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MOLECULAR CHARACTERIZATION OF *ACINETOBACTER BAUMANNII* AND METHICILLIN RESISTANT *STAPHYLOCOCCI* FROM SEPTICEMIA PATIENTS IN PESHAWAR

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Abstract

Septicemia is a leading cause of death worldwide. *Acinetobacter baumannii* and methicillin-resistant Staphylococci are common causative agents. It's really dangerous to the general population's health. The present state of bacterial isolates and their antibiotic resistance profile is crucial information for doctors and other healthcare practitioners to have in order to intervene effectively. Patients with septicemia in Peshawar were analyzed for the presence of methicillin-resistant *Staphylococci* and *Acinetobacter baumannii*. Patients at the Hayatabad Medical Complex in Peshawar who were thought to have septicemia had 100 blood samples obtained from them. Biochemical and phylogenetic analyses validated the isolated blood stains. They also took an antibiotic sensitivity test. Strains that were first validated biochemically were then confirmed by sequencing. Three *Acinetobacter baumannii* isolates (HB-1, HB-2, and HB-3) and four Staphylococcus isolates (S-1, S-2, S-3, and S-4) were sequenced to verify their authenticity. Of a total of 100 blood samples, 62% were positive for bacterial growth, whereas 38% were negative. There were 23 *Staphylococci* (37.09%), 11 *Acinetobacter baumannii* (17.74%), and 28 other species (45.16%) among the positive samples. A little neonatal majority was observed, with the required species being acquired from 20 (58.82%) male patients and 14 (41.7% female patients). Among male patients, those between the ages of 1 and 18 had the greatest rate of A. *baumannii*, whereas among female patients, those 50 and older had the lowest rate of A. *baumannii* isolates. xi Patients between the ages of 1 and 18 were found to have the highest rate of Staphylococci isolation, whereas patients between the ages of 18 and 50 had the lowest prevalence. Nine (81.8%) of the eleven (17.74%) A. *baumannii* tested were multidrug-resistant, while just two (18.18%) were responsive to antibiotics. Twenty-three of the bacteria (37.09%) were

Staphylococci, and all of them were resistant to methicillin. Among the Acinetobacter sequenced, HB-1 was determined to be *Acinetobacter junii*, HB-2 and HB-3 to be Acinetobacter baumannii, and S-1 to be *Staphylococcus hominis*, S-2 and S-4 to be Staphylococcus haemolyticus, and S-3 to be Staphylococcus arlettae.

Key words: MDR, *A. baumannii*, *Staphylococci*, Sequencing, *Staphylococcus arlettae*

Introduction

Infectious blood poisoning brought on by bacteria or their toxins is called septicemia [1], and it may be fatal if left untreated. An infection of the bloodstream caused by bacteria is called septicemia, sepsis, or a blood infection. Failure to treat bacteremia in a timely manner may result in this potentially fatal complication [2]. Symptoms of sepsis include fever, reduced urine output, chills, breathing difficulties, tachycardia, low blood pressure, poor blood perfusion, and altered mental function [3]. Septicemia is often brought on by bacteria like Methicillin-resistant Staphylococci or Acinetobacter baumannii. Due to its multi-drug resistance, Acinetobacter is rapidly becoming the most common causative agent of newborn septicemia. Once considered a minor pathogen, the gram-negative, non-fermenting coccobacillus Acinetobacter baumannii has emerged as a major cause of illness in both the general population and in healthcare facilities. It causes septicemia and pneumonia often in immunocompromised people. Due of chromosome-mediated genetic determinants, it is resistant to several antibiotic classes and thrives in the sterile conditions of hospitals [4]. Approximately 80% of all Acinetobacter infections are caused by A. baumannii, as reported by the Centers for Disease Control and Prevention (CDC). It is a leading source of infections in hospitals, particularly intensive care units (ICUs), worldwide [5]. Acinetobacter are common invaders of the skin and mucous membranes in hospitals, and they represent a major source of healthcare-associated illnesses. Three important variables contributing to the strength of the bacteria include resistance to essential antibiotics, desiccation, and disinfectants [6]. The mortality toll, hospitalization times, and healthcare expenses associated with antimicrobial resistance have all increased dramatically during the last decade. Multiple antibiotics are ineffective against between 50 and 70 percent of gram-negative bacteria. Multidrug- or even extensive-drug-resistant A. baumannii is a common occurrence. Staphylococcus is a member of the genus Staphylococcus and the family Bacillaceae. They cluster into grape-like globules that seem round or spherical under the microscope. A number of species of Staphylococcus are obligate anaerobes, meaning they can't live in an oxygenated environment [7]. Coagulase-negative Staphylococci and Staphylococcus aureus are common causes of hospital-acquired infections. Since these bacteria are part of the skin's natural flora, differentiating infections from contamination and colonization may be challenging in epidemiological studies of CNS illnesses. Infants may face a wide variety of CNS illnesses. Patients who have had a bone marrow transplant, children with cancer, and burn victims often get central nervous system infections. One of the most often found CNS species causing human infections is Staphylococcus epidermidis. One of the other Staphylococci implicated in a variety of human illnesses is methicillin-resistant Staphylococcus aureus. Urinary tract infections caused by this bacterium are rather prevalent in humans, especially among young girls [8]. Rising bacterial prevalence in recent years has led to a rise in the severity of infections caused by methicillin-resistant coagulase-negative Staphylococci (MRCNS) [9]. Since the 1940s, when antibiotics were first introduced, there has been an alarming rise in the creation of antibiotic-resistant bacteria [10]. Staphylococci develop methicillin resistance when they lose their penicillin-binding proteins, which reduces their sensitivity to beta lactam antibiotics. Staphylococcus aureus and central nervous system (CNS) isolates may have been responsible for the widespread dissemination of this gene [11]. Deadly hospital-acquired infections caused by methicillin-resistant Staphylococci (MRS) need the use of non-Beta lactam medicines. The severity of MRS has increased as the number of reported cases has grown. There is a pressing need to diagnose these illnesses early on and treat them before they become resistant to antibiotics or too expensive to cure [12]. Antibiotic resistance in CoNS, such as S. epidermidis, increases healthcare expenses and is associated with

significant morbidity and death. Antibiotic families such as aminoglycosides, macrolides, and glycopeptides, all of which are often employed, are losing their effectiveness against CoNS species. With the recent discovery of teicoplanin resistance in MR-CoNS in the United Kingdom and the United States, the question of how effective glycopeptides will be over the long run against these strains of bacteria has become timely. Several examples of glycopeptides-resistant CoNS in patients on long-term vancomycin therapy have surfaced since then, limiting treatment choices [13]. The advent of vancomycin resistance around the turn of the century led to the introduction of linezolid into clinical practice, and it was widely regarded as the antibiotic of choice for treating vancomycin-resistant S. aureus [14]. Interest in using macrolide-licosamide-streptogramin B (MLSB) to treat infections caused by S. aureus has increased in response to the growing prevalence of methicillin resistance in Staphylococci. drug resistance is a growing concern, hence this research aimed to examine drug susceptibility patterns and MDR phenotypic prevalence. In our case, the information presented here may aid in the quick selection of an appropriate antibiotic therapy for septicemia.

Methodology

Sample Collection

One hundred blood samples were collected from hospitalized patients and outpatients at the Hayatabad Medical Complex in Peshawar, Pakistan, and forwarded to the Microbiology Laboratory for culturing and susceptibility testing. Phenotypic characteristics were determined for the Staphylococci and A. baumannii that were isolated from processed blood samples. In addition, MDR Acinetobacter baumannii and methicillin-resistant Staphylococci were genotyped.

Sample processing

Specimens were inoculated onto a variety of media, including Blood agar, MacConkey agar, Mueller-Hinton agar, and mannitol salt agar, depending on the properties being examined. MacConkey agar was used to separate lactose fermenters from non-fermenters, whereas blood agar medium was employed to separate haemolytic from non-haemolytic colonies. There was a 24-hour incubation period in the incubator at 37°C for the cultivated plates. Following incubation, the isolated strains were verified using a battery of standard biochemical assays. Gram staining, colony morphology, incubation at certain temperatures, coagulase testing, TSI testing, oxidase testing, catalase testing, DNAase testing and indole testing are all common microbiological techniques used to characterize and identify isolates.

Antimicrobial susceptibility

Staphylococci and A. baumannii were tested to see how resistant they were to various classes of antibiotics. The disk diffusion technique was used to assess antibiotic susceptibility on Mueller-Hinton agar, as per standard laboratory procedure. Doxycycline (30 g), Amikacin (30 g), Meropenem (30 g), Ceftazidime (30 g), Cefotaxime (30 g), Piperacillin (110 g), Tazobactam (110 g), Meropenem (30 g), Imipenem (10 g), Ciprofloxacin (5 g), and Cefepime (30 g) susceptibilities were tested against A. Baumanni. Moxifloxacin (5 g), Gentamicin (120 g), Trimethoprim/sulfamethoxazole (25 g), Tigecycline (15 g), Linezolid (130 g), Cefoxitin (30 g), Chloramphenicol (30 g) and Amikacin (30 g), were tested for their effects on Staphylococci by disc diffusion. Multidrug-resistant microorganisms like A. baumannii and S. aureus are difficult to treat.

Molecular analysis of the isolated strains

The 16s rRNA gene is often used in bacterial phylogenetics as a genetic marker. 16s rRNA is considered the gold standard for molecular confirmation when biochemical tests fall short of elucidating an isolate's genus and species. Acinetobacter and methicillin-resistant Staphylococcus aureus pure colony strains were delivered to Macrogen Korea for sequencing. The findings of sequencing the 16s rRNA PCR products were recorded. Isolate sequences were trimmed using

Finchtv to provide clean reads for phylogenetic analysis. For 16s rRNA-based identification, these pristine reads were blasted in NCBI's nucleotide BLAST and compared to known rRNA sequences.

Results

3.1. Distribution of culture-positive and culture-negative samples

There was bacterial growth in 62 of 100 blood samples from patients with probable septicemia at Peshawar's Tertiary Care Hospitals, whereas there was no growth in 38 of the samples. Culture-positive and culture-negative blood smears for microorganisms are shown in Figure 3.1

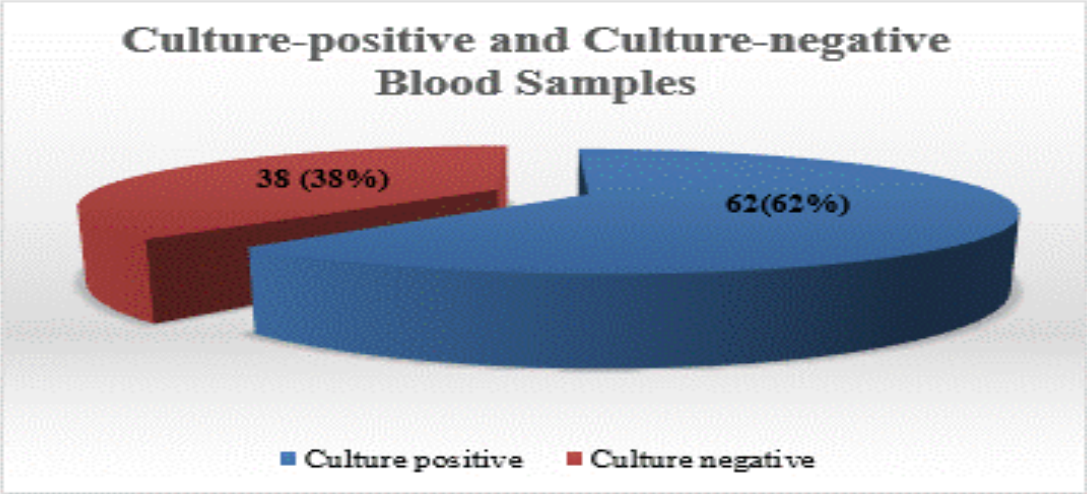

Fig. 3.1. Culture-positive and culture-negative blood samples

1.2. Bacterial characterization

Culture-positive samples were found to be Acinetobacter baumannii in 11 (17.74%), Staphylococci in 23 (37.09%), and other species in 28 (45.16%), as shown in Table 3.1 and Figure 3.2.

Table. 3.1. Bacterial Characterization

S. No	Bacterial isolates	No. of isolates	Percentage
01.	*A. baumannii*	11	17.74%)
02.	*Staphylococci*	23	37.09%
03.	Other species	28	45.16%

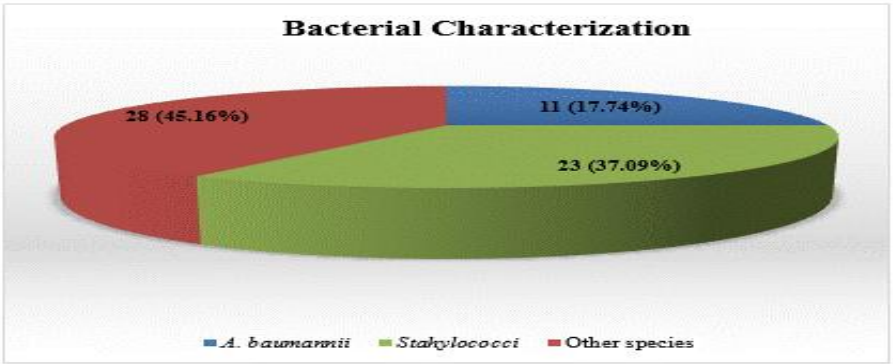

Figure. 3.2. Bacterial Characterization

3.3 Demographic distribution of blood samples

Twenty male (58.82%) and fourteen female (41.7%) individuals were included in the studies, with a little neonatal preponderance. Table 3.2 displayed demographic information about the samples, including sex and age distribution. Female patients over the age of 50 had the lowest incidence of A. baumannii isolates found, whereas male patients between 0-18 years had the greatest prevalence. Similarly, the isolation rate of Staphylococci was greatest in male patients aged 0-18 years and the lowest in female patients aged 18-50 years.

Table. 3.2. Distribution of *A. baumannii* and *Staphylococci* based on age and **Gender**-based scrutiny of samples

S. No	Gender	No. of samples	Percentage
1.	Male	20	58.82
2.	Female	14	41.7
Total		34	100

Bacteria species	Male/age (%)			Female/age (%)		
	0-18	18-50	>50	0-18	18-50	>50
A baumannii	4 (36.36)	3 (27.27)	----	3 (27.27)	---	1 (9.09)
Staphylococci	11 (47.82)	----	3 (13.04)	6 (26.08)	1 (4.34)	2 (8.69)

3.4 Identification of the isolated *A. baumannii* and *Staphylococci*

Under the microscope, colonies of A. baumannii were either rod-shaped (coccobacillary) or almost spherical, but those of Staphylococci had a deep violet or magenta hue anzsd had a cocci or circular form, appearing in clusters that evoked a bunch of grapes. Several biochemical assays were conducted to identify A. baumannii and Staphylococci, and their results are summarized in Table 3.3.

Table 3.3. Biochemical tests performed to detect *A. baumannii* and *Staphylococci.*

S. No	Biochemical tests	Bacterial isolate	
		A. baumannii	*Staphylococci*
1.	Catalase	+ ve	+ ve
2.	Coagulase	----	-ve
3.	DNase	----	-ve
4.	Mannitol fermentation	----	+ ve
5.	Oxidase	-ve	----
6.	TSI	K/K, H_2S -ve	----
7.	Citrate	+ ve	----
8.	Indole	-ve	----

3.5 Antibiotics Susceptibility Testing (AST) of *A. baumannii* and *Staphylococci*

After incubating the plates for 24 hours, we measured their diameters and those of the different zones. A. baumannii and Staphylococci antibiotic resistance profiles are shown in tables 3.4 and 3.5,

respectively. The results showed that 9 of the 11 (17.74%) A. baumannii were multidrug-resistant strains, whereas 2 (18.18%) were sensitive strains. Twenty-three of the bacteria (37.09%) were Staphylococci, and all of them were resistant to methicillin.

Table 3.4. Antibiotic resistance pattern of *A. baumannii*

S. No	Antibiotics	Codes	Sensitive	Intermediate	Resistant
1.	Cefepime (30 µg)	FEP	2 (18.18%)	----------	9 (81.81%)
2.	Ciprofloxacin (5 µg)	CIP	2 (18.18%)	2 (18.18%)	7 (63.63%)
3.	Cefotaxime (30 µg)	CTX	----------	----------	11 (100%)
4.	Meropenem (10 µg)	MEM	9 (81.81%)	----------	2 (18.18%)
5.	Amikacin (30 µg)	AK	1 (9.09%)	1 (9.09%)	1 (9.09%)
6.	Piperacillin-Tazobactam (110 µg)	TZP	2 (18.18%)	1 (9.09%)	8 (72.72%)
7.	Imipenem (10 µg)	IPM	1 (9.09%)	----------	10 (90.90%)
8.	Doripenem (30 µg)	DO	2 (18.18%)	----------	9 (81.81%)

Table. 3.5. Antibiotics resistance pattern of methicillin resistant *Staphylococci*

S. No	Antibiotics	Codes	Sensitive	Intermediate	Resistant
1.	**Moxifloxacin (5 µg)**	MXF	---------	10 (43.47%)	13 (56.52%)
2.	**Gentamicin (120 µg)**	CN	---------	4 (17.39%)	19 (82.60%)
3.	**Trimethoprim/sulphamethoxazole (25 µg)**	SXT	1 (4.34%)	3 (13.04%)	19 (82.60%)
4.	**Tigecycline (15 µg)**	TGC	4 (17.39%)	2 (8.69%)	17 (73.91%)
5.	**Amikacin (30 µg)**	AK	---------	7 (30.43%)	16 (69.56%)
6.	**Linezolid (130 µg)**	LZD	20 (86.95%)	3 (13.04%)	---------
7.	**Cefoxitin (30 µg)**	FOX	---------	1 (4.37%)	22 (95.65%)
8.	**Chloramphenicol (30 µg)**	C	---------	1 (4.37%)	12 (95.65%)

3.6 Molecular analysis of *Acinetobacter*

Shipments of Acinetobacter, including HB-1, HB-2, and HB-3, were made to Macrogen Korea. The aforementioned isolates' sequences were cleaned up using finchtv to provide high-quality reads for phylogenetic tree building. It was determined that HB-1 was in fact Acinetobacter junii, whereas HB-2 and HB-3 were both Acinetobacter baumannii. All of the isolates belong to the genus Acinetobacter, as shown by the results of the BLASTn search. Similarities between HB-1, HB-2, and HB-3 and 10 different Acinetobacter species were quite high. This returned FASTA file of sequences was then utilized to build a phylogenetic tree. To evaluate the evolutionary relationship of these isolates using the data from NCBI, we generated phylogenetic trees (Figures 3.3, 3.4, and 3.5) using the likelihood technique.

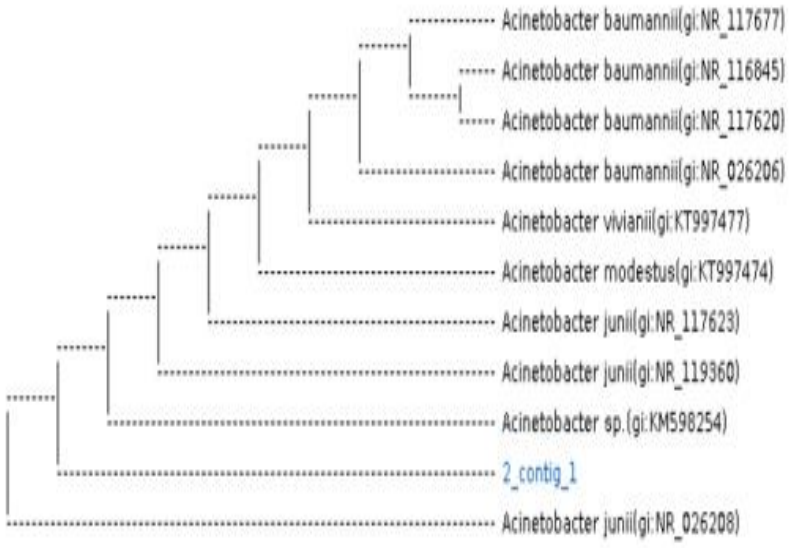

Figure 3.3. Phylogenetic tree constructed via likelihood approach demonstrating the query sequence being identified as *Acinetobacter junii*.

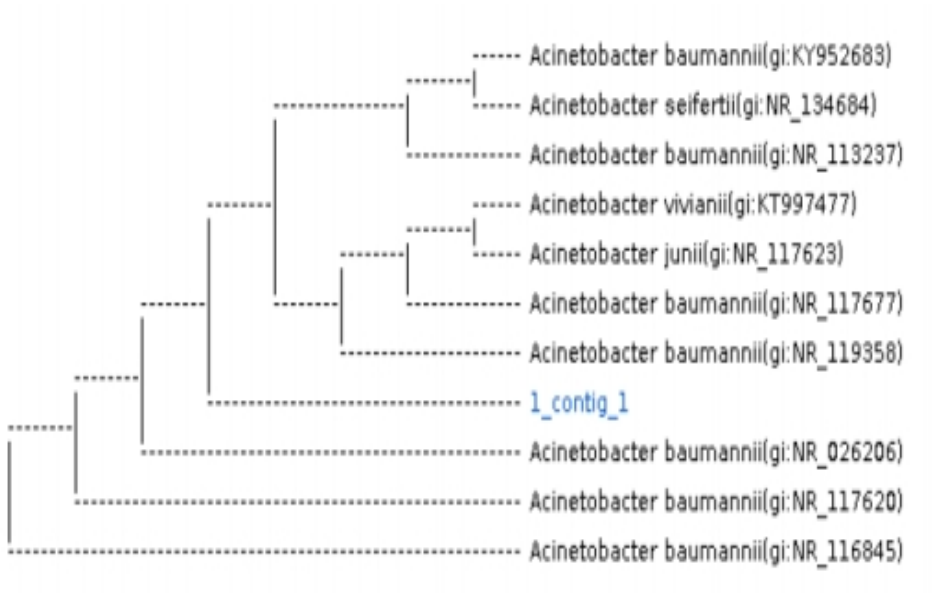

Figure 3.4. Phylogenetic tree constructed via likelihood approach demonstrating the query sequence being identified as *Acinetobacter baumannii*.

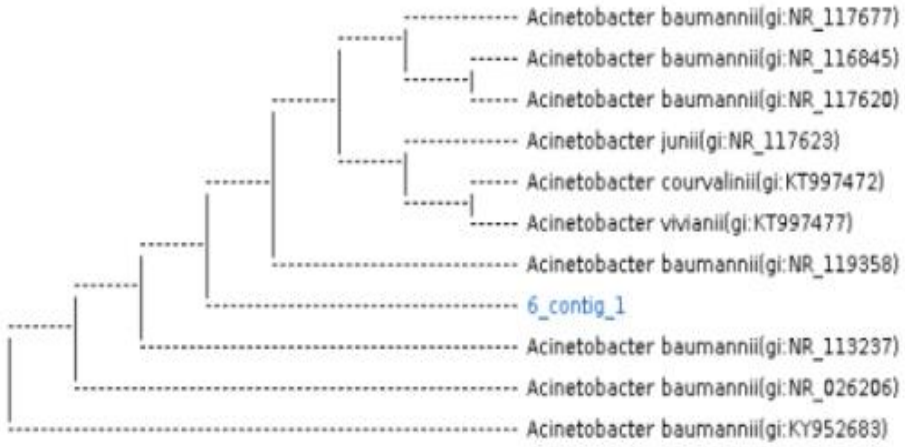

Figure 3.5. Phylogenetic tree constructed via likelihood approach demonstrating the query sequence being identified as *Acinetobacter baumannii*.

3.7 Molecular analysis of *Staphylococci*

Shipments of Staphylococci S-1, S-2, S-3, and S-4 were sent to Macrogen Korea. In order to generate a reliable phylogenetic tree, finchtv was used to clean the sequences of the aforementioned isolates. Staphylococcus hominis (S-1) was identified, Staphylococcus haemolyticus (S-2) and Staphylococcus arlettae (S-4) were both identified, and Staphylococcus arlettae (S-3) was identified. BLASTn analysis revealed that each isolate belonged to the Staphylococcus genus. There was a significant degree of similarity between S-1, S-2, S-3, and S-4 and 10 other Staphylococcus species. In order to create a phylogenetic tree, the sequences were downloaded in FASTA format. To evaluate the evolutionary relationship between these isolates and the data found in NCBI, phylogenetic trees were created using a likelihood technique, as shown in Figures 3.6, 3.7, and 3.8.

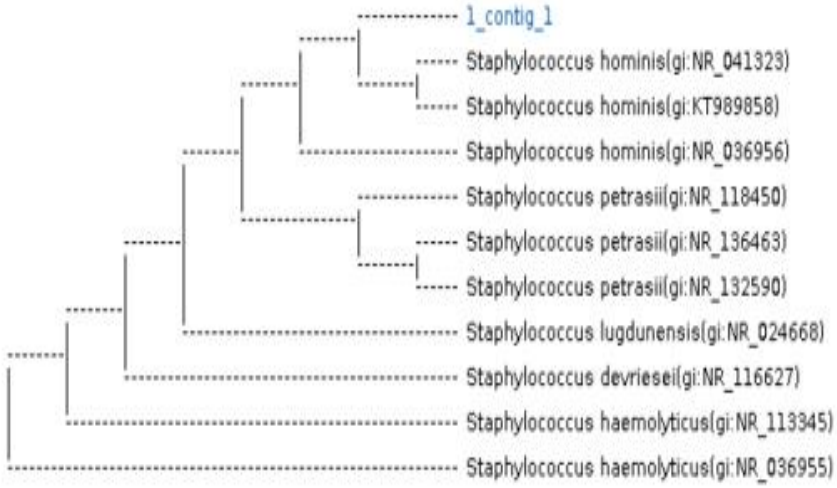

Figure 3.6. Phylogenetic tree constructed via likelihood approach demonstrating the query sequence being identified as *Staphylococcus hominis*.

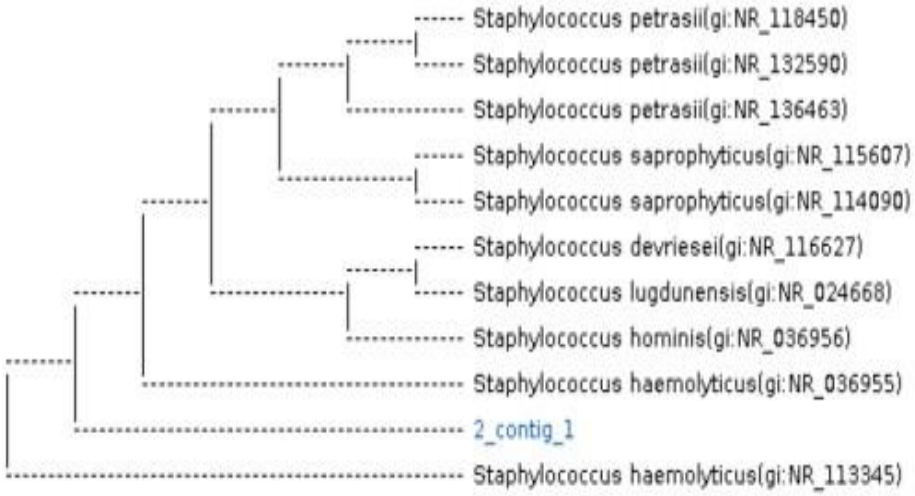

Figure 3.7. Phylogenetic tree constructed via likelihood approach demonstrating the query sequence being identified as *Staphylococcus haemolyticus*

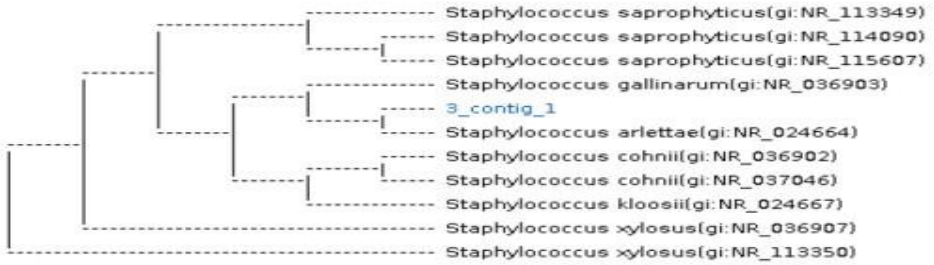

Figure 3.8. Phylogenetic tree constructed via likelihood approach demonstrating the query sequence being identified as *Staphylococcus arlettae.*

Discussion

Septicemia has a high mortality rate and is a worldwide health concern [15]. The germs that cause infections change through time, location, and patient age, therefore this area has to be monitored often [16]. It has been proven that prognosis may be greatly improved with the proper identification of patients, infectious agent, microbiological investigation, and therapeutic strategy [17]. Avoiding such epidemics requires prompt antibiotic selection and administration in patients with septicemia [18]. Acinetobacter baumannii and Staphylococci are two of the most common bacterial causes of severe sepsis. There is a growing public health concern due to the rising prevalence of antibiotic resistance among these bacterial pathogens [19]. The purpose of this research was to isolate, identify, and characterize Acinetobacter baumannii and methicillin-resistant Staphylococci from patients with septicemia. Our study indicated that out of 100 people, 62 (62%) had culture-positive sepsis and 38 (38%) did not. This percentage is quite similar to that reported by [20], who counted 1,133 (42% of total) instances in which cultures were positive. The same proportion of culture-positive cases (295) and culture-negative cases (164%) were reported [21]. Our study's proportion of culture-positive patients was quite close to the 31–47% range that has been reported in previous studies [22, 23]. Whereas 52% of cases were culture negative in Kim et al.'s (2019) research. Almost in accordance with the isolated 21.9% A. baumannii and 35% CoNS found in previous studies [24], we found 37.19% CoNS and 17.74% A. baumannii in our investigation. Our results are consistent with those of a Chinese investigation that also found CoNS (63.1%) [25]. Similarly, CoNS (15%) and A. baumannii (20%) were the most often recovered species from sepsis patients, with 48% CoNS being isolated

from these individuals. Similarly, 17.14% were found to be A. baumannii isolates. In our research, gram-positive isolates predominated over gram-negative ones by a large margin. However, gram-negative bacteria are more common than gram-positive ones [28]. There was a significant correlation between the occurrence of sepsis and a person's gender [29]. Our findings, showing a reduced risk of sepsis in females compared to men, provide conclusive evidence for this. Our research showed that male patients were more likely to be diagnosed with bacterial sepsis than female patients, with a little neonatal preponderance. Septicemia occurred more often in men (59%) than in females (41%). In addition, they stated that 35% of newborns had proven sepsis. In our research, we found that infants made up the vast majority of the population. We found numbers that were consistent with the widely reported 62% and 37% sepsis incidence rates in male and female patients, respectively. We found that A. baumannii (36.36%) and Staphylococci (47.82%) were more common in patients aged 0-18 years old and in patients aged 50 and older, respectively [30]. Men between the ages of 0 and 18 were more likely to have A. baumannii and 47% more likely to have CoNS. These results corroborate the widely reported 65% incidence rate of A. baumannii in male patients aged 0–18 years and the 7% incidence rate reported for female patients aged 41–60 years. It's in line with our findings, approximately [31]. Because it gives a clear picture of susceptibility to various classes of antibiotics, the antibiotic sensitivity assay is a very important and useful test for determining which medicines will work best against a given bacterial isolate [32]. Our data showed that 37.09 percent of the CoNS isolated from septic patients were resistant to methicillin. In a study using CoNS, similar to this one, 70% of the CoNS were resistant to methicillin. Our data revealed that 95% of CoNS were resistant to Cefoxitin. Consistent with our findings, this study found that 68% of the CoNS tested were resistant to Cefoxitin. In hospitals all throughout the globe, patients have become ill due to an outbreak of Acinetobacter baumannii. There is a growing fear that these isolates contribute to the epidemic of multidrug-resistant A. baumannii that has spread across hospitals. Our research found that 81.8% of A. baumannii were MDR strains and 18.18% were sensitive strains [33]. These findings are consistent with those of who also discovered that more than 75% of A. baumannii isolates were MDR. Cefotaxime- and imipenem-resistant strains of A. baumannii comprised 100% of the isolates in our investigation. Our research confirmed a similar 91% resistance. Antibiotic resistance among Acinetobacter species is worrisome because it might eventually spread to most currently used antibiotics. In particular, several multidrug-resistant Acinetobacter species have been found across the world. Our findings confirmed the presence of both A. baumannii and A. junii, as previously hypothesized [34]. A. baumannii was also found in individuals with sepsis. The most common CoNS that result in septicemia are Staphylococcus haemolyticus, Staphylococcus wareri, Staphylococcus epidermidis, and Staphylococcus hominis. During our investigation, we discovered S. epidermidis, S. haemolyticus, and S. hominis. In a similar vein, S. epidermidis was verified to have been isolated from sepsis patients in South Africa. S. epidermidis and S. haemolyticus were also isolated from septic patients, as we found in our research [35].

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