


# Antimicrobial Resistance Pattern of Bacterial Meningitis Among Patients in Quetta, Pakistan

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**Introduction:** Bacterial meningitis (BM) is a lethal infectious disease that persists as a substantial cause of morbidity and mortality. The antimicrobial drug resistance in BM pathogens poses a major threat to the community while raising challenges for clinicians. For the first time, we elucidate the incidence rate of BM and its drug resistance among the population of Quetta, Balochistan.

**Methods:** A cross-sectional study was conducted (from January 2018 to March 2021) among meningitis patients admitted to government hospitals in Quetta. The cerebrospinal fluid (CSF) specimen was collected and processed for microbiological and cytological analysis. Bacterial isolates were identified and confirmed using phylogenetic analysis of the 16S rRNA gene.

**Results:** We found 35.9% (321/894) confirmed cases of BM among the population of Quetta, Balochistan. The identified bacterial isolates comprised *Streptococcus pneumoniae* 14.7% (130/894), *Staphylococcus aureus* 6.9% (61/894), *Neisseria meningitidis* 5.7% (51/894), *Haemophilus influenzae* 2.5% (22/894), *Escherichia coli* 4.5% (40/894), and *Klebsiella pneumoniae* 1.9% (17/894). A high percentage in male patients of 20.8% (186/894) was identified as compared to female patients of 15.1% (135/894). Extreme age groups such as infants (age range: 1 month to 1 year) and adults (age range: 61 years to 99 years) with low immunity were critically affected by BM. Clinical parameters such as abnormal CSF appearance, pH, and high WBCs in BM patients with endocarditis as the underlying disease were significantly at elevated risk of fatal outcome. 16S rRNA gene phylogenetic analysis confirmed the evolutionary similarity of isolated strains with prevailing pathogenic strains in Asia. Furthermore, age was identified as a significant risk factor for clinical outcomes.

**Conclusion:** The majority of the identified BM patients belonged to rural areas with limited health-care facilities and paramedic staff. The high case fatality rate 11% demonstrates increased antibiotic resistance among BM isolates, thereby stimulating its devastation in underdeveloped regions. Timely detection and intensive treatment of BM may improve critical outcomes in patients.

**Keywords:** bacterial meningitis, cerebrospinal fluid, 16S rRNA, incidence rate, drug resistance

## Introduction

Bacterial meningitis (BM) is a serious global health challenge. BM involves inflammation of the meninges that protect the human brain and spinal cord. Bacterial infection, if left untreated, progresses to bacteremia and evades the immune system. It eventually infiltrates and disrupts the blood-brain barrier (BBB) and the nervous system.<sup>1</sup> The classical signs such as fever, headache and neck stiffness were frequently observed among BM patients.<sup>2,3</sup> The commonly

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known causative agents of BM are *Streptococcus pneumoniae*, *Haemophilus influenzae type b*, *Streptococcus group B*, *Staphylococcus aureus*, *Listeria monocytogenes* and *Neisseria meningitidis*.<sup>1</sup> The case-fatality rate of BM is high and varies over time, location, and host characteristics such as patient age, gender etc.<sup>4–7</sup> High morbidity and mortality rates are commonly reported among underdeveloped countries.<sup>6</sup> In untreated patients, the fatality ratio might increase by up to 70%, while one out of five survivors could be afflicted with neurological disabilities.<sup>6,8,9</sup> Early screening of pathogens with relevant treatment may prevent the case fatality rate and neurological complications. Antibiotics play an imperative role in reducing the fatality rate of infectious diseases. In spite of advancements in the healthcare system and therapeutics, the increased mortality rate is concerning.<sup>10</sup> For a long time, penicillin, cephalosporin, fluoroquinolones, or aminoglycosides were empirical therapy to treat BM. Nevertheless, these antibiotics were ineffective against resistant BM isolates.<sup>9</sup> For example, third-generation antibiotics (cephalosporin) are ineffective against *Escherichia coli* meningitis, as it increases mortality and morbidity rates as well as contributing to complications in 25 to 50% of survivors.<sup>5</sup> This prompt emergence of antimicrobial resistance (AMR) has raised substantial health concerns in developing and under-developed countries. Among diagnostic tests to identify BM, bacterial culturing remains a gold standard.<sup>5</sup> However, bacterial culturing of CSF sometimes lacks sensitivity owing to the use of antibiotics prior to lumbar puncture (LP), as well as low quantity and quality of CSF, which impedes the identification of causative agents. Molecular based identification is vital for confirmation of pathogens in biological samples. Targeting 16S rRNA genes (an evolutionarily conserved region) offers prompt detection of bacteria. Furthermore, 16S rRNA associated phylogenetic analysis of unknown isolates can identify evolutionary relationships between the reported strains. Monitoring molecular epidemiology and genetic variation of pathogenic strains helps to track microbial resistance and prevent future outbreaks of infectious diseases.<sup>11</sup> Therefore, periodic inspections are vital for evaluating fatal diseases.

Balochistan is the largest province in Pakistan with scarcity of health-care resources, impoverished population, with low socioeconomic status compared to its sister provinces. Inadequate epidemiological representation of bacterial infections and their emerging antibiotic resistance is a major challenge in Balochistan.<sup>12,14</sup> The

epidemiological data assists in making decisions in terms of therapeutic and curative approaches among BM patients. However, the data for meningitis are insufficient in Pakistan. Balochistan has inadequate resources and poses immense challenge in retrieving BM data; as a result, this disease remains neglected in the province with poor management and prognosis. Here, we for the first time explored the etiological cause of BM and its antibiotic resistance patterns among patients in Quetta City, Balochistan Province from 2018 to 2021. We aimed to evaluate clinical complications, risk factors and comorbid issues associated with BM in the region and its mortality rate among the patients.

## Materials and Methods

### Samples Collection

A total of 894 patients suspected of having BM were admitted to Bolan Medical Complex Hospital (BMCH) and District Hospital Quetta (DHQ) from 1st January 2018 to 31st March 2021. Both are government hospitals with tertiary medical health-care facilities located in Quetta, the provincial capital of Balochistan, Pakistan. Oral and written consent was taken from patients and from their parents/guardians in the case of children, before collecting CSF samples. One CSF sample (~1–3mL) from each patient was collected via LP. Samples were collected aseptically in a sterile tube according to the guidelines of the ethical committees of hospitals and institutions. This research was conducted according to the Helsinki declaration, the University of Balochistan (CAS/45/15-20), and the hospital's ethical committee's permission (E.C 18–12/2017). The collected samples were immediately processed for further microbiological processing at the Center for Advanced Studies in Vaccinology and Biotechnology (CASVAB), University of Balochistan, Quetta.

### Patients' Demographic Data

To identify risk factors among BM patients in Quetta, epidemiological data were collected. A questionnaire was constructed to record demographics such as gender, age group, and socioeconomic status. The following groups were included according to their salary/income in Pakistani Rupees (PKR) per month: upper class (>50,000 PKR), middle class (21,000–50,000 PKR) and lower class (<20,000 PKR). Educational status was identified as literate and illiterate. The clinical features of patients were

recorded as high fever, unconsciousness, seizures/partial fits (if age below 5 years), and neck stiffness.<sup>4</sup> Comprehensive examinations of all patients were done by professional doctors in an emergency, pediatrics, and neuro-medicine wards before LP.

## Laboratory Examination

Extensive investigation of CSF samples included: Gram staining, culture, CSF pH, appearance, WBC count, and glucose and protein level. Gram staining and culturing of CSF specimens were immediately performed. Delay in microbiological procedures and use of antibiotics before LP might affect the sensitivity of staining and culturing. Only positive samples in culture for bacterial isolates were considered in this study. Moreover, analysis for mycobacteria, fungal, or viral pathogens was performed at the doctor's discretion. The inclusion criteria for BM were a) identification of a specific bacterial agent (from CSF) in culture media, clinical history, and inflammation of the meninges b) Acute BM clinical signs and symptoms include fever, fits, vomiting, headache, neck stiffness, and unconsciousness. c) CSF has distinct characteristics such as increased WBCs, low glucose levels, and a high protein level.

## Isolation and Identification

The CSF samples were centrifuged, and the pellets containing isolates were inoculated on blood and chocolate agar plates. Inoculated plates were aerobically incubated with 5% CO<sub>2</sub> for approximately 48 hours at 37°C. After the incubation period, the colonies of bacteria were

identified and observed using standard methods such as gram staining and PCR identification.

## Sanger Sequencing and Phylogenetic Analysis of Isolates

PCR of the 16S rRNA gene was further confirmed. The total genomic DNA of isolates was extracted via a DNA purification kit (GeneAll, Korea). The 16S rRNA gene was used for identification with primer sequences for each specific primer. These primers were used to amplify their respective PCR fragments (Table 1). The sequencing of the 16S rRNA gene of all the isolates was shipped to the MacroGen Company. The ABI genetic analysis systems used the standard forward and reverse 16S rRNA primers for sequencing of the isolates. The obtained sequences were blasted with reported sequences in the NCBI database. Furthermore, the similarities among the reference sequences were analysed in bioedit software. The sequence consensus that had a similarity of 90% and above were included in this study.<sup>14</sup> Multiple sequence alignment was also performed. For the phylogenetic tree, the analysis was performed in MEGA X.<sup>15</sup> First, clustering was performed via the Neighbor-joining method. Further, the analysis of the tree was conducted by the maximum composite likelihood method. Finally, the cut-off values ( $\geq 75\%$ ) for each node<sup>15</sup> were determined.

## Antibiotic Susceptibility Test

The antibiotic susceptibility test was performed according to the standard method recommended by the CLSI. We used 12 antibiotics such as meropenem (10 µg), ceftazidime (30 µg),

**Table 1** The Primer Sequences for Diagnosis of Bacterial Isolates BM Patients

S. No.	Strain	Primer 16S rRNA	bps	Tm Values	Reference
1.	<i>E. coli</i>	Forward primer= GGGAGTAAAGTTAATACCTTTGC Reverse primer= CTCAAGCTTGCCAGTATCAG	204	58	46
2.	<i>S. pneumoniae</i>	Forward primer= CCACACTGGGACTGAGACAC Reverse primer= TCAACCTTGCGTCTACTC	598	59	This study
3.	<i>S. aureus</i>	Forward primer = ACGGTCTTGCTGTCACTTATA Reverse primer = TACACATATGTTCTCCCTAATAA	257	57	47
4.	<i>K. pneumoniae</i>	Forward primer= TCTGAGAGGATGACCAGCCA Reverse primer= GTTACGGCGTGGACTACCA	525	59	This study
5.	<i>N. Meningitidis</i>	Forward primer = TTTTGGACAATGGGCGCAAG Reverse primer = GGGTCCCCGTCAATTCCTTT	568	59	This study
6.	<i>H. influenzae</i>	Forward primer = ATACCCTGGTAGTCCACGCT Reverse primer = AGTTGCAGACTCCAATCCGG	528	60	This study

ceftriaxone (30 µg), erythromycin (15 µg), amoxicillin (25 µg), rifampicin (05 µg), oxytetracycline (30 µg), gentamicin (10 µg), vancomycin (30 µg), ciprofloxacin (05 µg), levofloxacin (05 µg) and amikacin (30 µg). The opacity of the inoculums was adjusted to 0.5 McFarland standards. The isolates were well spread over Mueller-Hinton Agar (MHA) (Oxoid Limited, England) plates whereas fastidious bacterial isolates were subcultured on 5% sheep blood supplemented MHA plates. The antibiotic discs were placed at an equal distance on the bacterial inoculated plates. Finally, the plates were incubated for 24 hours at 37°C with 5% CO<sub>2</sub> and results were recorded (mm).<sup>16</sup>

## Statistical Analysis

The demographics of the patients with their CSF characteristics and clinical features were analysed using multinomial regression and Pearson chi-square. The frequency of the antibiotic susceptibility test was analysed using SPSS version 20 (SPSS Inc., Chicago, IL, USA). Those values considered significant had a  $P \leq 0.05$ .

## Results

### Demographics of Patients

A total of 894 patients were enrolled in this study, out of which 35.9% (321/894) of the cases were BM positive (Figure 1). The majority of BM positive cases were from BMCH, 20.6% (184/894) and DHQ, 15.3% (137/894). Of 321 BM positive cases, high percentage of male patients 20.8% (186/894) were infected than 15.1% (135/894) females. BM were significantly found among children under one year old (range: 1 month-3 months; and 4 months-1 year) and in adults with age ranging between 61 and 99 years with 10.6% (94/894), 8.9% (80/894) and

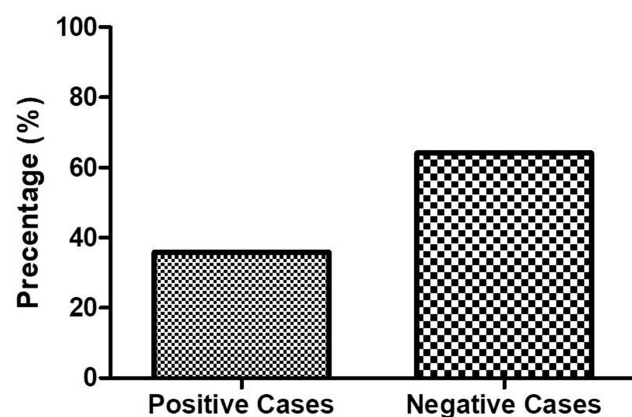


Figure 1 Frequency of positive bacterial meningitis cases.

5.7% (51/894) frequencies, respectively (Table 2). Mostly, affected patients belonged to the Pashtun ethnic group with 19.7% (176/894). The majority of the patients belonged to the rural areas of Balochistan 18.2% (163/894). The total case fatality rate was 11% (36/321) among the BM patients in 2018–2021 (Table 2).

Gram staining of the samples was compared to culture to identify the validity of the results (Table 3). The positive predictive value and negative predictive value of gram staining are 100% and 67.9%, respectively. The McNemar test determined that the slight agreement between gram staining and bacterial culture (Kappa = 0.191) was significant ( $p < 0.001$ ).

The persistent bacterial isolates from CSF of various age groups were *S. pneumoniae* 14.7% (130/894), *S. aureus* 6.9% (61/894), *N. meningitidis* 5.7% (51/894), *H. influenzae* 2.5% (22/894), *E. coli* 4.5% (40/894), and *K. pneumoniae* 1.9% (17/894). In CSF of children aged less than 1 year and older adult patients (61+ years), *S. pneumoniae*, *N. meningitidis*, *E. coli*, and *S. aureus* were widespread while *H. influenzae* and *K. pneumoniae* were dominant among ages 13 years to 60 years old (Table 4).

### Clinical Parameters

The CSF 24.4% (218/894) of patients was collected prior to antibiotic treatment, while in 11.5% (103/894) of patients the CSF was collected within 24 hours of antibiotic inception (Table 5). The majority of the cases 21.3% (190/894), had significantly elevated WBC counts ranging from 101 to 2000 cells/mm<sup>3</sup>. About 34.5% (308/894) patients had a raised protein level (>45 mg/dL), while 20.8% (186/894) patients had predominantly high neutrophil levels (range >61–90%) in CSF. The low glucose level predominates the cases with 27.7% (248/894), range from 0 to 44 mg/dL (Table 5). Furthermore, the frequently observed clinical features in all BM patients during the course of hospitalization were fever 35.9% (321/894), followed by headache 30.2% (270/894), unconsciousness 18.6% (167/894), and neck stiffness 16.7% (150/894) (Figure 2).

Majority of BM afflicted patients had underlying diseases such as 18% (161/894) had bacteremia, 17.3% (155/894) with severe pneumonia, followed by chronic obstructive pulmonary disease (COPD) 11.7% (105/894), and sinusitis 10.2% (91/894). The least comorbid diseases were diabetes mellitus 3.2% (29/894), otitis media 2.7% (24/894), and endocarditis 2.3% (21/894) (Table 5).

**Table 2** Demographics of Bacterial Meningitis Infected Patients

Characteristics	BM Cases n(%)	Non-BM Cases n(%)	Total n(%)	p-value
<b>Total</b>	321 (35.9%)	573 (64.1%)	894 (100%)	
<b>Gender</b>				
Male	186 (20.8%)	342 (38.3%)	528 (59.1%)	0.611
Female	135 (15.1%)	231 (25.8%)	366 (40.9%)	
<b>Among various age groups</b>				
1 month- 3 months	94 (10.6%)	42 (4.7%)	136 (15.2%)	0.001*
4 months- 1 year	80 (8.9%)	128 (14.3%)	208 (23.3%)	
2 years-12 years	46 (5.1%)	112 (12.5%)	158 (17.7%)	
13 years-19 years	13 (1.5%)	46 (5.1%)	59 (6.6%)	
20 years-40 years	17 (1.9%)	73 (8.2.4%)	90 (10.1%)	
41 years-60 years	20 (2.2%)	85 (9.5%)	105 (11.7%)	
61 years-99 years	51 (5.7%)	87 (9.7%)	138 (15.4%)	
<b>Resident</b>				
Rural	163 (18.2%)	215 (24.0%)	378 (42.3%)	0.001*
Urban	158 (17.7%)	358 (40.0%)	516 (57.7%)	
<b>Ethnicity</b>				
Pashtun	176 (19.7%)	306 (34.2%)	482 (53.9%)	0.343
Baloch	68 (7.6%)	113 (12.6%)	181 (20.2%)	
Punjabi	51 (5.7%)	117 (13.1%)	168 (18.8%)	
Hazara	26 (2.9%)	37 (4.1%)	63 (7%)	
<b>Socioeconomic status</b>				
Lower class <20K PKR per month	173 (19.4%)	243 (27.2%)	416 (46.5%)	0.003*
Middle class 21k-50k PKR per month	119 (13.3%)	255 (28.5%)	374 (41.8%)	
High class >50k PKR per month	29 (3.2%)	75 (8.4%)	104 (11.6%)	
<b>Education level</b>				
Illiterate	234 (26.2%)	361 (40.5%)	595 (66.6%)	0.003*
Literate	87 (9.7%)	212 (23.7%)	299 (33.4%)	
<b>Survival rate</b>				
Mortality	36 (4%)	13 (1.5%)	49 (5.5%)	0.001*
Survive	285 (31.5%)	560 (63%)	867 (94.5%)	
Case fatality rate	36 (11%)	-	36 (11%)	
<b>Hospital admitted</b>				
BMCH	184 (20.6%)	322 (36.0%)	506 (56.6%)	0.745
DHQ	137 (15.3%)	251 (28.1%)	388 (43.4%)	

**Note:** \*Statistically significant; 20K= 20,000; 50k= 50,000; PKR = Pakistani Rupee.

**Abbreviations:** BMCH, Bolan Medical Complex Hospital; DHQ, District Hospital Quetta.

The essential prognostic factors for the evaluation of fatal and non-fatal outcomes of BM diseases are listed in Table 5. The statistical analysis of variables such as CSF laboratory parameters, underlying diseases, and clinical features revealed significant prognostic factors such as

CSF pH ( $P \leq 0.01$ ), CSF appearance ( $P \leq 0.001$ ), CSF WBCs ( $P \leq 0.001$ ), and endocarditis ( $P \leq 0.05$ ). Furthermore, the regression showed that BM patients with abnormal CSF appearance, pH, and high WBCs were at higher risk of fatality compared to the reference

**Table 3** Comparative Analysis of Bacterial Culture and Gram Staining Among Bacterial Meningitis Cases

Test Performed	Bacterial Culture BM Cases		Total
	Positive Cases	Negative Cases	
Gram Staining positive cases	50	0	50
Gram Staining negative cases	271	573	844
Total	321	573	894

group. The likelihood of case fatality was increased even more if the BM patients had endocarditis as an underlying disease.

### Sanger Sequencing and Phylogenetic Analysis

The 16S rRNA PCR-based detection of bacteria in CSF of BM patients was confirmed the presence of *S. pneumoniae* 14.7% (130/984), *S. aureus* 6.9% (61/984), *N. meningitidis* 5.7% (51/984), *H. influenzae* 2.5% (22/984), *E. coli* 4.5% (40/984), and *K. pneumoniae* 1.9% (17/984) (Figure 3). We submitted the sequences to GenBank, NCBI, under following accession numbers: *S. aureus* (MW148201.1, MW148202.1, MW148203.1, MW148204.1, MT075573.1), *S. pneumoniae* (MW405781, MW405782, MW405783, MW405784, MW405785, MT075569.1), *H. influenzae* (MZ348556, MZ348557, MZ348558, MZ348559, MZ348560) *N. meningitidis* (MZ318247, MZ318248, MZ318249, MZ318250, MZ318251), *E. coli* (MT075572, MT916768, MT916769, MT916770); *K. pneumoniae* (MW208813, MW208814, MW208815, MW208816, MW208817, MT102939). The phylogenetic analysis of 16S rRNA gene sequences showed the genetic relationship between our

isolated strains and the reference strains (Figure 4). Isolated *E. coli* strains showed a cluster with two reference strains (KY655034 and KY655046) isolated from the blood cultures of Turkish patients (Figure 2). Isolated *K. pneumoniae* strains clustered with pathogenic *K. pneumoniae* strain (KT261771 and KT261773) isolated from Chinese patients. *N. meningitidis* strains showed similarity with reference strain isolated from American patient's CSF (NR\_104946.1). *H. influenzae* strains showed similarity with Japanese (AB377164.1) and Nigerian (AM420172.1) reference strains. *S. aureus* strains clustered with Saudi Arabian and Pakistani reference strains isolated from hospitalized patients. The isolated *S. pneumoniae* strains had high similarity with Japanese (NR\_114172.1) and Hong Kong (AY525795.1) strains separated from the sputum of patient. The phylogenetic tree showed the genetic and evolutionary relationship between our isolates in addition to pathogenic strains prevailing in Asia (Figure 4).

### Antibiotic Susceptibility Test

We estimated antibiotic susceptibility of all bacterial isolates from BM patients (see Table 6). *S. aureus* isolates showed highest resistant to erythromycin 90.2% (55/61) and oxytetracycline 65.6% (40/61), while demonstrated high sensitivity against ciprofloxacin 96.7% (59/61) and levofloxacin 73.8% (45/61). *S. pneumoniae* isolates were 100% (130/130) susceptible to both levofloxacin and vancomycin. However, it showed highest resistant against amoxicillin 100% (130/130) and rifampicin 59% (77/130). All *H. influenzae* isolates were 100% (22/22) susceptible to meropenem, ceftazidime and levofloxacin. *N. meningitidis* isolates were highly susceptible to meropenem 100% (51/51), rifampicin 98% (50/51), ceftriaxone 96.1% (49/51) and ciprofloxacin 92.2% (47/51). *E. coli* isolates demonstrated high resistance rate against

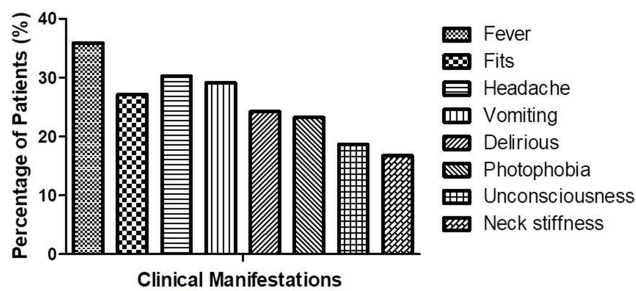
**Table 4** Incidence Rate of Isolated Bacterial Meningitis Pathogens Among Various Age Groups

Bacteria Causing Meningitis	Among Various Age Groups							Total n(%)
	1 Month–3 Months n(%)	4 Months–1 Year n(%)	2–12 Years n(%)	13–19 Years n(%)	20–40 Years n(%)	41–60 Years n(%)	61–90 Years n(%)	
<i>E. coli</i>	25 (2.8%)	12 (1.3%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	3 (0.3%)	40 (4.5%)
<i>S. aureus</i>	12 (1.3%)	9 (1.0%)	13 (1.5%)	7 (0.8%)	6 (0.7%)	3 (0.3%)	11 (1.2%)	61 (6.8%)
<i>K. Pneumoniae</i>	5 (0.6%)	7 (0.8%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	5 (0.6%)	17 (1.9%)
<i>S. pneumoniae</i>	17 (1.9%)	27 (3.0%)	32 (3.6%)	6 (0.7%)	11 (1.2%)	17 (1.9%)	20 (2.2%)	130 (14.5%)
<i>H. influenzae</i>	5 (0.6%)	8 (0.9%)	1 (0.1%)	0 (0%)	0 (0%)	0 (0%)	8 (0.9%)	22 (2.5%)
<i>N. meningitidis</i>	30 (3.4%)	17 (1.9%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	4 (0.4%)	51 (5.7%)

**Table 5** Prognostic Factors of Bacterial Meningitis Infected Patients

Characteristics	Total BM Cases n(%) / Out of 894	Non-Fatal BM Cases n (%)	Fatal BM Cases n (%)	P value	Odd Ratio	95% Confidence Interval	
						Lower	Upper
Male	35 (61.4%)	159	27	0.512	0.634	0.0162	2.476 <sup>a</sup>
Female	22 (38.6%)	126	9				
<b>Antimicrobial therapy before LP</b>							
Within 24hrs	103 (11.5%)	89	14	0.909	1.097	0.225	5.341 <sup>b</sup>
After 24hrs	218 (24.4%)	196	22				
<b>CSF pH</b>							
7.32–7.5	90 (10.1%)	69	21	0.01*	0.129	0.026	0.645 <sup>c</sup>
7.6–8	231 (25.8%)	216	15				
<b>CSF appearance</b>							
Clear	120 (13.4%)	112	8	0.001*	22.847	3.830	136.287 <sup>d</sup>
Turbidity	201 (22.5%)	173	28				
<b>CSF protein level (Range)</b>							
14–45mg/dL	13 (1.5%)	11	2	0.993	1.015	0.038	27.049 <sup>e</sup>
> 45mg/dL (Range; 46–2500mg/dL)	308 (34.5%)	274	34				
<b>CSF glucose level (Range)</b>							
45–100mg/dL	64 (7.2%)	58	6	0.449	2.205	0.285	17.058 <sup>f</sup>
>100mg/dL (Range; 100–250 mg/dL)	9 (1%)	9	0	0.998.	6017.95	6015.951	6021.951 <sup>f</sup>
Low ≤45 mg/dL (Range; 0–44 mg/dL)	248 (27.7%)	218	30				
<b>CSF WBCs (Range)</b>							
21 to 100 cells/mm <sup>3</sup>	86 (9.6%)	73	13	0.117	4.694	0.678	32.518 <sup>g</sup>
101 to 2000 cells/mm <sup>3</sup>	190 (21.3%)	178	12	0.001*	24.177	3.932	148.644 <sup>g</sup>
≥2001 cells/mm <sup>3</sup>	45 (5%)	34	11				
<b>Neutrophils</b>							
>10–≥30%	34 (4%)	25	9	0.118	0.184	0.022	1.535 <sup>h</sup>
>31–≥60%	101 (11.1%)	85	16	0.476	0.572	0.123	2.656 <sup>h</sup>
>61–≥90%	186 (20.8%)	175	11				
<b>Underlying diseases</b>							
Diabetes mellitus	29 (3.2%)	23	6	0.918	0.841	0.031	23.017
Hypertension	39 (4.4%)	34	5	0.944	1.082	0.119	9.855
COPD	105 (11.7%)	95	10	0.337	1.862	0.524	6.625
Liver disease	33 (3.7%)	28	5	0.765	0.735	0.097	5.544
Smoker	36 (4.02%)	29	7	0.220	0.092	0.002	4.170
Severe pneumonia	155 (17.3%)	134	21	0.513	0.518	0.072	3.720
Bacteremia	161 (18%)	139	22	0.861	0.853	0.145	5.028
Sinusitis	91 (10.2%)	75	16	0.557	0.633	0.138	2.906
Endocarditis	21 (2.3%)	17	4	0.05*	316.44	0.810	1235
Otitis media	24 (2.7%)	21	3	0.997	1.010	0.005	194.55

**Notes:** <sup>a</sup>relative to female gender; <sup>b</sup>relative to antimicrobial therapy not started within 24hrs; <sup>c</sup>relative to high pH; <sup>d</sup>relative to turbidity of CSF; <sup>e</sup>relative to High protein level; <sup>f</sup>relative to low glucose level; <sup>g</sup>relative to high WBCs (≥2001 cells/mm<sup>3</sup>); <sup>h</sup>relative to high neutrophils level (>61–≥90%); \*p < 0.05 (significant value).

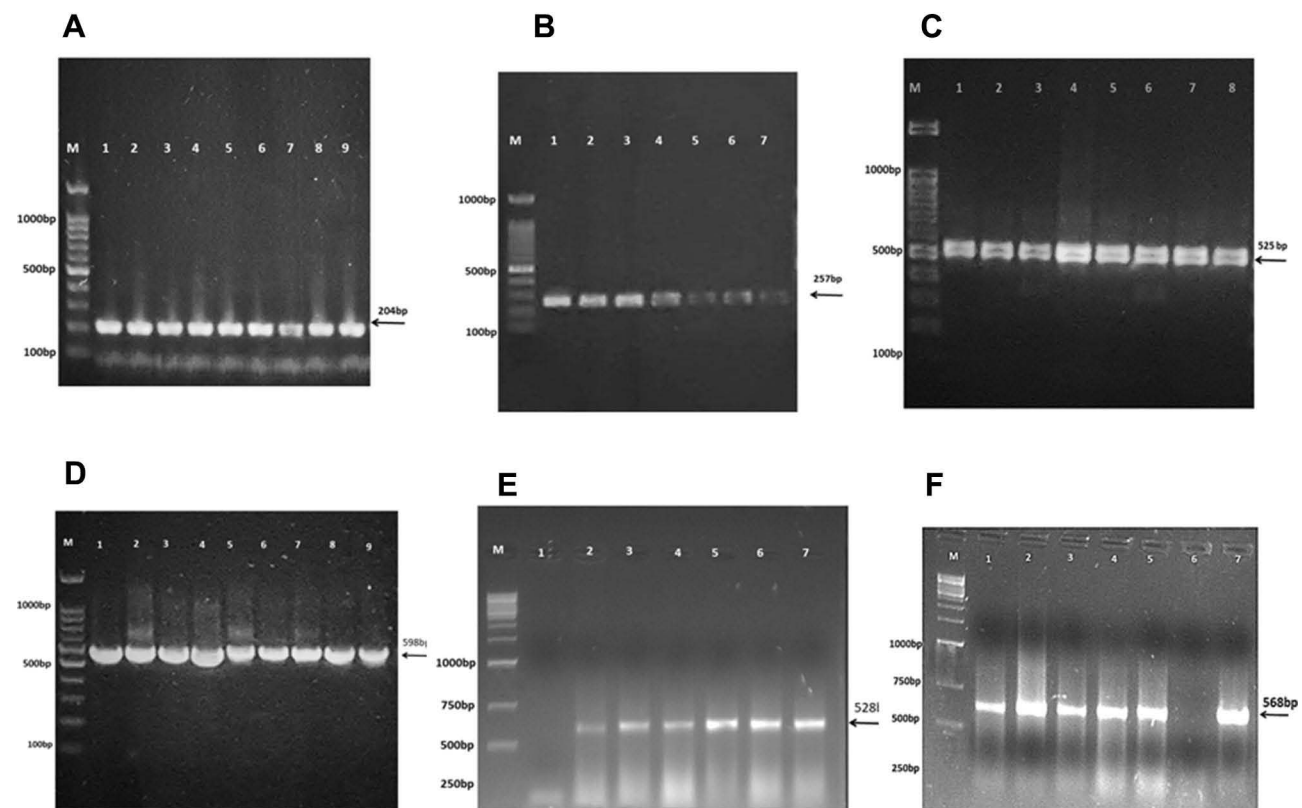


**Figure 2** Clinical manifestations of bacterial meningitis patients.

amoxicillin 80% (32/40) and rifampicin 77.5% (31/40). *E. coli* showed highest susceptibility to meropenem 92.5% (37/40) and amikacin 97.5% (39/40). *K. pneumoniae* isolates were 100% (17/17) resistant against amoxicillin, whereas 88.2% (15/17) resistant to erythromycin and oxytetracycline. Highest susceptible drugs against *K. pneumoniae* were amikacin with 88.2% (15/17), levofloxacin and gentamicin 64.7% (11/17), ceftriaxone 52.9% (9/17), ciprofloxacin and meropenem 58.8% (10/17), respectively (Table 6).

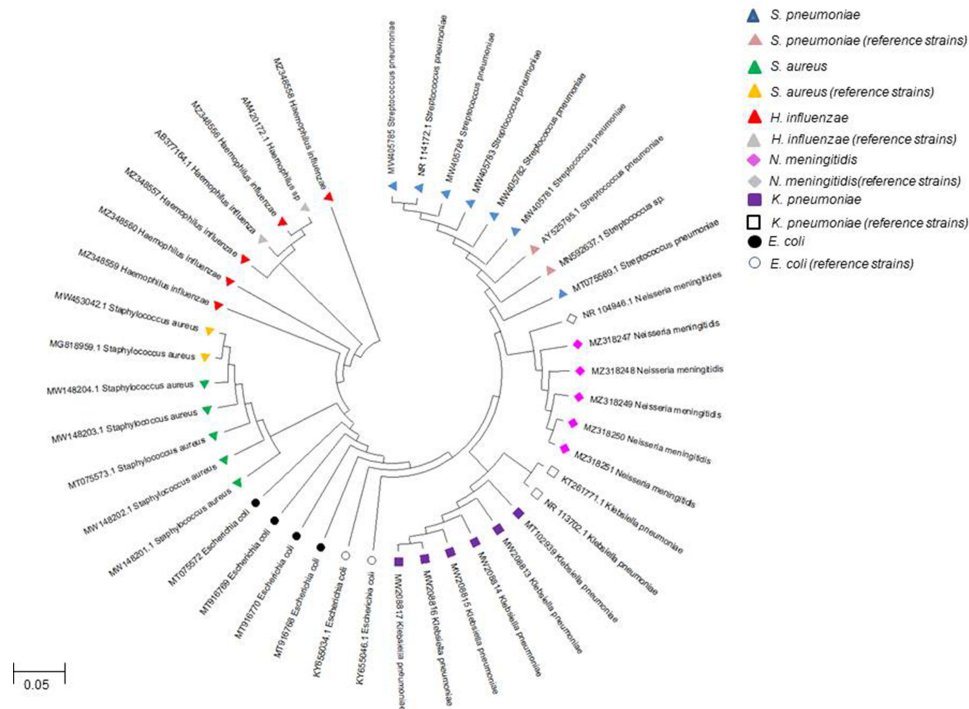
## Discussion

In this study, we identified predominant pathogens of bacterial meningitis among the population of Quetta, Balochistan. The study showed *S. pneumoniae* (14.5%), *S. aureus* (6.8%), *N. meningitidis* (5.7%), *H. influenzae* (2.5%), *E. coli* (4.5%) and *K. pneumoniae* (1.9%) were the main causative agents. Despite variations in frequency of each pathogen, these agents remain the prime cause of BM among population.<sup>17–19</sup> Several studies suggested that *S. pneumoniae*, *S. aureus*, *E. coli* and *K. pneumoniae* were commonly isolated pathogens from CSF of Pakistani children.<sup>20–22</sup> Previously, similar studies conducted in developing and under-developed countries reported prevalence of these pathogenic strains as dominant cause of infections in CSF.<sup>4,20,21,23</sup> There are several factors that influence the specific etiology of the disease such as immune function, status of immunization, age, genetic makeup and geographical location of patient.<sup>17,24</sup> In this study *S. pneumoniae* and *S. aureus* were the predominant pathogens among all the age groups. *S. pneumoniae* globally accounts for 25.1–41.2% of BM



**Figure 3** 16S rRNA gene PCR-based identification bacteria isolated from CSF of bacterial meningitis patients. (A) *E. coli* isolates with 204bp amplified fragments; (B) *S. aureus* isolates 257bp amplified fragments; (C) *K. pneumoniae* isolates 525bp amplified fragments; (D) *S. pneumoniae* isolates 598bp amplified fragments; (E) *H. influenzae* isolates 528bp amplified fragments; (F) *N. meningitidis* isolates 568bp amplified fragments. M= molecular weight marker (1kb).





**Figure 4** Phylogenetic relationship of bacterial isolates from bacterial meningitis patients with their reference strains. *S. pneumoniae* (MW405781, MW405782, MW405783, MW405784, MW405785, MT075569.1); *E. coli* (MT075572, MT916768, MT916769, MT916770); *K. pneumoniae* (MW208813, MW208814, MW208815, MW208816, MW208817, MT102939); *S. aureus* (MW148201.1, MW148202.1, MW148203.1, MW148204.1, MT075573.1); *H. influenzae* (MZ348556, MZ348557, MZ348558, MZ348559, MZ348560); *N. meningitidis* (MZ318247, MZ318248, MZ318249, MZ318250, MZ318251) isolated from BM patients of Balochistan were compared using 16S rRNA gene sequence with other reference strains using neighbor-joining phylogenetic tree. The bootstrap values were based on 1000 replicates in MEGA X software.

cases across all age groups and geographic regions.<sup>25</sup> This high incidence of *S. pneumoniae* is due to endogenous transmission from nasopharynx region to the meninges. The patients infected with pneumococcal meningitis were mostly immunocompromised, had immature nasopharynx (in children), or during dry season when the nasal cavity is particularly susceptible to damage and cracks.<sup>26</sup>

We found weak relationship between ethnicity and BM cases. We found high proportion of Pashtun ethnic group affected with BM as compared to other ethnic groups. Geographically, Balochistan shares its borders with Afghanistan and Iran and consequently receives high influx of patients from these countries. Another reason for the enormous proportion of BM cases was due to the large population of Paktoon living in rural areas of Balochistan, with inadequate health-care facilities, deplorable hygiene, a poor literacy rate, low socioeconomic status, and many clans living in congested houses. Despite inception of the Expanded Program for Immunization (EPI), the incidence of the pathogenic strains has amplified in Balochistan comparative to other regions of Pakistan.<sup>20–22</sup> Although in many cities of Pakistan, vaccination coverage has contributed to

a drastic reduction (72%) in *H. influenzae* related meningitis cases,<sup>20–22,27</sup> whereas in this study we found 2.5% of *H. influenzae* related BM cases. Though, EPI launched the Hib and Pneumococcal Conjugate Vaccine (PCV) programs in 2009 and 2012 respectively. The low frequency of *H. influenzae* in CSF of meningitis patients suggested that initiative of vaccination of Hib in the region has influenced the incidence rate and promoted herd immunity. While we found *S. pneumoniae* is the most common causative agent in BM patients. A high incidence rate of *S. pneumoniae* was observed among children who were unvaccinated or partially vaccinated due to low coverage of vaccination, higher urbanization among the rural population, which led to negligence and low vaccination rate among the population.<sup>12,28</sup> According to USAID report of Pakistan, 1 in every 5 children remains unvaccinated in cities while rural areas have high rate of unvaccinated children (ie 2 out of 3).<sup>13</sup> We also found significantly high percentage of children (less than one year of age) and elderly patients afflicted with *E. coli* and *K. pneumoniae* associated BM. Likewise, Jiang and coworkers found that 28.5% of *E. coli* associated cases in CSF of Chinese children. Another research demonstrated

**Table 6** The Antimicrobial Susceptibility Test Against Bacteria Isolated Bacterial Meningitis Patients

Antibiotics	Conc (µg)	Bacterial Isolates											
		<i>E. coli</i>		<i>K. pneumoniae</i>		<i>S. aureus</i>		<i>S. pneumoniae</i>		<i>H. influenzae</i>		<i>N. meningitidis</i>	
		R n/N (%)	S n/N (%)	R n/N (%)	S n/N (%)	R n/N (%)	S n/N (%)	R n/N (%)	S n/N (%)	R n/N (%)	S n/N (%)	R n/N (%)	S n/N (%)
Amikacin	30	1/40 (2.5%)	39/40 (97.5%)	2/17 (11.8%)	15/17 (88.2%)	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Amoxicillin	25	32/40 (80%)	8/40 (20%)	17/17 (100%)	0/17 (0%)	61/61 (100%)	0/130 (0%)	130/130 (100%)	n.a.	n.a.	n.a.	n.a.	n.a.
Ceftazidime	30	7/40 (17.5%)	33/40 (82.5%)	11/17 (64.7%)	6/17 (35.3%)	n.a.	n.a.	n.a.	n.a.	2/22 (10%)	20/22 (90%)	n.a.	n.a.
Ceftriaxone	30	11/40 (27.5%)	29/40 (72.5%)	8/17 (47.1%)	9/17 (52.9%)	n.a.	n.a.	n.a.	n.a.	3/22 (13.6%)	19/22 (86.4%)	2/51 (3.9%)	49/51 (96.1%)
Ciprofloxacin	5	12/40 (30%)	28/40 (70%)	7/17 (41%)	10/17 (58.8%)	2/61 (3.3%)	59/61 (96.7%)	n.a.	n.a.	1/22 (4.5%)	21/22 (95.5%)	4/51 (7.8%)	47/51 (92.2%)
Erythromycin	15	18/40 (45%)	22/40 (55%)	15/17 (88.2%)	2/17 (11.8%)	55/61 (90.2%)	6/61 (9.8%)	84/130 (64.6%)	46/130 (35.4%)	n.a.	n.a.	n.a.	n.a.
Gentamicin	10	7/40 (17.5%)	33/40 (82.5%)	6/17 (35.3%)	11/17 (64.7%)	13/61 (21.3%)	48/61 (78.7%)	35/130 (27%)	95/130 (73%)	n.a.	n.a.	n.a.	n.a.
Levofloxacin	5	5/40 (12.5%)	35/40 (87.5%)	6/17 (35.3%)	11/17 (64.7%)	16/61 (26.2%)	45/61 (73.8%)	0/130 (0%)	130/130 (100%)	0/22 (0%)	22/22 (100%)	n.a.	n.a.
Meropenem	10	3/40 (7.5%)	37/40 (92.5%)	7/17 (41.2%)	10/17 (58.8%)	n.a.	n.a.	n.a.	n.a.	0/22 (0%)	22/22 (100%)	0/51 (0%)	51/51 (100%)
Oxytetracycline	30	22/40 (55%)	18/40 (45%)	15/17 (88.2%)	2/17 (11.8%)	40/61 (65.6%)	21/61 (34.4%)	27/130 (20.8%)	103/130 (79.2%)	5/22 (22.7%)	17/22 (77.3%)	n.a.	n.a.
Rifampicin	05	n.a.	n.a.	n.a.	n.a.	34/61 (55.7%)	27/61 (44.3%)	77/130 (59%)	53/130 (41%)	3/22 (2.6%)	19/22 (86.4%)	1/51 (2%)	50/51 (98%)
Vancomycin	30	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0/130 (0%)	130/130 (100%)	n.a.	n.a.	n.a.	n.a.

**Abbreviations:** Conc, concentration; R, resistance; S, susceptible; n/N(%), observed value/total tested value (percentage); n.a., not applicable.

*E. coli* (51.3%) and *K. pneumoniae* (1.7%) as common causes of BM among young infants.<sup>4,23</sup> The universally known risk factors responsible for meningitis in children, especially among infants includes: premature birth, low weight, and infection caused by pathogenic bacteria.<sup>29,30</sup> Children less than 1 year (such as infants and neonates) are at higher risk of BM, because maternal antibodies are unable to cross the placenta (after 32 week gestation) or immune systems weaken by the neutrophils and monocyte's phagocytic activity. Moreover, bacterial infections caused by *E. coli* are responsible for meningitis and sepsis in very low birth weight babies at early stage of life.<sup>18,31</sup> However, this disparity in our data could be due to a difference in sample size, mode of collection of CSF, or use of antibiotics prior to LP. Additionally, we found high ratio of male infants afflicted by BM infection as compared to female patients, this imbalance is due to the fact that female infants have strong immune system than male.<sup>32</sup> Additionally, female's estrogen enhances level of Th1 providing stronger immune response against bacterial pathogens while testosterone diminishes the cytokines (interleukin 4 and interferon  $\gamma$ ) generation in T-cells.<sup>32,33</sup>

The common signs and symptoms closely associated with BM include fever, respiratory distress or jaundice, vomiting, and lethargy.<sup>3</sup> We also observed headache, vomiting, fits, fever, and neck stiffness as common symptom among all BM patients. However, the seizures complicate the condition of BM patients, especially in children.<sup>4</sup> These clinical features along with CSF parameters and bacterial culture increase the accuracy, sensitivity, and specificity of the test by many folds in diagnosing BM.<sup>34</sup> In BM patients, elevated numbers of CSF cells, proteins, and reduced levels of glucose are hallmarks of diagnosis. In this study, inflammatory changes such as protein count, glucose level, WBCs, neutrophils, and lymphocytes were prominent in CSF among BM patients. We found underlying chronic diseases and infections such as bacteremia, diabetes mellitus, COPD, severe pneumonia, otitis media and endocarditis as predisposing risk factors of meningitis in patients. Previous studies reported *K. pneumoniae* as a concomitant factor among patients with critical medical conditions such as meningitis, septicemia, liver abscess, pneumonia, and urinary tract inflammation leading to serious ailments and high mortalities (33.3–48.5%).<sup>35,36</sup> Several studies have reported that immuno-compromised conditions such as HIV, diabetes, and post-neurological states may contribute to the progression of bacterial meningitis.<sup>35</sup> Similarly,

endocarditis caused by *S. pneumoniae* and *S. aureus* increases the risk of BM. Another study reported that 2% of BM patients with endocarditis had higher chances (63%) of fatal outcomes.<sup>37</sup> In agreement with this, we found that endocarditis substantially magnifies the risk of fatality in BM patients. We observed significantly higher case fatality (11%) rate among BM patients. According to Government of Pakistan meningitis claims 23,000 children's lives annually,<sup>27</sup> with BM-related case fatalities around 10.1% in 2012.<sup>22</sup> Even though medical research has advanced rapidly with accessibility to effective therapies and potent drugs, the case fatality rates of BM (16–32%) have exponentially accelerated in developing countries.<sup>38,39</sup> This rapid increase in fatality rates is a consequence of lack of basic healthcare facilities, absence of professional paramedics' staff, and the shortage of medical equipments.<sup>12</sup> The bacterial culturing of the CSF is gold standard and routinely performed for diagnosis, yet it is a laborious and time-consuming method. The molecular detection method is suitable for BM patients with single-cell pathogens in CSF. We performed 16S rRNA PCR and phylogenetic based confirmation of bacterial isolated in CSF of BM patients. The amplification and sequencing approach helped to identify taxonomy of isolates. Hence, the particular level of genetic distances and similarities within species of BM isolates and their reference strains suggested conserved close similarity via 16S rRNA sequences. Therefore, early identification of the causative agents is imperative for timely therapeutic and treatment purposes.

Major challenge faced by current healthcare system is continuous rise of antibiotics resistant bacteria. We evaluated AMR and antimicrobial susceptibility (AMS) in all isolated bacterial pathogens from the CSF BM patients. The first (amoxicillin, oxytetracycline), second (levofloxacin, ciprofloxacin), third generation (ceftriaxone, ceftazidime) and other groups of antibiotics were utilized to access resistance. We found *S. pneumoniae*, *E. coli* and *K. pneumoniae* resistant to amoxicillin, erythromycin, and oxytetracycline. Additionally, some isolates showed resistance to 3rd generation antibiotics such as ceftazidime and ceftriaxone. We found *S. pneumoniae* significantly susceptible to vancomycin and levofloxacin. This increasing resistance to 3rd generation antibiotic is alarming as it has intensified infection among people of Balochistan. The antibiotic-resistant of *E. coli* aggravates the occurrence of infections especially in mothers and infants. Majority of infants were affected with *E. coli* associated

meningitis was found 85% resistant to ampicillin.<sup>40</sup> A meta-analysis study revealed that various regions of Pakistan, pathogenic *E. coli* and *Klebsiella species* isolated from several hospitalized patients were highly resistant to 1st generation antibiotics (penicillin 100%, ampicillin 91% and amoxicillin 85%), 2nd and 3rd generation antibiotics such as cefotaxime (82%) and cefaclor (100%) respectively.<sup>41</sup> This resistance is an emerging problem due to misuse and overuse of extended-spectrum cephalosporin drugs.<sup>10</sup> The emerging resistance in *K. pneumoniae* was due to its ability to produce an enzyme that degrades the carbapenem and beta-lactam ring structures.<sup>44</sup> We found *S. aureus* resistant to amoxicillin, erythromycin, rifampicin, oxytetracycline, and gentamicin. Similarly, in various region of Pakistan, *S. aureus* showed high resistance to penicillin (98%), cefoxitin (83%) and levofloxacin (80%). In a recent study, Tigabu and coworkers reported high resistance (66.80%) in *S. aureus* against erythromycin and amoxicillin among adult BM patients in Gondar.<sup>42</sup> Another study conducted by Assegu Fenta and his group declared that high resistance of *S. aureus* was reported against amoxicillin (100%).<sup>43</sup> The high resistance occurs due to exposure of broad range of antibiotics, self-medication, and incomplete dosage of antibiotics, leading *S. aureus* to develop new cellular processes to evade effects of drugs.<sup>44</sup> In this study we found all isolates of *N. meningitidis* and susceptible to all tested antibiotics whereas we found *H. influenzae* is resistance to (2–22%) first, second, and third generation of antibiotics. While Tigabu and group reported 100% susceptibility of *N. meningitidis* isolates against amoxicillin and erythromycin.<sup>42</sup> The variation in antibiotic susceptibility trend among isolates might be due to late diagnostic tests and misuse of antibiotics. Hence, the increasing antibiotic resistance among various pathogens is a serious health problem. The AMR in bacteria utilizes various defense mechanisms such as restricting and pumping out drug from the bacterial cell, degrade or alter the antibiotic's structure to limit its target function.<sup>44</sup> Despite the fact that AMR is natural process, the extensive use and misuse of antibiotics has accelerated emergence of multidrug resistance in pathogens. The AMR led antibiotics to least effective against various infectious diseases. Eventually, AMR increases economic burden due to prolonged hospital admission, expensive medical treatments and high mortality rate.<sup>45</sup> Therefore, the emergence of antibiotic resistance in clinical isolates should be taken into account before prescribing the appropriate drug. On a broad

glance, awareness is required to educate people regarding vaccination courses, personal hygiene, and self-medication. Hence, taking these obligatory steps is vital to control the prevalence of infectious diseases and their antimicrobial resistance in the community.

## Conclusion

This study for the first time investigated the incidence rate of BM in Balochistan, Pakistan. *S. pneumoniae* and *S. aureus* were the leading pathogens for BM and commonly found among all age group. Males were highly affected as compared to females. Extreme age groups such as infants (age range: 1 month–1 year) and adults (age range: 61 years–99 years) with low immunity were at higher risk. High case fatality rate was observed among BM patients. The increasing antibiotic resistances of causative agents are major challenge in treating patients with prudent use of empiric antibiotics. To control BM incidence and its fatality rate, it is important to promote AMR surveillance in all clinics and hospitals to reduce future threats. Timely detection and intensive treatment of BM may improve critical outcomes in patients. Further research is required to track the varying epidemiology of causative agents and risk factors of BM.

## Institutional Review Board Statement

The research protocol was approved by the institutional research ethics committee of hospitals and institutions. This research was conducted according to Helsinki declaration, University of Balochistan (CAS/45/15-20) and hospital ethical committee's permission (E.C 18-12/2017).

## Data Sharing Statement

Data of this study are available from corresponding author on reasonable request.

## Informed Consent Statement

A written consent form was taken from patients and from parents/guardians in case of children to publish this paper. For illiterate participants, the verbal consent was obtained.

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## Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

## Disclosure

All authors declared that they have no competing interests in this work.

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