	Total reads	Frequency (Sample Number)
Escherichia	2626	9
Enterococcus	2150	6
Pseudomonas	1178	8
Lactobacillus	966	6
Lactococcus	822	2
Neisseria	636	2
Gluconacetobacter	606	1
Gardnerella	317	3
Yersinia	245	4
Facklamia	187	2
Corynebacterium	149	9
Pediococcus	127	4
Megasphaera	125	1
Prevotella	93	7
Enterobacter	68	8
Peptoniphilus	66	6
Atopobium	58	2
Tolumonas	56	3
Proteus	55	2
Psychrobacter	51	2
Variovorax	46	5
Clostridium	44	3
Aerococcus	41	2
Staphylococcus	32	6
Stenotrophomonas	27	5
Helcococcus	27	1
Alkaliphilus	25	2
Streptococcus	24	3
Acinetobacter	19	4

Nevskia	17	4
Rickettsia	15	7
Bifidobacterium	14	4
Alloscardovia	14	3
Providencia	13	3
Vagococcus	12	2
Paucibacter	11	3
Methylobacterium	11	2
Anaerococcus	11	1

Table 8: The most common micro-organisms obtained by NGS DNA sequence analysis from urine samples that showed no significant bacterial growth. Bacterial classification based on species.

Bacterial species	Total reads	Frequency (Sample Number)
Escherichia coli	1991	9
Enterococcus faecalis	843	3
Enterococcus faecalis	843	3
Lactococcus raffinolactis	673	1
Lactobacillus iners	540	4
Pseudomonas lundensis	522	3
Pseudomonas fragi	408	2
Gardnerella vaginalis	314	3
Neisseria mucosa	303	2
Enterococcus lactis	226	3
Yersinia frederiksenii	129	3
Neisseria lactamica	121	1
Lactobacillus ultunensis	97	3
Lactobacillus taiwanensis	84	5
Pediococcus stilesii	84	4
Lactobacillus crispatus	81	2
Pseudomonas azotoformans	74	1
Enterococcus durans	71	1
Prevotella timonensis	61	6
Tolumonas auensis	56	3
Lactobacillus jensenii	52	2
Yersinia kristensenii	47	1
Variovorax paradoxus	42	4
Proteus penneri	30	2
Corynebacterium flavescens	30	1
Aerococcus christensenii	29	1
Yersinia massiliensis	29	1
Pediococcus cellicola	27	4

Psychrobacter pulmonis	27	2
Pseudomonas tremae	24	1
Prevotella bivia	22	3
Enterobacter aceae	21	5
Facklamia tabacinasalis	21	1
Helcococcus sueciensis	21	1
Peptoniphilus asaccharolyticus	19	4
Corynebacterium simulans	17	1
Enterobacter hormaechei	16	3
Alkaliphilus peptidifermentans	16	2
Escherichia coli	13	3
Peptoniphilus gorbachii	12	4
Vagococcus teuberi	12	2
Lactobacillus acidophilus	11	2

Table 9: The most common micro-organisms obtained by NGS DNA sequence analysis from urine samples that showed no bacterial growth. Bacterial classification based on genus.

Bacterial genus	**Total reads**	**Frequency (Sample Number)**
Klebsiella	381	4
Variovorax	182	4
Escherichia	55	4
Providencia	35	4
Corynebacterium	19	4
Microvirus	6	4
Sphingopyxis	103	3
Methylobacterium	71	3
Enterobacter	57	3
Bacillus	49	3
Delftia	48	3
Pseudomonas	46	3
Enterococcus	44	3
Paucibacter	38	3
Serratia	30	3
Prevotella	23	3
Limnobacter	20	3
Nevskia	16	3
Heliorestis	4	3
Staphylococcus	578	2
Lactobacillus	194	2
Sphingomonas	48	2

Rickettsia	34	2
Acinetobacter	27	2
Streptococcus	19	2
Faecalibacterium	18	2
Azohydromonas	18	2
Yersinia	17	2
Polaromonas	16	2
Tolumonas	13	2
Burkholderia	11	2

Table 10: The most common micro-organisms obtained by NGS DNA sequence analysis from urine samples that showed no bacterial growth. Bacterial classification based on speics.

Bacterial species	**Total reads**	**Frequency (Sample Number)**
Klebsiella variicola	244	3
Variovorax paradoxus	168	3
Lactobacillus ultunensis	94	1
Lactobacillus crispatus	54	1
Delftia lacustris	48	3
Sphingopyxis witflariensis	44	1
Aerococcus viridans	43	1
Klebsiella pneumoniae	38	1
Escherichia coli	36	4
Methylobacterium marchantiae	31	2
Sphingomonas oligophenolica	25	1
Staphylococcus caprae	24	1
Novosphingobium aromaticivorans	23	1
Providencia rettgeri	17	4
Klebsiella granulomatis	17	2
Enterobacter amnigenus	16	3
Methylobacterium goesingense	16	2
Enterococcus faecalis	16	2
Providencia sneebia	16	2
Variovorax boronicumulans	14	4
Nevskia ramosa	14	3
Yersinia frederiksenii	14	2
Polaromonas jejuensis	14	2
Sphingomonas echinoides	14	1
Tolumonas auensis	13	2
Methylobacterium adhaesivum	12	2
Ralstonia pickettii	12	1
Sphingopyxis chilensis	11	2

Enterobacter nickellidurans	11	2
Bacillus litoralis	11	1

Table 11: The most common micro-organisms obtained by NGS DNA sequence analysis from CSF samples that showed positive bacterial growth. Bacterial classification based on genus.

Bacterial genus	**Total reads**	**Frequency (Sample Number)**
Staphylococcus	2702	3
Sphingomonas	655	3
Variovorax	552	3
Microbacterium	524	2
Micrococcus	373	2
Methylobacterium	346	1
Polaromonas	158	3
Prevotella	142	1
Paucibacter	131	3
Lactobacillus	127	1
Escherichia	111	3
Delftia	107	2
Rathayibacter	105	1
Oerskovia	97	1
Faecalibacterium	85	1
Streptococcus	75	2
Citricoccus	73	1
Ureaplasma	71	1
Pseudomonas	53	3
Bacillus	52	2
Ralstonia	44	1
Serratia	43	2
Acinetobacter	43	2
Sediminibacterium	40	1
Bacteroides	39	1
Oscillospira	38	1
Kocuria	37	2
Rhodococcus	35	1
Succinivibrio	27	1
Pediococcus	23	1
Arthrobacter	22	1
Corynebacterium	19	2
Veillonella	17	1
Agrobacterium	16	2
Blautia	15	2

Porphyromonas	14	1
Clostridium	13	2
Paracoccus	13	1
Rickettsia	12	3
Providencia	12	2
Parabacteroides	11	1
Ruminococcus	11	1

Table 12: The most common micro-organisms obtained by NGS DNA sequence analysis from CSF samples that showed positive bacterial growth. Bacterial classification based on species.

Bacterial species	Total reads	Frequency (Sample Number)
Variovorax paradoxus	486	3
Sphingomonas oligophenolica	307	3
Polaromonas jejuensis	150	3
Staphylococcus haemolyticus	664	2
Micrococcus yunnanensis	312	2
Sphingomonas echinoides	261	2
Staphylococcus massiliensis	146	2
Delftia lacustris	107	2
Escherichia coli	96	2
Microbacterium maritypicum	92	2
Serratia entomophila	43	2
Microbacterium marinilacus	37	2
Microbacterium hydrocarbonoxydans	31	2
Kocuria gwangalliensis	26	2
Staphylococcus caprae	25	2
Acinetobacter tjernbergiae	12	2

Table 13: The most common micro-organisms obtained by NGS DNA sequence analysis from CSF samples that showed negative bacterial growth. Bacterial classification based on genus.

Bacterial genus	Total reads	Frequency (Sample Number)
Variovorax	1074	8
Pseudomonas	805	9
Sphingomonas	655	9
Methylobacterium	392	7
Enterobacter	340	9
Escherichia	282	9
Staphylococcus	255	9
Lactobacillus	255	6
Polaromonas	235	7
Delftia	214	8

Paucibacter	199	5
Rickettsia	187	8
Prevotella	139	5
Chryseobacterium	126	3
Gardnerella	116	3
Serratia	114	9
Acinetobacter	94	9
Rathayibacter	90	4
Providencia	86	8
Corynebacterium	81	8
Ureaplasma	77	1
Klebsiella	76	5
Stenotrophomonas	72	6
Faecalibacterium	65	6
Bacillus	64	8
Limnobacter	60	5
Streptococcus	59	6
Nevskia	50	7
Pediococcus	48	4
Erwinia	42	3
Ruminococcus	40	5
Blautia	39	5
Azohydromonas	39	4
Peptoniphilus	38	5
Veillonella	38	3
Enterococcus	36	5
Bacteroides	35	5
Streptomyces	34	8
Tolumonas	33	5
Bifidobacterium	33	3
Micrococcus	32	3
Clostridium	29	6

Table 14: The most common micro-organisms obtained by NGS DNA sequence analysis from CSF samples that showed negative bacterial growth. Bacterial classification based on species

Bacterial species	Total reads	Frequency (Sample Number)
Variovorax paradoxus	967	8
Pseudomonas xanthomarina	463	2
Sphingomonas oligophenolica	288	8
Sphingomonas echinoides	241	6
Escherichia coli	234	9

Polaromonas jejuensis	218	7
Delftia lacustris	214	8
Methylobacterium marchantiae	195	5
Lactobacillus iners	151	5
Pseudomonas stutzeri	136	1
Gardnerella vaginalis	116	3
Methylobacterium adhaesivum	106	6
Variovorax boronicumulans	97	5
Enterobacter hormaechei	77	6
Prevotella copri	75	3
Enterobacter amnigenus	67	6
Enterobacter nickellidurans	64	4
Providencia rettgeri	59	8
Ureaplasma parvum	59	1
Enterobacter ludwigii	51	6
Ralstonia pickettii	47	7
Chryseobacterium bovis	47	1
Rathayibacter caricis	45	4
Klebsiella oxytoca	44	2
Limnobacter thiooxidans	38	4
Erwinia billingiae	37	2
Pediococcus stilesii	35	4
Tolumonas auensis	33	5
Nevskia ramosa	30	6
Ralstonia detusculanense	30	4
Stenotrophomonas pavanii	29	4
Stenotrophomonas geniculata	29	3
Veillonella montpellierensis	28	3
Enterobacter asburiae	27	1
Acinetobacter johnsonii	26	5
Micrococcus yunnanensis	25	3
Rathayibacter tritici	24	4
Lactobacillus ultunensis	23	4
Pseudomonas chloritidismutans	23	3
Bacillus litoralis	22	6
Acinetobacter tjernbergiae	22	5
Lactobacillus taiwanensis	21	4

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Comparison between cultivation and 16S rRNA

sequencing –based approaches analysis for bacteria in urine and cerebrospinal fluid

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يعتبر اختيار الطريقة الامثل والاكثر دقة وحساسية في الكشف عن البكتيريا من أهم التحديات في علم الاحياء الدقيقة ، وخصوصا في حالات الطوارئ والامراض المنتشرة والمعدية .

يتم في مختبرات المستشفيات جمع عينات (كالدم وسوائل الجسم) من المرضى وفي هذه الدراسة تم التركيز على عينات البول و عينات سائل النخاع الشوكي ، وتعتبر من اهم العينات التي تساعد في الكشف عن حالة المرضى ،لا سيما عندما تكون الاعراض غير واضحة لدى الاطباء وخاصة عند الاطفال وكبار السن ، لان اغلب الطرق تفشل في تحديد مسبب المرض .

تمثل الاصابة بمرض التهاب السحايا البكتيري ، من الامراض التي تهدد حياة الانسان ويمكن ان تؤدي الى الوفاة او حصول مضاعفات تؤثر على حياة الانسان ، ولذلك يجب اختيار طريقة لتشخيص هذه البكتيريا باسرع وقت وتحديد العلاج المناسب لها.

فحص زراعة البكتيريا يعتبر من الطرق التقليدية للكشف عن البكتيريا ، ويمتلك العديد من التحديات كطريقة جمع العينات ،حجم العينة، الوقت المستهلك لزراعة العينة وظهور النتائج ومقاومة بعض السلالات البكتيرية للمضادات الحيوية بالاضافة الى السلالات التي لا يمكن زراعتها في المختبر.

ان الهدف الرئيس لهذه الدراسة هو ايجاد طرق تشخيصية جديدة ذات دقة وحساسية عالية للكشف عن العدوى البكتيرية في عينات البول وسائل النخاع الشوكي ومقارنتها بالطرق التقليدية.

تم مقارنة طريقة زراعة البكتيريا التقليدية (الكلاسيكية) مع تقنية(ن جي اس) والتي تعتمد على الجيل التالي من تحديد الحمض النووي للكشف عن معظم الخلايا البكتيرية في العينات المجموعة.

إن تقنية الجيل القادم من تحديد تسلسل الحمض النووي (NGS) ، هي تقنية جديدة نسبيا تسمح بتحديد شامل لتسلسل الحمض النووي وتمكن من انتاج مجموعة من المعلومات الوراثية من الكائنات الحية بشكل متوازي وتوفر قياسا كميا مفصلا لكل جزء من اجزاء DNA المتسلسلة ، حيث تم استخدام بادئات ترتبط على هذه التسلسلات لزيادة اعداد مقاطع الجينات) (16 S rRNAلانواع البكتيرية.

تمت هذه الدراسة، بعد جمع 50 عينة بول (30 عينة ايجابية الاصابة بالبكتيريا، 15 عينة لا نمو كبيرعليها و 5 عينة سلبية الاصابة بالبكتيريا) و 14 عينة من سائل النخاع الشوكي (4 عينات ايجابية الاصابة بالبكتيريا و 10 عينات سلبية الاصابة بالبكتيريا) من مختبر الأحياء الدقيقة في مستشفى المقاصد - القدس - فلسطين. تم جمع نتائج الزراعة البكتيرية من نفس مختبر مستشفى المقاصد . وقد تم استخراج عينة من الحمض النووي(DNA)في مختبرات جامعة القدس، وبعد ذلك تم تحليل

تسلسل الحمض النووي (DNA) بموائمة احدى الطرق المستخدمة ضمن استراتيجيات شركة Illumina MiSeq لتحليل الحمض النووي . DNA تم إجراء ما مجموعه 64 عينة تم تجميعها وفق الجيل التالي .(NGS)
لاحقا تم الحصول على البيانات كملفات FASTQ ثم تم تحويلها الى ملفات FASTA ثم تم تحليلها وفقا للتصنيف البكتيري للجنس والأنواع، حيث يستند الى طول التسلسل.
تمكنا في هذه الدراسة ، اولا : من تحديد أنواع البكتيريا المسببة لالتهاب المسالك البولية لدى الانسان ، الموجودة في عينات البول وأهم الأنواع التي تم تحديدها وهي:

Escherichia coli, Pseudomonas, Klebsiella, Lactobacillus, Enterobacter, streptococcus Group B, Acinetobacter, Staphylococcus, Rickettsia and Gardnerella.

هذه الأنواع موجودة كبكتيريا مسببة للأمراض في المسالك البولية لدى الانسان، والأكثر وفرة وأهمية هي أنواع *Escherichia coli* التي تسبب التهاب المسالك البولية، حيث ظهرت هذه البكتيريا في كلا الطريقتين الحديثة والتقليدية.
ثانيا : اهم الانواع التي تم الكشف عنها في عينات سائل النخاع الشوكي (CSF) ، وهي:

Pseudomonas, Enterobacter, Escherichia, Staphylococcus and Lactobacillus.

تعتبر *Staphylococcus haemolyticus* من اكثر انواع البكتيريا وفرة في عينات سائل النخاع الشوكي التي تم جمعها والتي تتسب بحدوث اللاتهابات في الجهاز العصبي . وايضا ظهرت هذه البكتيريا في كلتا الطريقتين.

وفي الختام ، تعتبر NGS طريقة سريعة وحساسة جدا وطريقة خاصة نستطيع من خلالها الكشف عن انواع البكتيريا التي يصعب كشفها بالطرق التلقليدية : كالبكتيريا بطيئة النمو و الانتهازية و يمكنها الكشف عن خلايا البكتيرية قليلة التركيز في العينات حيث تصل دقتها للكشف عن خلية واحدة في العينة.