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Original Article

Microbiology Section

Clinicomicrobiological Profile and Antibiotic Susceptibility Pattern of *Burkholderia cepacia* Complex Isolates from a Tertiary Care Hospital in Southern India: A Cross-sectional Study

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ABSTRACT

Introduction: *Burkholderia cepacia* Complex (BCC) is a group of Gram negative betaproteobacteria with complex taxonomy that causes healthcare-associated infections and hospital outbreaks. BCC is the fourth most pathogenic non fermentative Gram negative bacilli worldwide, following *Burkholderia cepacia*, *Acinetobacter baumannii*, and *Stenotrophomonas maltophilia*, with a prevalence ranging between 10-20% for non fermentative Gram negative bacilli and 5-15% for BCC. Human infections are caused by 22 known species and 14 novel species. Pulmonary BCC infections lead to "Cepacia syndrome," a fatal illness that results in progressive respiratory failure and necrotising pneumonia, leading to early death in 20% of cases.

Aim: To emphasise the disease burden and clinical outcomes of BCC infections, as well as to assess the performance of various methods for BCC detection.

Materials and Methods: A cross-sectional study was conducted at PSG Institute of Medical Sciences and Research, Coimbatore, Tamil Nadu, India. A total of 91,778 samples were received between April 2021 and December 2022, over a period of one year and nine months, to determine the disease burden of BCC. The identification of BCC was carried out using manual culture and sensitivity, VITEK®-2 ID/AST system, Matrix Assisted Laser Desorption Ionisation-Time of Flight Mass Spectrometry (MALDI-ToF-MS), *recA* gene virulence determinant by Polymerase Chain Reaction (PCR), and 16S Ribosomal ribonucleic acid (rRNA) sequencing. Out of 115 manually identified BCC isolates, 56 (48.70%) underwent automated Vitek® 2 ID/AST, MALDI-ToF-MS, *recA* gene PCR, and 16S rRNA sequencing for identification and characterisation. The results were entered into Microsoft Excel, and statistical analysis was performed using the International Business Machines (IBM) Statistical Package for the Social Sciences (SPSS) software version 28.0.

Results: Culture positivity was observed in 16,949 samples (18.47%), among which 3,387 (29.25%) were non fermentative gram negative bacilli. The incidence of *Burkholderia* spp. isolation was 115 (3.4%) out of 3,387 non fermentative gram negative bacilli. The prevalence of BCC among the study population was 115 (0.13%) out of 91,778.

Conclusion: BCC, causing a wide array of infections, results in profound morbidity and mortality, especially in hospital settings. Early identification using Vitek-2 and MALDI-ToF-MS, along with molecular methods like PCR and 16S rRNA sequencing, could be the key to confirming the diagnosis and initiating appropriate management.

Keywords: Bacterial sensitivity test, Genetic testing, Matrix-assisted laser desorption-ionisation mass spectrometry, Multidrug resistance, Sequence analysis

INTRODUCTION

The BCC is a significant healthcare-associated pathogen, causing a plethora of multidrug-resistant infections, namely bacteraemia, urinary tract infection, prosthetic joint infections, septic arthritis, peritonitis, and respiratory tract infections, especially among inpatients. These species can thrive in fluid environments, where they colonise and establish infections, especially in immunocompromised patients. BCC belongs to a complex group of Gram negative Betaproteobacteria, which are aerobic, motile, non sporing, yellow-pigmented, catalase-positive, oxidase-positive, indole-positive, citrate-positive, lysine decarboxylation-positive, polymyxin B-resistant, non lactose fermenting bacteria. BCC encompasses nine distinct species identified as *B. cepacia*, *B. multivorans*, *B. cenocepacia*, *B. stabilis*, *B. vietnamiensis*, *B. dolosa*, *B. ambifaria*, *B. anthina*, and *B. pyrrocinia* [1,2]. BCC causes several hospital outbreaks primarily due to contaminated disinfectants, nebuliser solutions, mouthwash, medical devices, and also intravenous solutions due to contamination of lipid emulsion stoppers [3,4].

Globally emerging multidrug-resistant BCC hampers the prognosis and patient outcomes. Therefore, adequate infection control measures need to be implemented to significantly contain the associated morbidity and mortality of BCC infections. The worldwide prevalence of non fermentative gram negative bacilli infections especially in hospitalised patients is generally higher and ranges between 10-20%, and the incidence of BCC infections in tertiary care hospitals were found to be 5-15% especially among immuno-compromised patients [5-7]. BCC identification by conventional culture and biochemical tests is typically imprecise and inappropriate when it comes to characterising species due to the high similarity between different species. Genus and species levels of the BCC members could be misidentified using commercial systems, where strains of these various species were incorrectly identified as BCC-like organisms like *B. gladioli*, *R. pickettii*, *Alcaligenes* spp., *Pseudomonas* spp., *S. maltophilia*, *Flavobacterium* spp., and *Chryseobacterium* spp. [8,9]. Selective isolation of BCC isolates could be attempted on *B.cepacia* Selective Agar (BCSA), *Pseudomonas Cepacia* (PC) agar, or Oxidation-Fermentation Polymyxin-Bacitracin

Lactose (OFPBL) agar. Automated identification systems, including Phoenix, VITEK-2 ID/AST System, and MALDI-ToF-MS, identify BCC and non BCC species at different specificities. MALDI-ToF-MS could correctly identify most BCC species and exhibited 100% concordance for genus identification and 82% species-level identification, respectively [8].

Several molecular typing techniques are used for precise BCC species differentiation. For example, 16S rRNA gene analysis could be used to distinguish between BCC and non BCC organisms. However, these species exhibit upto 28 intra-species diversities in their 16S rRNA gene sequences, making it unreliable to identify them at the species level through a simple comparison of complete 16S rRNA gene sequences [10]. Among the popular techniques are *recA* PCR Restriction Fragment Length Polymorphism (*recA* PCR-RFLP) and sequence-based analysis. The BCC *fur* gene (ferric uptake regulator protein) is a potential virulence factor for differentiating virulent BCC strains from environmental isolates. Currently, a polyphasic method is used for accurate identification of BCC members [10,11].

Misidentification of non fermentative gram negative bacilli can interfere with patient management and seriously compromise infection control measures, confounding efforts to understand the epidemiology and natural history of infection. Genus-specific BCC *recA* gene PCR is used to detect the virulence factor *recA* gene, which encodes recombinase A [12,13]. *recA* gene PCR-RFLP can help distinguish *Burkholderia* from non *Burkholderia* species. BCC *fur* gene PCR can differentiate clinical isolates from environmental isolates. BCC PCR-RFLP can serve as an epidemiological tool to study the source and aid in early detection. Early detection is crucial for initiating appropriate antibiotic treatment, which poses a practical challenge for treating physicians aiming to improve clinical outcomes [14,15].

Emphasising the disease burden and clinical outcomes of BCC infections, as well as evaluating the performance of various methods for BCC detection, are of paramount importance for effective management. Reports studying the prevalence and molecular detection of BCC in this part of Southern India are scarce. The aim of this study was to emphasise the disease burden and clinical outcomes of BCC infections, and to assess the performance of various methods for BCC detection. The study objectives were to evaluate the clinicomicrobiological profile of BCC and its susceptibility using phenotypic identification methods such as Vitek-2 ID and AST, protein signature identification methods like Bruker Biotyper (MALDI-ToF-MS), and molecular detection of targets through *recA* gene PCR followed by 16S rRNA PCR and sequencing.

MATERIALS AND METHODS

A cross-sectional study was conducted in the Diagnostic Microbiology Laboratory, with a total of 91,778 samples received from April 2021 to December 2022 at the Department of Microbiology, PSG Institute of Medical Sciences and Research, Coimbatore. Institutional Human Ethics Committee clearance (PSG/IHEC/2021/Appr/Exp/077) and waiver of informed written consent were obtained for this project (No. 21/083) as patients were not directly involved in this project.

Inclusion criteria: The study population included specimens sent for culture and susceptibility from suspected or diagnosed BCC infections among patients attending the hospital during the study period.

Exclusion criteria: Respiratory samples from sputum-positive Pulmonary Tuberculosis (PTB) and Influenza (Inf A) Subtype H1N1 (Swine flu)/H3N2 cases were excluded from the study.

Sample size calculation: The required sample size (N) was determined using the formula $N=Z^2P(1-P)/d^2$, where CI is the confidence interval (95%), d is the margin of error (5%), P is the prevalence (9.4% [16]), and Z is 1.96 for a 95% CI. The calculated sample size was 129.93, rounded up to 130.

BCC isolates were identified phenotypically by conventional culture methods based on colony morphology on Blood agar plates, MacConkey agar plates, *Burkholderia cepacia* Selective Agar (BCSA), with incubation at 37°C for 24-48 hours. Growth was demonstrated at 42°C in BCSA, and further confirmation was done by routine diagnostic methods as per standard operating procedures [8,9]. Automated identification and susceptibility testing were performed using the biomeriux Vitek-2 ID and AST System. The clinical BCC isolates were sent to Bruker MALDI-ToF-MS, Microbiological Laboratory, Coimbatore, Tamil Nadu, for identification. Antibiotic susceptibility testing was conducted using the Kirby-Bauer disc diffusion method to evaluate the susceptibility of BCC to ceftazidime, meropenem, minocycline, and cotrimoxazole, following CLSI 2021, M100 32 Edition [17]. Automated identification and susceptibility testing were also performed using the VITEK-2 ID and AST System, and MICs for levofloxacin and chloramphenicol were established. Quality Control (QC) was performed using ATCC 25416 *Burkholderia cepacia* reference strain and ATCC 27853 *Burkholderia cepacia*. QC strains were procured from Hi Media Laboratory Private Limited, India.

Molecular detection of BCC by conventional *recA* PCR involved the following steps: Bacterial colonies were suspended in 50 µL of deionised water and incubated at 94°C for 10 minutes. The suspension was then centrifuged at 13,000g for four minutes, and 25 µL of the supernatant was transferred into Eppendorf tubes. DNA extraction was performed using the Qiagen Extraction kit, following the manufacturer's instructions [15,18]. The DNA extraction kit was procured from HiMedia Laboratory Private Limited, a product of Germany. The extracted DNA was assessed for presence and quantitation using a NanoDrop spectrophotometer. It was then used directly as a template for PCR or stored at -20°C for long-term storage [19].

For amplification of the *recA* gene, a Veriti 96-well Conventional Thermal cycler (Applied Biosystem - Thermo Fisher Scientific) was used. PCR was performed under appropriate thermal conditions for the *recA* gene, following standard reference methods. The primers were procured from HiMedia Laboratory Private Limited, India. To amplify the DNA, 1 µL of the forward primer 5'-TGA CCGCCGAGAAGAGCAA-3' and 1 µL of the reverse primer 5'-CTCTTCTTCGTCCATCGCCTC-3' were added to 13 µL of the master mix (containing dNTPs, PCR buffer 10X, $MgCl_2$, and Taq polymerase), along with 10 µL of the extracted DNA, resulting in a final reaction volume of 25 µL. The specific thermal conditions for *recA* gene amplification were as follows: a) initial denaturation at 94° for three minutes; b) followed by 30 cycles at 94° for one minute; c) annealing at 67° for one minute; d) extension at 72° for two minutes; e) final extension at 72° for 10 minutes [18].

The amplified PCR products of the *recA* gene were analysed by gel electrophoresis. A 1.2% agarose gel prepared in 1X Tris-Borate EDTA buffer with 0.5 µg/mL ethidium bromide was used for this purpose, following the standard method. A 1kb DNA ladder was used as a reference for measuring the amplicons. Additionally, amplified products of ATCC 25922 *Escherichia coli* served as the negative control, while ATCC 25416 *Burkholderia cepacia* served as the positive control for each run. To the rest of the wells, 3 µL of the amplified product and 2 µL of loading dye (bromophenol blue) were added. Electrophoresis of the PCR amplicons was performed at 50 volts for one hour and 30 minutes, and the gel was examined using a Gel Doc EZ imager documentation system by Bio-Rad [19].

A total of 20 *recA* gene-positive BCC isolates were sent for Microbial Identification using 16S rRNA gene Sequencing and Phylogenetics after DNA extraction, ©Hi-Gx360® HiMedia Labs Pvt., Ltd. The gold standard approach, chain termination-based sequencing, also known as Sanger's sequencing technology, was employed. Universal primers were used to PCR-amplify the housekeeping target genes for quick species identification. The amplified PCR product was further purified using salt precipitation. Agarose gel electrophoresis was performed to assess the quality of the PCR amplicons, and the PCR products were subsequently purified. After purification,

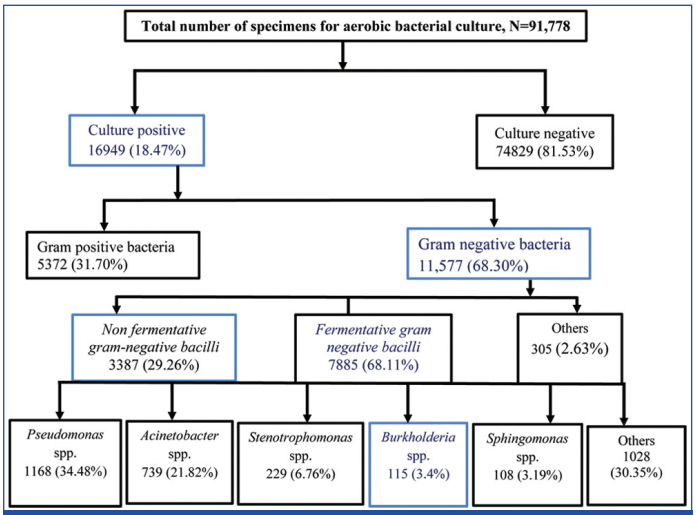
cycle sequencing using BDT v3.1 chemistry was performed on the purified amplicons, and the results were read on an ABI 3500XL Genetic Analyser. The DNA sequencing results, along with database searches and phylogenetic analysis, were used to identify the organism of interest [20,21]. Demographic data, clinical presentation, clinical condition, co-morbid conditions, specimen distribution, culture and susceptibility results, automated Vitek-2 Identification and susceptibility, Bruker Biotyper (MALDI-ToF-MS) identification, *recA* gene PCR, and 16S rRNA PCR and sequencing results of the study population with suspected BCC infections were collected.

STATISTICAL ANALYSIS

The data generated was entered into Microsoft Excel, and statistical analysis was conducted using IBM SPSS statistical software version 28.0. The data was then presented as descriptive statistics.

RESULTS

The male-to-female ratio of the study population was 3.1:1. Out of these samples, 44,365 (48.34%) were received from the blood section, 23,073 (25.14%) from the urine section, 12,629 (13.76%) from the miscellaneous section, and 11,711 (12.76%) from the respiratory section. Culture positivity was observed in 16,949 (18.47%) samples, while the remaining 74,829 (81.53%) specimens were culture negative. Among the positive cultures, Gram-positive bacteria accounted for 5,372 (31.70%) out of 16,949, and Gram negative bacteria accounted for 11,577 (68.30%) out of 16,949. Among the Gram negative bacteria, 3,387 (29.26%) were identified and reported as non fermentative gram negative bacilli, while the remaining 7,885 (68.11%) were fermentative gram negative bacilli, and 305 (2.63%) fell into other categories out of the total 11,577. In this study, the reported members of non fermentative gram negative bacilli were *Pseudomonas* spp. 1168 (34.48%) out of 3,387, *Acinetobacter* spp. 739 (21.82%) out of 3,387, *Stenotrophomonas* spp. 229 (6.76%) out of 3,387, *Burkholderia* spp. 115 (3.4%) out of 3,387, and *Sphingomonas* spp. 108 (3.19%) out of 3,387, as depicted in [Table/Fig-1].



[Table/Fig-1]: Bacterial culture and sensitivity results of the study population with suspected or diagnosed BCC infections.

The incidence of BCC was found to be 115 (3.4%) out of 3,387 non fermentative gram negative bacilli. The prevalence of BCC among the study population was 115 (0.13%) out of 91,778. Among the culture-positive organisms, BCC was detected in 115 (0.68%) out of 16,949. The mean age of male and female patients with BCC infections ranged from 41 to 50 and 51 to 60, respectively. The distribution of the male-to-female ratio in BCC infections showed a ratio of 1.8:1, indicating a male preponderance. Due to financial limitations, only 338 non duplicate clinical isolates were selected using a convenient sampling method from a total of 3,387 non fermentative gram negative bacilli culture-positive isolates for further testing to identify and characterise BCC. The specimen distribution of the 56 BCC isolates is as follows: 35 (62.5%) out of 56 from blood, 6 (10.71%) out of 56 from urine, and so on, as depicted in [Table/Fig-2].

S. No.	Specimen	N (%)	S. No.	Specimen	N (%)
1	Blood	35 (62.5)	6	Sputum	2 (3.57)
2	Urine	6 (10.71)	7	Pus	1 (1.79)
3	Tracheal aspirate	4 (7.14)	8	Bile	1 (1.79)
4	Broncho alveolar lavage	3 (5.36)	9	Tissue	1 (1.79)
5	Pleural fluid	3 (5.36)	Total		56 (100)

[Table/Fig-2]: Specimen distribution of patients with BCC isolates selected for further testing by culture, Vitek-2 and MALDI-ToF-MS and *recA* gene PCR and 16s rRNA sequencing (N=56).

Demographic details, such as the age and sex distribution of 338 conveniently selected non fermentative gram negative bacilli patient isolates suspected of having BCC infections, are depicted in [Table/Fig-3a]. The age and sex distribution of the 56 BCC isolates selected for further characterisation among the study population is shown in [Table/Fig-3b]. These selected BCC isolates were included for further testing using phenotypic and genotypic tests to identify and characterise them.

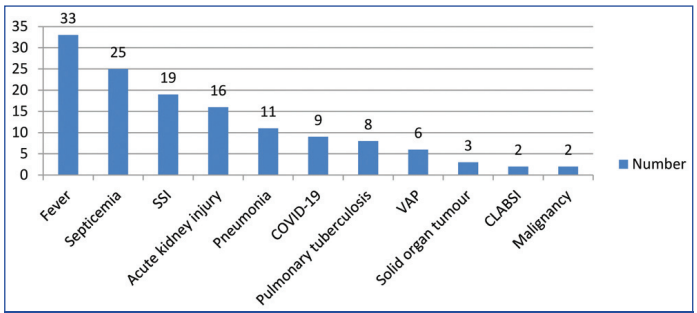
Age in years	Sex		Total N (%)
	Male N (%)	Female N (%)	
<18	16 (7.2)	17 (14.66)	33 (9.76)
18-40	39 (17.56)	20 (17.24)	59 (17.46)
40-65	106 (47.74)	37 (31.90)	143 (42.31)
>65	61 (27.47)	42 (36.20)	103 (30.47)
Total	222 (65.68)	116 (34.32)	338 (100)

[Table/Fig-3a]: Age-sex-wise distribution of 338 conveniently selected out of total non fermentative Gram-negative bacilli patient isolates with suspected BCC infections as the study population (N=3387).

Age in years	Sex		Total N (%)
	Male N (%)	Female N (%)	
<18	3 (8.33)	5 (25)	8 (14.29)
18-40	8 (22.22)	3 (15)	11 (19.64)
40-65	20 (55.56)	6 (30)	26 (46.43)
>65	5 (13.89)	6 (30)	11 (19.64)
Total	36 (64.28)	20 (35.72)	56 (100)

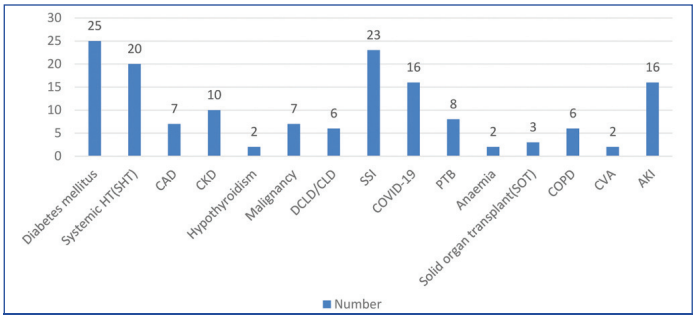
[Table/Fig-3b]: Age-seX distribution of BCC isolates selected and subjected for further characterisation among the study population (N=56).

Patients diagnosed with BCC infections presented with more than one clinical condition, namely fever for evaluation in 33 (58.93%) cases, septicaemia in 25 (44.64%) cases, pneumonia in 11 (19.64%) cases, infections following instrumentation including surgery in 19 (33.93%) cases, ventilator-associated pneumonia in 6 (10.71%) cases, central line-associated bloodstream infection in 2 (3.57%) cases, and COVID-19 infection associated with pneumonia in 9 (16.07%) cases. Additionally, 3 (5.36%) patients were organ transplant recipients, and 2 (3.57%) patients had haematological malignancy, as shown in [Table/Fig-4].



[Table/Fig-4]: Clinical presentation of study population (N=56).
SSI: Surgical site infection; VAP: Ventilator associated pneumonia; CLABSI: Central line associated blood stream infection

The co-morbid conditions associated with BCC infection were Diabetes which was found to be the major risk factor in 25 (44.64%) cases, Surgical Site Infections (SSI) in 23 (41.07%) cases, Systemic Hypertension (SHT) in 20 (35.71%) cases, COVID-19 in 16 (28.57%) cases, Acute Kidney Injury (AKI) in 16 (28.57%) cases, Chronic Kidney Disease (CKD) in 10 (17.86%) cases, PTB in 8 (14.29%) cases, Malignancy in 7 (12.5%) cases, Coronary Artery Disease (CAD) in 7 (12.5%) cases, Decompensated Liver Disease (DCLD) in 6 (10.71%) cases, Chronic Obstructive Pulmonary Disease (COPD) in 6 (10.71%) cases, Anaemia in 2 (3.57%) cases, Cerebrovascular accident in 2 (3.57%) cases, Hypothyroidism in 2 (3.57%) cases, and others as listed in [Table/Fig-5].



[Table/Fig-5]: Co-morbid conditions among study population (N=56).
CAD: Coronary artery disease; CKD: Chronic kidney disease; DCLD: Decompensated liver disease; CLD: Chronic liver disease; SSI: Surgical site infection; PTB: Pulmonary tuberculosis; COPD: Chronic obstructive pulmonary disease; CVA: Cerebro vascular accident; AKI: Acute kidney injury

Out of the 115 manually identified BCC isolates, 56 (48.70%) were subjected to growth on BCSA, automated Vitek® 2 ID/AST, MALDI-ToF-MS, *recA* gene PCR, and 16S rRNA sequencing for identification and characterisation. Among the 56 culture-positive BCC isolates identified by conventional culture, 42 (75%) grew on BCSA agar, as shown in [Table/Fig-6a,b].

was considered significant. Among the BCC isolates, 29 (51.78%) out of 56 were identified as *B. cenocepacia* at the species level, followed by 15 (26.79%) out of 56 as *B. cepacia*, 4 (7.14%) out of 56 as *B. pseudomallei*, and others as shown in [Table/Fig-7a].

A comparison was made between MALDI-ToF-MS and 16S rRNA sequencing methods. Since only 20 species were identified by 16S rRNA sequencing, the comparison was done for those 20 species only, as shown in [Table/Fig-7b].

Due to financial considerations, only 20 (35.71%) out of the 36 *recA* gene-positive BCC isolates were sent for 16S rRNA Sanger sequencing. These BCC isolates were identified as follows: *B. cenocepacia* (6, 30%); *B. pseudomallei* (3, 15%); *B. seminalis* (3, 15%); *B. cepacia* (2, 10%); *B. diffusa* (1, 5%); *B. arboris* (1, 5%); *B. multivorans* (1, 5%); and *B. mallei* (1, 5%). Among them, 3 (15%) isolates initially identified as *B. pseudomallei* by Bruker Biotyper (MALDI-ToF-MS) showed varying results with 16S rRNA sequencing. The latter confirmed 2 (66.67%) isolates as *B. pseudomallei* and 1 (33.33%) as *B. mallei*. Additionally, 1 (5%) out of the 20 isolates initially identified as *B. cenocepacia* with a log score of 1.98 was confirmed as *B. pseudomallei* by 16S rRNA Sanger sequencing technique. Two BCC species identified by MALDI-ToF-MS were confirmed as *Achromobacter* spp. by 16S rRNA sequencing. Although MALDI-ToF-MS allows species-level identification, there is a discrepancy when compared to 16S rRNA results, which can be considered a confirmatory tool for BCC detection. The results of MALDI-ToF-MS and 16S rRNA sequencing are depicted in [Table/Fig-7b].

Out of the 56 isolates, approximately 31 (55.36%) were found to be sensitive to all antibiotics, while the remaining 25 (44.64%) were found to be resistant to one or more antibiotics. The antibiotic susceptibility of BCC isolates (N-56) is depicted in [Table/Fig-8a].

BCC isolates	Manual identification as BCC by colony morphology and biochemical reactions		Growth of BCC on BCSA agar		Vitek® 2 ID/AST identification		MALDI-ToF - MS identification	
Total N (%)	Yes N (%)	No N (%)	Yes N (%)	No N (%)	Yes N (%)	No N (%)	Yes N (%)	No N (%)
56 (100)	56 (100)	0	42 (75)	14 (25)	50 (89.29)	6 (10.71)	52 (92.86)	4 (7.14)

[Table/Fig-6a]: Comparison of conventional and automated methods for BCC detection (N=56).
BCC: *Burkholderia cepacia* compleX; BCSA: *Burkholderia cepacia* selective agar; ID/AST: Identification and antibiotic susceptibility testing; MALDI-ToF-MS: MatriX assisted laser desorption ionisation-time of flight mass spectrometry

BCC isolates	MALDI-ToF-MS identification			
		Positive	Negative	Total
BCSA	Positive	42 (80.77%)	0	42 (75%)
	Negative	10 (19.23%)	4 (100%)	14 (25%)
	Total	52 (92.86%)	4 (7.14%)	56 (100%)

[Table/Fig-6b]: Comparison of BCC isolates growth on BCSA and Automated Identification by MALDI-ToF-MS for BCC detection (N=56).

Fifty (89.28%) out of the 56 manually identified BCC isolates were subjected to the automated Vitek® 2 ID/AST system and were identified as BCC. Among these, 20 (40%) showed very good identification (95%), and the remaining 30 (60%) showed excellent identification (99%) by the Vitek-2 automated system, as depicted in [Table/Fig-6a].

Conventional PCR detected the BCC *recA* virulence gene as positive in 36 (64.29%) out of 56 BCC isolates, while it was negative in the remaining 20 (35.71%) out of 56 isolates, as shown in [Table/Fig-6c]. The performance of *recA* gene PCR for BCC was compared with manual identification by culture, automated identification methods like Vitek-2 and MALDI-ToF-MS, as depicted in [Table/Fig-6d-h].

Furthermore, all 56 BCC isolates were subjected to MALDI-ToF-MS, as shown in [Table/Fig-6a]. It identified 52 (92.86%) out of 56 isolates as BCC, while the remaining 4 (7.14%) out of 56 isolates were identified as *Burkholderia pseudomallei*, as shown in [Table/Fig-7a]. All 56 isolates had an identification log score of ≥1.77, which

BCC isolates	*recA* gene PCR			
		Positive	Negative	Total
Vitek 2 identification	Positive	30 (83.33%)	20 (100%)	50 (89.29%)
	Negative	6 (16.67%)	0	6 (10.71%)
	Total	36 (64.29%)	20 (35.71%)	56 (100%)

[Table/Fig-6c]: Comparison of Vitek-2 ID and Genotypic *recA* gene conventional PCR for BCC detection (N=56).
PCR: Polymerase chain reaction

BCC isolates	*recA* gene PCR			
		Positive	Negative	Total
MALDI-ToF-MS identification	Positive	32 (88.89%)	20 (100%)	52 (92.86%)
	Negative	4 (11.11%)	0	4 (7.14%)
	Total	36 (64.29%)	20 (35.71%)	56 (100%)

[Table/Fig-6d]: Comparison of MALDI-ToF-MS and recA PCR for BCC detection (N=56).

BCC isolates	Vitek 2 identification			
		Positive	Negative	Total
Manual identification	Positive	50 (100%)	6 (100%)	56 (100%)
	Negative	0	0	0
	Total	50 (89.29%)	6 (10.71%)	56 (100%)

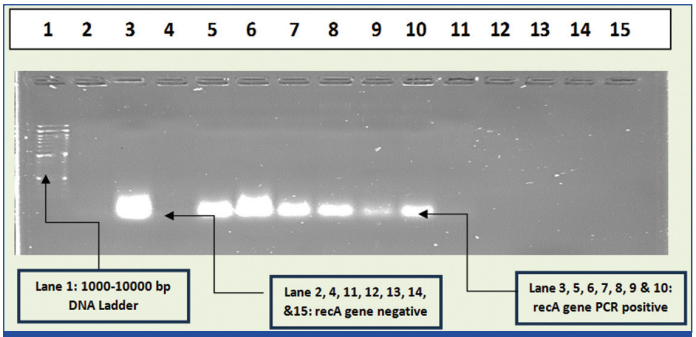
[Table/Fig-6e]: Comparison of manual identification and VITEK 2 ID/AST System for BCC detection (N=56).

BCC isolates	MALDI-ToF-MS identification			
		Positive	Negative	Total
Vitek 2 identification	Positive	46 (88.46%)	4 (100%)	50 (89.29%)
	Negative	6 (11.54%)	0	6 (10.71%)
	Total	52 (92.86%)	4 (7.14%)	56 (100%)

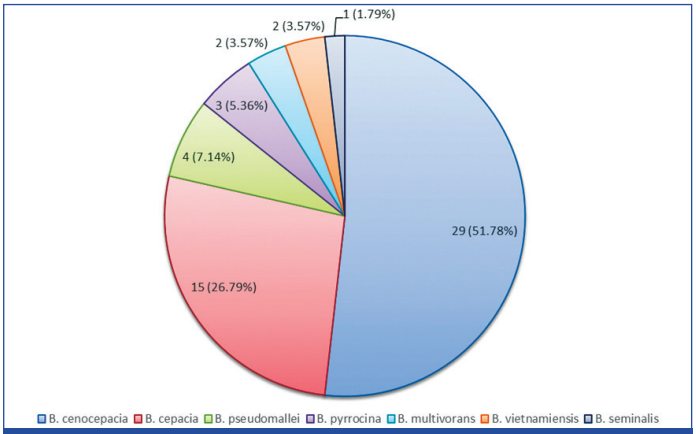
[Table/Fig-6f]: Comparison of VITEK 2 ID/AST System and MALDI-ToF-MS for BCC detection (N=56).

BCC isolates	*recA* gene PCR			
		Positive	Negative	Total
Manual identification	Positive	36 (100%)	20 (100%)	56 (100%)
	Negative	0	0	0
	Total	36 (64.29%)	20 (35.71%)	56 (100%)

[Table/Fig-6g]: Comparison of manual ID and conventional *recA* gene PCR for BCC detection (N=56).



[Table/Fig-6h]: *recA* virulence gene PCR 1020bp positive image under Gel doc system.



[Table/Fig-7a]: Frequency of various BCC species detected by MALDI-TOF-MS (N=56).

In patients with BCC infections, 51 (91.07%) out of 56 patients were admitted to the inpatient ward, while 5 (8.93%) out of 56 were out-patients. Among the inpatients, 36 (70.59%) out of 51 were non ICU patients admitted to the wards, and 15 (29.41%) out of 51 were admitted to intensive care units. The distribution of BCC-infected patients (N-56) by location and the mortality rate are depicted in [Table/Fig-8b].

Out of the total 56 BCC-infected patients, 36 (64.28%) were cured, and 7 (12.50%) showed clinical improvement after receiving appropriate medical therapy. The mortality rate was observed in 9 (16.07%) out of 56 patients with BCC infections. The remaining 4 (7.14%) out of 56 patients had persistent infections as repeat cultures were positive, which could be due to therapeutic failure, as depicted in [Table/Fig-8c].

DISCUSSION

The BCC is a known opportunistic pathogen and a contaminant in hospital environments, causing serious outbreaks and fatal illness, especially in immunocompromised individuals. Its high antibiotic and disinfectant resistance make BCC a potential threat to patients and treating physicians. However, accurate identification of BCC is challenging for conventional microbiologists due to its taxonomic complexity and phenotypic similarity to other non fermentative Gram negative bacilli.

The study population showed a male preponderance, with a male-to-female ratio of 1.8:1. The majority of cases were in the 41-50 years and 51-60 years age groups, which was similar to an outbreak study conducted in India by Rastogi N et al., [22]. Another study by Adan FN et al., reported a high male preponderance, with a male-to-female ratio of 2.45:1 among 160 confirmed cases of hospital-acquired pneumonia due to non fermentative gram negative bacilli infection [23]. *Burkholderia* spp. was the fourth most common isolate among non fermentative Gram negative bacilli infections in this study, which aligns with the study by Gautam V et al., [24]. Additionally, *Burkholderia* spp. was isolated in 115 cases (3.4%) out of 3387 non fermentative Gram negative bacilli. Another study reported a higher prevalence (44%) of global burden of *B. pseudomallei* in South Asia alone, which could be attributed to geographical variation and sample size of the study population, among other factors [25].

Among automated protein signature identification methods, MALDI-ToF-MS aids in the accurate identification of non fermentative Gram negative bacilli. In contrast, Vitek-2 ID and AST are less reliable due to phenotypic variations and slower growth rates. Commercial

S. No.	MALDI-ToF-MS identification and log score	Identification by 16S rRNA sequencing	S. No.	MALDI-ToF-MS identification and log score	Identification by 16S rRNA sequencing
1.	*B. cenocepacia* (2.03)	*B. cepacia*	11.	*B. cenocepacia* (2.35)	*B. cenocepacia*
2.	*B. multivorans* (1.94)	*B. multivorans*	12.	*B. pseudomallei* (1.85)	*B. pseudomallei*
3.	*B. pyrrocinia* (2.23)	*B. cenocepacia*	13.	*B. cenocepacia* (1.98)	*B. cenocepacia*
4.	*B. seminalis* (2.06)	*B. seminalis*	14.	*B. cepacia* (1.97)	*Achromobacter aegrifaciens*
5.	*B. cepacia* (2.05)	*B. seminalis*	15.	*B. cepacia* (2.09)	*B. diffusa*
6.	*B. cenocepacia* (1.98)	*B. pseudomallei*	16.	*B. cenocepacia* (2.03)	*B. cenocepacia*
7.	*B. cenocepacia* (2.07)	*B. cenocepacia*	17.	*B. pseudomallei* (2.06)	*B. mallei*
8.	*B. cenocepacia* (2.11)	*Achromobacter ruhlandii*	18.	*B. cenocepacia* (2.35)	*B. cenocepacia*
9.	*B. cenocepacia* (2.05)	*B. arboris*	19.	*B. pseudomallei* (2.22)	*B. pseudomallei*
10.	*B. cepacia* (2.05)	*B. cepacia*	20.	*B. multivorans* (1.94)	*B. seminalis*

[Table/Fig-7b]: MALDI-ToF-MS vs 16S rRNA Sequencing results among *recA* gene positive BCC isolates.

Antibiotics	Susceptible N (%)	Resistant N (%)	Antibiotics	Susceptible N (%)	Resistant N (%)
Meropenem	48 (85.71)	8 (14.29)	Cotrimoxazole	34 (60.71)	22 (33.29)
Ceftazidime	47 (83.93)	9 (16.07)	Chloramphenicol	38 (67.86)	18 (32.14)
Minocycline	50 (89.28)	6 (10.71)	Levofloxacin	35 (62.5)	21 (37.5)

[Table/Fig-8a]: Antibiotic susceptibility of BCC isolates (N=56).

Study population (N-56)	Out-patients (OP) N (%)	In-patients (IP) N (%)				Total N (%)
		Critical care units N (%)			Ward (Non-ICU) N (%)	
		MICU N (%)	HPBLT N (%)	PICU N (%)		
Mortality	0	8 (14.29)	1 (1.79)	0	0	9 (16.07)
Total cases with BCC infections	5 (8.93)	13 (23.21)	1 (1.79)	1 (1.79)	36 (64.28)	56 (100)

[Table/Fig-8b]: Location-wise distribution and mortality rate among BCC-infected patients (N=56).
MICU: Medical intensive care unit; HPBLT: Hepato-pancreato-biliary liver transplantation unit; PICU: Paediatric intensive care unit

Type of patients		Cured N (%)	Clinically improved after appropriate therapy N (%)	Therapeutic failure-persistent infection N (%)	Dead N (%)	BCC infections N (%)
IP	ICU	1 (3.22)	3 (42.86)	2 (50)	9 (100)	15 (26.78)
	Non-ICU	30 (96.78)	4 (57.14)	2 (50)	0	36 (64.28)
	Total IP	31 (86.11)	7 (100)	4 (100)	9 (100)	51 (91.07)
OP		5 (13.89)	0	0	0	5 (8.93)
Total		36 (64.28)	7 (12.50)	4 (7.14)	9 (16.07)	56 (100)

[Table/Fig-8c]: Clinical outcome among BCC-positive patients (N=56).
IP: In-patients; ICU: Intensive care units; Non-ICU: Ward patients; Total IP-ICU and ward in-patients; OP: Outpatients; AMA: Against medical advice

phenotypic databases are often outdated and lack current taxonomy, unless proven otherwise [26,27].

In this study, 56 manually confirmed BCC isolates were subjected to the automated Vitek® 2 ID/AST system, and only 50 (89.29%) isolates were identified as BCC. Among them, 30 (60%) showed excellent identification (99%), and 20 (40%) showed very good identification (95%), highlighting the excellent concordance and quality of manual identification compared to the commercial automated phenotypic detection system like Vitek-2. Manual identification is labourious and time-consuming but cost-effective compared to Vitek-2, which requires special equipment and is costly. However, Vitek-2 provides faster results, aiding clinicians in initiating appropriate definitive therapy. The time required for Vitek-2 to produce a final identification result for all gram negative bacilli is 3 to 5 hours.

Among the 56 bacterial isolates subjected to MALDI-ToF-MS, 52 out of 56 (92.86%) were identified as BCC, and approximately 3 (5.35%) out of 56 were identified as *Burkholderia pseudomallei* with a significant score of ≥1.77. These results were similar to studies conducted elsewhere [28,29]. Among the 52 MALDI-ToF-confirmed BCC isolates, 29 (51.78%) were identified as *B. cenocepacia*, which was the predominant *Burkholderia* species in this study, followed by *B. cepacia* (15 isolates, 26.79%), *B. pyrrocinia* (3 isolates, 5.36%), *B. multivorans* (2 isolates, 3.57%), *B. vietnamiensis* (2 isolates, 3.57%), and *B. seminalis* (1 isolate, 1.79%). There was a discrepancy in the identification of *B. pseudomallei* and *B. mallei* by MALDI-ToF-MS. One out of the three *B. pseudomallei* isolates identified by MALDI-ToF-MS was later confirmed as *B. mallei* through 16s rRNA sequencing.

MALDI-ToF-MS can be used as a supplemental test to 16s rRNA sequencing and PCR, which are the gold standard tests for the detection and confirmation of BCC up to the species level. A study conducted by Mahenthiralingam E et al., reported that *recA* gene sequencing was superior in identifying different genomovars of BCC [30]. Specific primers are available for the detection of all genomovars of BCC. The identification of the BCC *recA* gene, using BCR1 and BCR2 specific primers for Genomovar I (*B. cepacia*), had a sensitivity of 71.8% in different infections (CF and non-CF) [31], which was similar to the findings of this study. MALDI-ToF-MS is a fast, reliable, and cost-effective technique that has the potential to replace conventional phenotypic identification for most bacterial isolates in clinical microbiology laboratories. MALDI-ToF-MS demonstrated 100% concordance in genus-level identification and 82% concordance in species-level identification with respect to BCC isolates from clinical specimens [9].

In this study, antibiotic susceptibility testing was conducted according to the CLSI 2021 M100 Edition 32. Out of 56 isolates, approximately 31 (55.36%) were found to be sensitive to all antibiotics, namely meropenem, chloramphenicol, ceftazidime, minocycline, levofloxacin, and cotrimoxazole. Additionally, 25 (44.64%) out of 56 isolates were found to be resistant to one or more antibiotics. Minocycline demonstrated sensitivity in 50 (89.28%) out of 56 cases, meropenem in 48 (85.71%) out of 56 cases, ceftazidime in 47 (83.93%) out of 56 cases, chloramphenicol in 38 (67.86%) out of 56 cases, levofloxacin in 35 (62.5%) out of 56 cases, and cotrimoxazole in 34 (60.71%) out of 56 cases. The study showed that BCC exhibited maximum susceptibility to minocycline, followed by ceftazidime, which was consistent with the findings of a study conducted by Dutta S et al., where high sensitivity to ceftazidime was reported [32]. However, the sensitivity of cotrimoxazole was lower compared to the results of a five-year study conducted by Bhavana MV et al., where maximum susceptibility was observed with cotrimoxazole [33].

Infections due to BCC remain a challenge to manage. Trimethoprim-Sulfamethoxazole (TMP-SMX) and ceftazidime are considered first-line options for BCC infections. BCC is intrinsically resistant to antimicrobial agents like polymyxin, aminoglycosides, first and second-generation cephalosporins, and anti-pseudomonal penicillins. Cefiderocol, which is a siderophore cephalosporin, could be given for healthcare-associated BCC infections. Cefepime-Taniborbactam should be used in adults with complicated urinary tract infections. The combination of meropenem/vaborbactam or ceftazidime/avibactam or cefepime with AAA 101 (Enmetazobactam), or the combination of plazomycin with meropenem can be used for bloodstream infections and healthcare-associated infections.

Phage Antibiotic Synergy (PAS) therapy, a combination of Phage KS12 with minocycline and meropenem, is under trial and could combat antimicrobial resistance and improve penetration into biofilms. PAS could be a promising alternative to antimicrobial therapy to treat severe prosthetic joint infections caused by multidrug resistant BCC, especially in immunocompromised individuals. This might significantly reduce the loss of function. Globally, phage therapy 2.0 should be integrated with industrial-academic partnerships to perform clinical trials and understand its potential benefits, and vice-versa [34,35]. Phage-antibiotic synergy is termed as synography and can be well applied to evaluate synergism, additivism, and antagonism for all classes of antibiotics across clinically achievable stoichiometries [36]. Phages provide an adjuvant effect by lowering the MIC for drug-resistant strains. Phage therapy is a promising alternative for bacterial control and environmental safety, serving as a biotechnological tool against pathogenic bacteria, including those

resistant to antibiotics. It could be used as a potent weapon against pandemic drug-resistant clonal groups of pathogenic bacteria. A promising solution to antimicrobial resistance is the introduction of combined phage antibiotic therapy, which can potentiate existing antibiotics by augmenting, prolonging, or even restoring their activity against specific bacteria [37].

Limitation(s)

Troubleshooting the standardisation of DNA extraction and gel electrophoresis for *recA* and 16S rRNA PCR was a concern. In this study, *recA* PCR-RFLP was not performed. Including *recA* gene and *fur* gene PCR-RFLP would aid in better differentiation of clinical and environmental isolates. Closely related species, such as the BCC belonging to other Betaproteobacteria genera (including *Pandoraea* and *Ralstonia*), may be misidentified as *Burkholderia* species. Although Multi-Locus Sequence Typing (MLST) and Whole Genome Sequencing (WGS) are highly precise, they are costly and were not used for the identification of BCC.

CONCLUSION(S)

The BCC is an important bacterial pathogen causing a wide array of infections with high morbidity and mortality, especially due to the surge in antibiotic resistance. MALDI-ToF or Vitek-2 identification could identify BCC at the genus or species level, which is vital for effective diagnostic and antimicrobial stewardship practices to improve clinical outcomes.

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