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The efficiency of molecular methods compared to traditional methods in identifying bacteria from blood and cerebrospinal fluid samples

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ABSTRACT

Aims: This study was aimed to directly diagnose bacterial pathogens from clinical specimens by amplifying and sequencing their 16S rRNA gene compared with traditional methods.
Methodology and results: A total of 100 specimens, including blood (50 samples) and cerebrospinal fluid CSF (50 samples) were collected from patients hospitalized in Mosul city hospitals for the period from December 2021 to April 2022. Traditional methods based on cultural characteristics and simple biochemical tests were used to detect pathogens from these samples, as followed by hospital laboratories. Bacterial genomic DNA (deoxyribonucleic acid) was extracted directly from the samples and PCR (polymerase chain reaction) was conducted to amplify their 16S rRNA gene using 16S universal primers. PCR products from samples that showed positive results were purified and sent for sequencing. Sequences were compared to counterpart sequences submitted in NCBI to identify the bacterial pathogen. Our results showed that traditional methods for bacterial isolation and identification from CSF detected 5 positive bacterial cultures out of 50 samples tested (10%), while the remaining cultures (90%) did not show any bacterial growth after 7 days of incubation. The 45 negative samples were subjected to molecular identification, and it was found that 28/45 (62.2%) successfully amplified 16S rRNA bands indicating the presence of bacterial genomic DNA. Sequencing results showed that the highest prevalent genera in CSF samples were *Bacteroides* (41.1%), followed by *Lactobacillus* (17.6%), *Pseudomonas* (11.7%), *Janibacter* (11.7%), *Achromobacter* (11.7%) and *Planococcus* (5.8%). Traditional methods for bacterial isolation and identification from blood were capable of isolating 2 pathogens from 50 samples tested (4%). When testing the 48 negative samples using molecular methods, 25/48 (52%) successfully amplified 16S rRNA bands. The 16S rRNA sequences belonged to 9 different genera, the most dominant was *Pseudomonas* (16.6%) and the least dominant was *Bacteroides* (5.5%).
Conclusion, significance and impact of study: Our results clearly show the power of molecular diagnosis compared to traditional methods used at hospitals and show the danger of misdiagnosis. The study showed that the molecular approach is much more efficient than the conventional methods in identifying pathogens as it identified new pathogens that were not previously detected.

Keywords: Blood culture, CSF culture, *Janibacter* spp., *Plancococcus* spp.

INTRODUCTION

Bacteria live in complex communities in nature and human bodies. Bacterial infections are one of the top ten causes of death worldwide (Abayasekara *et al.*, 2017). Bacteria, including both pathogenic and non-pathogenic, are found in clinical specimens acquired from various regions of the human body. Early detection of pathogens in clinical samples is necessary for improved diagnosis and successful management of diseases (Ismail *et al.*, 2022). To identify bacterial causing infections and determine antibiotic susceptibility, clinical bacteriology laboratories have historically depended on cultural characteristics, biochemical tests and phenotypic approaches (Török and Peacock, 2012).

However, culture-dependent approaches may fail in detecting some pathogens, particularly those that are slow-growing, have stringent culture requirements or are non-cultivable. Another factor that inhibits pathogen isolation is the administration of antimicrobial drugs before collecting the clinical sample (Ismail *et al.*, 2022).

Rapid pathogen detection in clinical samples is critical to ensure better levels of health care (Bonilla *et al.*, 2011; Abayasekara *et al.*, 2017). On the other hand, conventional methods for identifying fastidious bacteria are time-consuming, require special expertise and some

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bacteria might vary in the growth factors they require, therefore, will not grow unless added (de Melo Oliveira *et al.*, 2013).

In the last ten years of the twentieth century, a rapid expansion in knowledge of molecular biology techniques was observed, which had far-reaching repercussions and enabled significant advancements in various fields, including bacteriology (Grif *et al.*, 2012). Introducing PCR tests in clinical laboratories was a significant step that helped directly detect bacteria from clinical specimens and find genes encoding antibiotic resistance (Török and Peacock, 2012). Since its discovery, 16S rRNA-based method has been universally recognized as a superior method for bacterial identification that has gradually taken the place of traditional morphological and biochemical methods (Abayasekara *et al.*, 2017). DNA sequencing of 16S rRNA genes has occasionally resulted in the discovery of entirely unexpected microbes, therefore, may be used to identify unknown or well-known bacteria in unusual infectious foci (Barghouthi, 2011).

To our knowledge, most of the CSF and blood specimens sent to the laboratory for bacterial culture turn out to give negative results. We predicted that traditional bacterial isolation methods are insufficient for this purpose. Therefore, this study aimed to detect pathogens from CSF and blood specimens from in-patients and out-patients that attended public hospitals in Mosul-Iraq by direct isolation of genomic DNA from these samples and sequencing their 16S rRNA gene in comparison with traditional techniques.

MATERIALS AND METHODS

Collection of clinical specimens

A total of 100 samples (50 blood and 50 CSF) were collected from different clinical cases of hospitalized patients using sterile containers during the period from December 2021 to April 2022. Both blood and CSF samples were transported to the laboratory in cooled containers and subjected to traditional isolation methods for identifying bacteria.

Traditional culture methods for bacterial isolation

To isolate bacteria from blood and CSF samples, sample CSF were plated on blood agar, chocolate agar and MacConkey agar by the steaking method. The plates were incubated for 24-48 h at 37 °C. Samples were initially diagnosed based on phenotypic, morphological and cultivar characteristics of CSF on media, its odor and its ability to analyze blood, colony, color and height. Pure cultures were stained with a chromium dye to observe the shape of cells and their interaction with the dye. Basic biochemical tests, including IMVC, oxidase and catalase tests were performed when required (Vandepitte *et al.*, 2003).

DNA extraction

Genomic DNA was extracted directly from blood and CSF samples using a genomic DNA isolation kit supplied by the Geneaid company. Steps were followed as recommended by the manufacturer. A single lysis step was added as a modification to the kit when dealing with blood samples. Concentration and purity of genomic DNA was measured then DNA was stored at -20 °C until further used.

Polymerase chain reaction

PCR was conducted in a 20 µL volume reaction using GoTaq G2 Green Master Mix supplied by Promega (USA). The universal primers 27F AGAGTTTGATCMTGGCTCAG and 1522R AAGGAGGTGATCCARCCGCA were used to amplify the full region of the 16S rRNA gene (Abdulrazzaq and Faisal, 2022). The concentration of primers (1 µM each) and the total amount of template DNA (100 ng) were added as recommended by the manufacturer. The PCR program for the 16S rRNA gene was set as follows: initial denaturation at 95 °C for 3 min followed by 30 cycles of amplification including a denaturation step at 95 °C for 30 sec, annealing at 55 °C for 30 sec and extension at 72 °C for 1 min. A final extension step was set at 72 °C. PCR products were separated on 1% agarose gel and stained with Midori Green Advance DNA stain. A 100 bp DNA marker (New England Biolabs, UK) was used as a molecular weight marker.

DNA sequencing and homology search

PCR products for the 16S rRNA gene were purified and sent for sequencing at Psomagene sequencing company (USA). Retrieved sequences were searched for homology against published genes submitted in GenBank using the BLAST tool at NCBI.

RESULTS AND DISCUSSION

Traditional methods for isolation

Traditional methods for bacterial isolation and identification from CSF detected 5 (10%) positive bacterial cultures out of 50 samples tested, while the remaining cultures (90%) did not show any bacterial growth after 7 days of incubation (Table 1). The bacterial species identified on blood agar, MacConkey agar and chocolate agar were diagnosed as *Escherichia coli* (4) and *Pseudomonas aeruginosa* (1). One of the most frequent bacterial pathogens responsible for extra-intestinal illnesses such as newborn meningitis (NM), septicemia and urinary tract infections is *E. coli*. In a study conducted in eastern China mortality rates ranged from 10-15%, especially during the newborn period. *Escherichia coli*-related NM is still a significant public health issue in industrialized nations. Out of 182 NM cases detected, 69 were caused by *E. coli* (Liu *et al.*,

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Table 1: Comparison of traditional and molecular methods for bacterial identification.

Sample	No. of cases	Traditional methods		Molecular method		Percentage of miss-identification by traditional methods
		Positive	Negative	Positive	Negative	
CSF	50	5 (10%)	45 (90%)	33 (66%)	17 (34%)	28/45 (62.2%)
Blood	50	2 (4%)	48 (96%)	27 (54%)	23 (46%)	25/48 (52%)
Total	100	7 (7%)	93 (93%)	60 (60%)	40 (40%)	53/93 (56.98%)

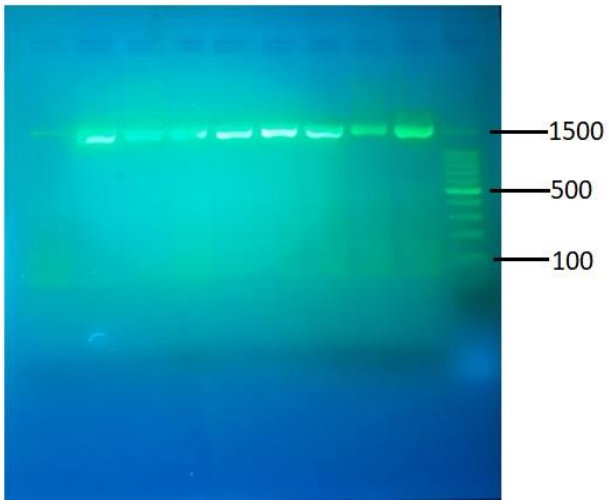

Figure 1: An example of 16S rRNA gene amplification from bacterial genomic DNA isolated from CSF and blood samples.

2021). On the other hand, traditional methods diagnosed one *P. aeruginosa* strain. This Gram-negative bacilli is a rare causative agent of meningitis (Gallaher *et al.*, 2017). Meningitis caused by *P. aeruginosa* is more likely to occur as a post-infection for neurosurgical procedures. This pathogen was isolated from a patient after 5 years of neurosurgery (Singla and Singh, 2021). Infections with *P. aeruginosa* occur due to the use of implantation of an additional ventricular brain or shunt. This pathogen was shown to cause 1-18% of nosocomial meningitis infections (Pai *et al.*, 2015).

Traditional methods for isolating bacteria from blood samples identified only 2 (4%) pathogens out of 50 blood samples tested (Table 1). Blood cultures frequently yield negative results, and this could be due to non-bacteremia illnesses or more likely, to the low presence of bacteria in the blood. Another possibility is that pathogenic bacteria are weak, fastidious, nonviable, slow growing at low densities or uncultivable in a blood culture medium. Another main factor that should be addressed is the patient intake of antibiotics prior to sample collection, which may destroy or decrease the growth of pathogens resulting in a false negative case.

Several other factors, including improper sample handling, organism mortality or growth inhibition, could result in false negative cases (Ter *et al.*, 2021). So far, the history of infectious diseases demonstrates that no human bacterial pathogen is uncultivable: the main question appears to be whether we can discover the environmental parameters necessary for prokaryotic agents to grow (Houpikian and Raoult, 2002).

Identification of the two bacterial strains isolated showed that they belonged to *Staphylococcus epidermidis* and *Klebsiella pneumonia*. In a study conducted by Gogoi *et al.* (2021), MRSA, *Acinetobacter baumannii* and *K. pneumonia* were the most common antimicrobial-resistant bacteria found in blood cultures of the patients, accounting for 31.47% (79/251), 23.90% (60/251) and 17.93% (45/251) from all antimicrobial-resistant positive cases, respectively (Gogoi *et al.*, 2021). Gram-positive bacteria, particularly *Staphylococcus aureus* have overtaken Gram-negative bacteria as the main causes of sepsis and sepsis-related mortality in the latter half of the 20th century. Sepsis is also frequently attributed to coagulase-negative staphylococci (CNS), with the predominant species being *S. epidermidis*, especially in neonates. The identification of CNS in blood samples is frequently due to contamination rather than an actual infection, but because they are common commensals on human skin, it is challenging to make a microbiological diagnosis of an actual CNS blood infection (Otto, 2017).

Molecular method for isolation

Amplification of the 16S rRNA gene from the genomic DNA isolated directly from CSF and blood samples showed that samples that gave positive results using traditional methods all showed positive results using molecular techniques, as expected. Results for sequencing the 16S rRNA gene showed that there was a match between diagnosis by conventional methods and molecular methods for all seven isolates except one sample from CSF. This single sample was diagnosed as *E. coli*, while molecular methods diagnosed this bacterium as *Bacteroides*.

However, when examining the negative samples from CSF and blood using molecular methods, results showed that 28/45 (62.2%) CSF samples and 25/48 (52%) blood samples were positive, accordingly these samples produced a 1.5 kb band that corresponds to the complete fragment of 16S rRNA gene (Figure 1 and Table 2).

The PCR products containing the 16S rRNA gene detected from both CSF and blood samples used in this study were subjected to gene sequencing and their sequences were compared to sequences submitted to the NCBI database to identify the type of bacteria. Analysis of 16S rRNA gene sequences obtained from CSF samples diagnosed 17/28 (60.71%) bacteria to the genus level, as

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Table 2: Number and percentage of bacterial isolated by sequencing and comparing the sequence to the NCBI database.

	Isolated bacteria	Number of bacteria	Percentage of bacteria %
CSF sample	*Lactobacillus* sp	3	17.6
	Planococcus sp	1	5.8
	Pseudomonas sp	2	11.7
	Janibacter sp	2	11.7
	Bacteroides sp	7	41.1
	Achromobacter sp	2	11.7
Total	Total of positive	17	60.7
	Total of negative	11	39.3
	Total of all sample	28	100
Blood samples	*Lactobacillus* spp.	2	11
	Bacteroides spp.	1	5.5
	Pseudomonas spp.	3	16.6
	Klebsiella spp.	2	11
	Citrobacter spp.	1	5.5
	Janthinobacterium spp.	2	11
	Staphylococcus spp.	1	5.5
	Enterobacter spp.	1	5.5
	Escherichia coli	2	11
Total	Total of positive	15	60
	Total of negative	10	40
	Total of all sample	25	100

shown in Table 2. Negative samples of CSF were identified by molecular methods to contain 6 different bacterial genera, as seen in Figure 2, including *Lactobacillus* spp. (3), *Planococcus* spp. (1), *Pseudomonas* spp. (2), *Janibacter* spp. (2), *Bacteroides* spp. (7) and *Achromobacter* spp. (2).

Lactobacillus spp. strains are commonly utilized as probiotics, yet it has been suggested that they may have pathogenic roles. A case was reported of *Lactobacillus plantarum*-induced meningoencephalitis in a 63-year-old man with newly diagnosed metastatic Plano epithelial lung cancer. *Lactobacillus plantarum* was cultivated from the patient's blood and cerebrospinal fluid. Lactobacilli are now known to be a cause of infection, particularly bacteremia. This case was the fourth known case of *Lactobacillus*-associated neuro infection and the second case detected worldwide in adults (Biesiada *et al.*, 2019).

Our study detected the presence of *Plancococcus* spp. in CSF for the first time worldwide. No other cases were recorded worldwide; however, this pathogen was detected from blood samples in the Czech Republic at the beginning of 2022, where they isolated *Planococcus glaciei* from the blood of a 57-year-old man suffering meningitis using an anaerobic cultivation bottle (Mališová *et al*., 2022). Such results indicate that molecular tools of identification are much more effective in isolating bacteria that fail to be isolated using traditional methods used in hospitals.

Pseudomonas (but not *P. psychrophila*), a Gram-negative bacillus, is an uncommon cause of meningitis. Patients with a history of neurosurgical procedures are more likely to acquire *P. aeruginosa* meningitis. However, cases of patients who had community-acquired chronic meningitis caused by *P. aeruginosa* 5 years after undergoing otosclerosis surgery have been reported (Gallaher *et al.*, 2017). The reason behind the detection of *Pseudomonas* by molecular methods and not by traditional methods could be due to improper sample handling, storing, time the sample was left before culture, use of antibiotics or a low number of bacteria in the sample (Arabestani *et al.*, 2015).

The clinical importance of *Janibacter hoylei* was not yet noticed until 2017, when this bacterium was first isolated from an 8-week-old male infant suffering from bacteremia. Conventional methods for isolation failed in diagnosing these Gram-negative bacilli; therefore, this bacterium was identified using 16S rRNA gene sequencing (Lim *et al.*, 2017). Later in 2021, *J. hoylei* was isolated from a blood sample of a 52-year-old woman with a *Clostridioides difficile* infection history. *Janibacter hoylei* requires nine days to grow aerobically in blood culture (Moktan *et al*., 2021) and this might be the reason behind the failure of hospital microbiology labs to isolate this pathogen from blood. This study was the first to identify *Janibacter* spp. from CSF samples. The presence of this pathogen in CSF samples could be due to contamination from blood, as it has been reported that bacteremia is a risk factor in patients that acquire external ventricular drains or lumbar drains. These medical devices could be a route where bacteria can enter the CSF of these patients (Vrettou *et al*., 2022).

Meningitis caused by anaerobic bacteria is somehow rare and when it occurs, is hard to diagnose using traditional culturing methods (Chatzopoulos *et al*., 2020). In a study that spanned 30 years by Lee *et al*. (2018), they included 540 meningitis patients and only 13 (2.4%) showed the presence of anaerobic bacteria. These bacteria belonged to *Bacteroides fragilis*, *Prevotella* spp.,

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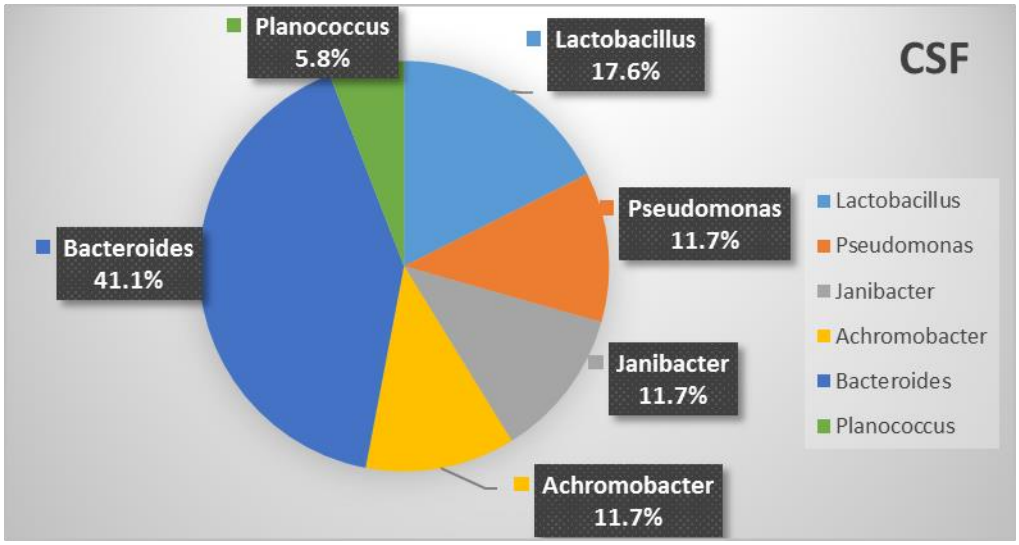

Figure 2: The percentages of bacteria isolated from CSF samples using molecular methods.

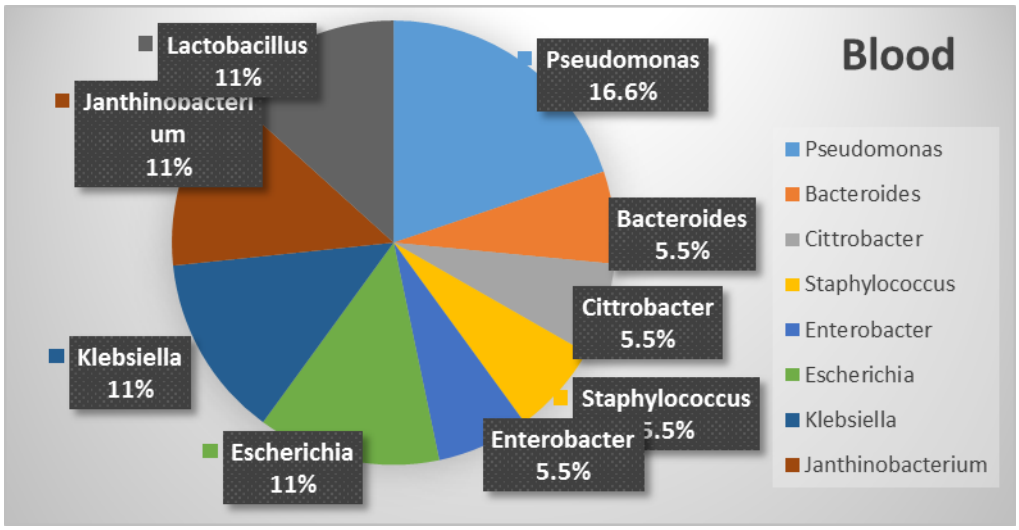

Figure 3: The percentages of bacteria isolated from blood samples using molecular methods.

Fusobacterium nucleatum, *Parvimonas micra* and *Propionibacterium acnes* (Lee *et al*., 2018). In a case report from the USA, *B. fragilis* was detected by sequencing the 16S rRNA directly from the CSF of a 7-year-old male suffering a history of headache, fever and seizure. The direct culturing of the CSF did not show the presence of any bacterial infection, even though the symptoms of meningitis were suspected; accordingly, 16S rRNA sequencing was used for proper identification (Shah *et al*., 2022). In a different case study, a different species of *Bacteroides*, *Bacteroides thetaiotaomicron* meningitis was also identified from the CSF of a 45-year-old man. This species from *Bacteroides* was the first time isolated in pure culture (Feuillet *et al.*, 2005).

Our method for isolation detected two isolates of *Achromobacter* spp. from CSF. This bacterium is a rare causative agent of meningitis; however, *A. xylosoxidans* is the most reported species from this genus, especially in patients acquiring sepsis after neurosurgical operations (Borni *et al*., 2021).

Even though our method successfully isolates a number of different pathogens that are not isolated by traditional methods used at hospitals, this method failed to detect 11/28 (39.28%) positive samples, i.e., samples that showed specific bands for 16S rRNA gene on the gel.

As for the positive blood samples, we had 15 positive samples with a percentage of 60%, while the negative samples were 10 samples with a percentage of 40%, as shown in table (2). Our method isolated nine different genera from blood samples, including *Lactobacillus* spp. (11%), *Bacteroides* spp. (5.5%), *Pseudomonas* spp. (16.6%), *Klebsiella* spp. (11%), *Citrobacter* spp. (5.5%),

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Janthinobacterium spp. (11%), *Staphylococcus* spp. (5.5%), *Enterobacter* spp. (5.5%) and *E. coli* (11%), as seen in Figure 3.

Lactobacillus can cause serious infections, such as bacteremia and infectious diseases, under specific conditions and in the presence of specific risk factors. Peritonitis, liver abscesses, pulmonary infections, pyelonephritis, endocarditis (IE) and meningitis. *Lactobacillus gasseri* was reported for the first time to cause bacteremia and liver abscesses simultaneously (Ramos-Coria *et al*., 2021).

The most frequent anaerobic bacteria found in the clinical laboratory following mono- and polymicrobial infections is *B. fragilis*. *Bacteroides fragilis* is relatively resistant to β-lactams, tetracycline, fluoroquinolones and macrolides. *Bacteroides fragilis* is an important and commonly isolated anaerobic bacterium in clinical laboratories, alongside other aerobic and facultative anaerobic bacteria, and is a well-known component of the normal colonic microbiota. Only around 5% of the gastrointestinal microflora is made up of *B. fragilis*, yet despite having infection rates of 60-80%, it is thought to be the most common species in the *Bacteroides* genus (Yekani *et al.*, 2022).

There are several prominent case reports of bacteremia caused by *P. aeruginosa* in the literature. Most of the patients infected with this bacterium are usually immuno-compromised patients (Singh *et al*., 2021). *Pseudomonas luteola* is another species that is now increasingly regarded as a significant contributor to hospital-acquired infections (HAIs). According to reported cases, it may be the causative agent for different bacterial infections such as bacteremia, endocarditis, peritonitis, endophthalmitis, osteomyelitis or cutaneous infections (Alhalimi *et al*., 2022).

Klebsiella pneumonia is one of the most common causes of HAIs around the world, including UTI, pneumonia, wound infections and sepsis. Recent research has shown that it is a major contributor to newborn sepsis in Africa and Asia (Gorrie *et al*., 2022).

Another member of the Enterobacteriaceae, *Citrobacter*, is a causative agent of nosocomial infections of the urinary tract, liver, biliary tract, peritoneum, intestines, bone, respiratory tract, endocardium, wounds, soft tissue and meningitis (Rahman *et al*., 2022). *Citrobacter brakii* is a very rare causative agent of bacteremia. However, cases have been reported in immunocompromised patients (Prasanna *et al*., 2022).

Janthinobacterium is a member of Proteobacteria and is one of the most abundant skin microbiota after *Pseudomonas* (Grice *et al*., 2008). The presence of *Janthinobacterium* in 11% of our positive blood samples could be related to contamination from the skin while collecting the blood sample. This bacterium was detected in our method and not by traditional methods, most likely because *Janthinobacterium* requires NaCl for growth which is not provided in cultures used for culturing blood samples (Baricz *et al*., 2018). *Janthinobacterium* has also been detected as the most abundant bacteria in blood samples of depressive episode patients and was decreased after treatment with antidepressants (Ciocan *et al*., 2021).

Staphylococcus aureus is one of the leading causes of life-threatening bloodstream infections, such as sepsis and endocarditis. This bacterium has been shown to be a leading cause of superficial skin and soft tissue infections, invasive infections, sepsis and mortality in the community and in healthcare (Kwiecinski and Horswill, 2020).

Enterobacter spp. is a frequent pathogen in a number of infections, including bloodstream and intra-abdominal infections, the majority of which are healthcare-associated. After *E. coli* and *K. pneumonia*, *Enterobacter* spp. is the third most prevalent human pathogen from Enterobacteriaceae, causing blood infections (Wu *et al*., 2021). *Escherichia coli* is the most prevalent Gram-negative pathogen in humans, which also causes sepsis, newborn meningitis and urinary tract infections (Hasanli *et al*., 2022). As a result, the detection of *E. coli* in 11% of our samples is expected.

CONCLUSION

16S genome technology is the most effective method used to identify bacteria from different sources. Our work showed that this method was highly sensitive to detecting the bacterial presence in blood and CSF. The method was effective in identifying fastidious and rare pathogens that are not being diagnosed by traditional methods used at hospitals, thereby causing negative effects on the treatment method for patients. According to our results, it is suggested to add molecular methods to medical laboratories at hospitals to provide better health care for patients.

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