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Burkholderia cepacia Complex, an Emerging Nosocomial Pathogen at Health Care Facilities in Sebha, Libya

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ABSTRACT

Introduction: *Burkholderia cepacie* complex (Bcc) is an emerging multidrug-resistant gram-negative bacteria frequently isolated from health care facilities worldwide. The present study investigated the prevalence of Bcc in health care settings in Sebha, Libya. **Methods:** Two hundred swabs were initially collected. Forty-seven nosocomial Bcc isolates were identified from three medical care facilities, i.e., 40 (20%) from Sebha Medical Center, five from the Sebha Infertility Treatment Center, and two from Althanweya Clinic. The isolates were identified using a combination of biochemical tests and USP chapter <60> Microbiological Examination of Non-Sterile Products Tests for *Burkholderia* Cepacia Complex guidelines. A UPGMA dendrogram was used to examine the biochemical relationship of isolates. Some of the putative virulence factors contributing to the pathogenicity of the isolates were also explored. **Results:** Of the 47 isolates, 29.79% were *B. cepcia*, 23.40% *B. cenocepcia*, 12.77% *B. thailandensis*, 8.51% *B. vietnamiensis*, 6.38% *B. ambifaria*, *B. pyrrocinia*, and *B. stabilis* each, 4.26% *B. anthina*, and 2.13% *B. arboris*. A variation in virulence factors was observed among isolates; all (100%) isolates produced siderophore, 91% had capsules, 91% produced lipase, 89% formed a biofilm, and 49% produced alkaline protease. The UPGMA dendrogram revealed that Bcc species shared substantial phenotypical identity among themselves. **Conclusion:** In developing countries with limited resources, diagnostic challenges in identifying Bcc species can be resolved using selective media and USP chapter <60> guidelines.

INTRODUCTION

Burkholderia cepacia complex (Bcc) are opportunistic gram-negative bacteria related to non-lactose fermenting bacilli that cause lung infections in cystic fibrosis immune-compromised patients, leading to severe complications like cepacia syndrome [1]. The nosocomial infections caused by these bacteria have increased during the last decades, and they are frequently isolated from health care facilities [2], which raises concerns about new emerging nosocomial gram-negative bacilli infections.

Many virulence factors in these bacteria contribute to disease severity and antibiotic resistance, e.g., extracellular enzymes like protease, hemolysin, lipase, and biofilm formation. Also, the production of siderophores assists Bcc in competing for iron with host iron-binding proteins [3]. For instance, extracellular enzymes, including protease, hemolysin, and lipase, and biofilm formation., Also, the production of siderophores helps Bcc to compete for iron with host iron-binding proteins [3]

Phylogenetically, these bacteria are closely related to *Pseudomonas* spp. and phenotypically similar to each other, which may cause misidentification [4]. This explains limited reports on *B. cepacia* infections in Libya and other developing countries. Hence, there is no precise data about the prevalence of nosocomial Bcc in North Africa, including Libya.

In general, the identification of Bcc is still a controversial issue; commercial methods such as API20NE and Vitek test lack the accuracy for identifying these bacteria [5]. Some authors reported that commercial practices need to be supplemented with a specific biochemical test to be more reliable [6]. Recently, the United States Pharmacopeia (USP) chapter<60> recommends a new guideline involving isolation and identification of Bcc.

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This study investigated the identification and prevalence of nosocomial Bcc in three medical facilities using USP chapter<60> guidelines [7] and biochemical tests. This work also compared the Bcc phenotypic characterizations and focused on virulence factors in these bacteria and their relation to their pathogenicity.

MATERIAL AND METHODS

Bacterial strains. Two hundred swabs were collected from Sebha Medical Center (Intensive Care Unit, Neonate Department, Operation Theatre, Outpatient Department), Sebha Infertility Treatment Center, and Althanwey Clinic from September 2019 until January 2020. Swabs were collected from tables, equipment, medical devices, sinks, and beds according to USP chapter <60> guidelines [7]. The swabs were first recovered on soybean casein digest medium (Oxoid Ltd, England) for 72 h at 35° C. Each specimen was then cultured on *B. cepacia* selective agar (BCSA) (Oxoid Ltd, England) [8] supplemented with polymyxin B 600000 IU, Gentamicin 10 mg, and Vancomycin 2.5mg (Honeywell Fluka, USA) pH 7.2 ± 0.2. All recovered isolates were stored in -20 until used.

Phenotypic identification of Bcc. The isolates were subjected to biochemical tests: ***a***. oxidase activity (Himedia Ltd, India) was performed by a single colony culture on blood agar containing 5% sheep blood; ***b***. *a* motility test was done by hanging drop method using liquid bacterial culture; ***c***. single colony growth in 42°C, ***d***, blood hemolysis was observed by streaking single colonies on blood agar (Oxoid Ltd, England) followed by incubation at 37 °C for 48 h, ***e,*** Poli-β-hydroxybutyrate (PHB) was detected using Sudan Black B dye with slight modification, ***f,*** oxidation-fermentation of carbohydrates was done according to others [9]. Utilization of sorbitol, trehalose, L-Arabinose, adipate, Gelatin, and mannitol was done by phenol red broth fermentation assay (Himedia Ltd, India) [10]. Bile esculin, urease (Oxoid Ltd, England) was done according to the manufacturer's instructions. Starch hydrolysis test was performed by streaking test organisms on brain heart infusion agar (Oxoid Ltd, England) supplemented with 2% starch, followed by incubating at 37 °C for 24-48 h; then plates were flooded with an iodine solution for 30 s, the clear zone around the bacterial growth was considered a positive result for starch hydrolysis [11]. Lysine decarboxylase and Arginine hydrolase was done according to others [12]. According to the manufacturer's instructions, the phenylalanine deaminase test was done using a phenylalanine agar medium (Honeywell Fluka, USA). Capsule stain test was done according to others [13] using negative stain with Congo red. The interpretation of the results and identification of strains was made according to Bergey's Manual of Systematic Bacteriology [14] and Koneman's Color Atlas and Textbook of Diagnostic Microbiology [15]

Phenotypic detection of virulence factors

1- Alkaline Protease Detection

A single colony was cultured on skim milk agar plates with 0.0015% Bromocresol green dye and incubated at 37 °C for 24- 48 h.

2- Lipase Production test

Lipolytic enzyme assay was done using phenol red agar (PRA) supplemented with olive oil by mixing the following: phenol red (0.01% w/v), olive oil (0.1% v/v) Tween 20 (0.1%v/v), $CaCl_2$ (0.1% w/v), and agar (2% w/v). The bacteria were subcultured on PRA and incubated at 37 °C overnight. Color change to yellow in PRA indicated a positive result due to the production of lipase enzyme

3- Biofilm detection test

Briefly, 1×10^7 CFU/ml of organisms were inoculated in a polystyrene test tube with 10 mL of trypticase soy broth (Himedia Ltd, India) containing 1% glucose. The tubes were incubated at 37 °C for 24 h. The tubes were then washed five times with phosphate buffer saline (pH 7.3) (Sigma Aldrich, USA) and allowed to dry. Tubes were then stained with safranine (0.1%) for an hour, washed with deionized water, and dried at room temperature. Biofilm formation was considered positive when a visible film lined the wall and the bottom of the tube.

4- *Burkholderia* siderophore production test

For siderophore production, iron-depleted minimal medium (IDMM Agar) (Himedia Ltd, India) was used; first, Bcc colonies were cultured on brain heart infusion (BHI) agar plates, then subcultured on MM9 agar, and plates were incubated at 37C° for 24 h. The bacteria growth indicated a positive result for siderophore production.

Dendrogram. The unweighted pair group method with arithmetic mean **(**UPGMA) dendrogram was performed using cluster analysis by PAST software 2.09 [16] for biochemical numerical analysis of Bcc isolates.

RESULTS

Prevalence and identification of Bcc. Out of 200 swabs collected from three different medical facilities, only 47 isolates could grow on selective media. The majority of isolates, 40 (20%), were from Sabha Medical Center, 5 (10%) from the Sebha Infertility Treatment Center, and 2 (2%) from Althanweya Clinic (Table 1).

All 47 isolates were Gram-negative. The biochemical tests used for isolates' identification were interpreted according to Bergey's Manual of Systematic Bacteriology [22] and Koneman's Color Atlas, and Textbook of Diagnostic Microbiology [23] (Table 2). The majority of isolates were *B. cepcia* (29.79%), followed by *B. cenocepcia* (23.40%), *B. thailandensis* (12.77%), *B. vietnamiensis* (8.51%), *B. ambifaria*, *B. pyrrocinia*, and *B. stabilis* (6.38%), *B.anthina* (4.26%) and *B. arboris (*2.13%) (Fig. 1 and 2).

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Table 1. Distribution of Bcc isolates among different health care facilities at Sebha, Libya

Medical facility	*No. of samples collected*	*No. of positive Bcc species (percentage)*
Sebha Medical Center	100	40 (20%)
Sebha Infertility Treatment Center	50	5 (10%)
Althanweya Clinic (Local)	50	2 (2%)
Total	200	47 (23.5%)

Table 2. Percentage of virulence factors produced by Bcc isolates

Virulence factors	Positive	Percentage
Alkaline Protease production	23	49 %
Lipase Production	43	91%
siderophore production	47	100%
Capsule production	43	91%
Biofilm formation	42	89%

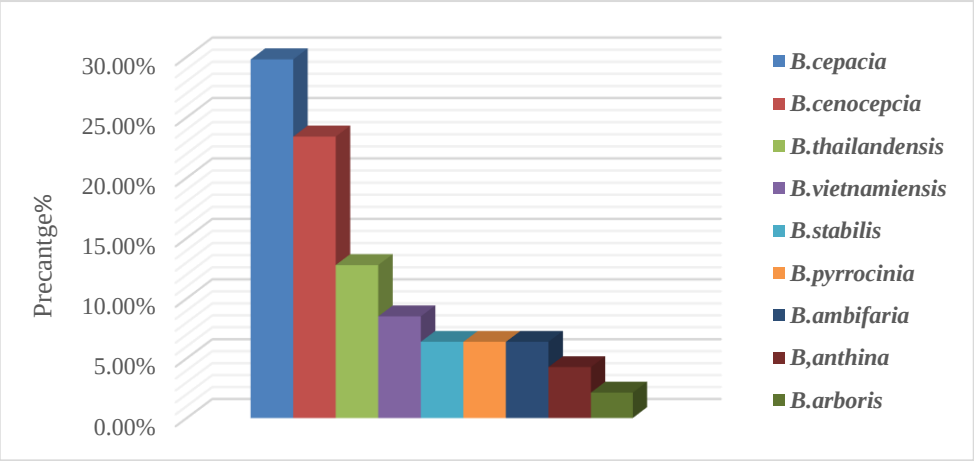


Fig. 1. The frequency of Bcc isolates detected in this study

Cluster anaylsis (UPGMA). UPGMA dendrogram using PAST software was constructed based on biochemical characteristics. In the dendrogram, all 47 isolates were grouped into two clusters, group1 (G1) and 2 (G2). The G1 is clustered into four subgroups (4, 5, 6, and 7), including *B. ambifaria*, *B. arboris*, *B. thailandensis*, *B. vietnamiensis*, *B.pyrrocinia*, and *B. anthina*, with 99%-100% similarity. The G2 clustered into three subgroups (1, 2, and 3), including *B. cepacaia*, *B. cenocepcia*, and *B. stabilis*, exhibiting 100% similarity. Interestingly, one *B. vitamenasis* isolate was more related to *B. cepacia* isolates than other *B.vitamenasis* isolates (Fig. 3).

Virulence factors production. All Bcc isolates (100%) produced siderophore, 91% had capsules, 91% produced lipase, 89% formed biofilms, and 49% produced alkaline protease. Notably, only *B. cepacia* isolates could produce all the virulence factors mentioned above (Table 3).

DISCUSSION

During the last decades, Bcc has been associated with many nosocomial infections and is frequently isolated from health care facilities [2, 17, 20]. The pathogenicity of these bacteria is linked to their various virulence factors [21]. Identifying these bacteria is the cornerstone of choosing an effective treatment strategy.

Studying nosocomial pathogens at Sebha Medical Center has become of great interest to many researchers in this city. Previous reports indicated outbreaks in this medical center [22, 23] and the highest Bcc isolates rates compared to other medical facilities. The patients and healthcare workers could be the primary source of Bcc species similar to other nosocomial infections [24]. In this study, we highlighted a newly emerging pathogen in medical centers of Libya. The bacteria were mainly isolated from contaminated surfaces and devices. Previously, Bcc prevalence was reported in India and Turkey [25, 26], where these bacteria were mainly isolated from blood, sputum, and urine [25, 26]. In this study, *B. cepacie* isolates constituted 14 (29.79%) of all isolates, consistent with other reports where an outbreak with this agent occurred in neonate intensive care units [18].

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Table 3. Biochemical Result of Bcc isolates

		Biochemical Features				Hydrolysis of									Assimilation of											
Species	Number	LDC	ADH	ONPG	ODC	BE	CU	UT	PD	PHB	NIT	H2S	Starch	Gelatin Hydrolysis	Trehalose	OF glucose	OF lactose	Fructose	OF Sucrose	OF Xylose	L-Arabinose	Mannitol	Malonate	Sorbitol	Adipate	MaC Growth	42°C Growth
B. Cepacia	14	+	-	+	-	-	+	+	-	+	-	-	-	+	+	+	+	+	+	-	+	+	+	+	+	+	+
B. Cenocepcia	11	+	-	+	+	-	+	-	-	+	-	-	-	-	+	+	+	+	+	+	+	+	-	+	+	+	+
B. thailandensis	6	-	+	-	-	+	+	-	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B. vietnamiensis	4	+	-	+	-	-	+	+	-	+	-	-	-	-	+	+	+	+	+	+	+	+	+	-	+	+	+
B. Stabilis	3	+	-	-	+	-	+	-	-	+	-	-	-	+	+	+	+	+	-	-	+	+	+	+	+	+	+
B. pyrrocinia	3	-	-	-	-	-	+	-	-	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	-
B. ambifaria	3	+	-	+	-	+	+	-	-	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
B. anthina	2	-	-	-	-	+	+	-	-	+	-	-	-	-	+	+	+	+	-	+	+	+	+	+	+	+	-
B. arboris	1	+	-	+	-	+	+	-	-	+	+	-	-	-	+	+	+	+	+	-	+	-	+	+	+	+	-

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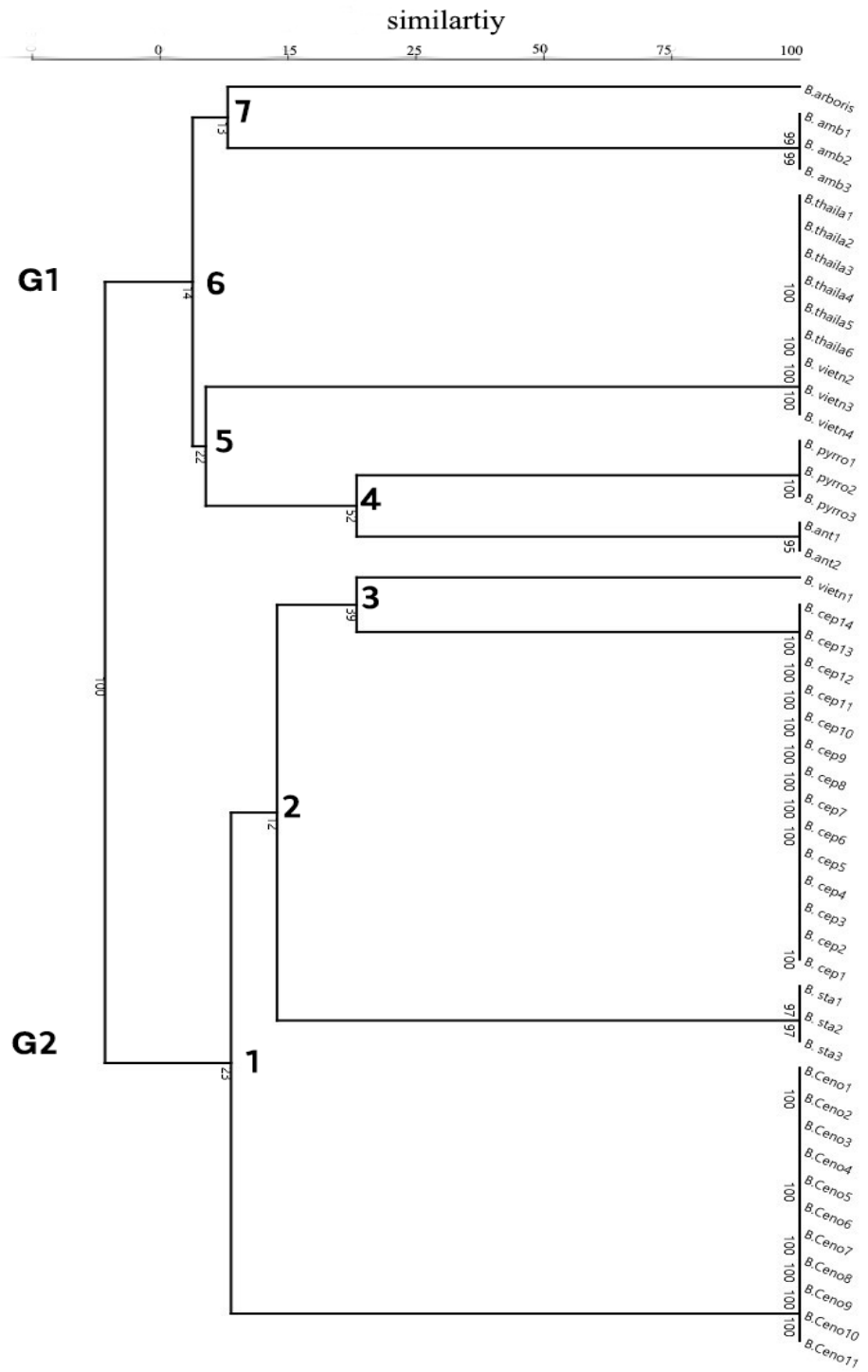


Fig. 2 . UPGMA dendrogram generated by BioNumerics software PAST showing the biochemical relationships among Bcc isolates

Moreover, *B.cenocepacia* was responsible for nosocomial pulmonary tract infections in a French intensive care unit [27]. These bacteria were also isolated from immunocompromised patients in oncology centers [19]. These findings support our result about the *B. cenocepacia* prevalence (23.40%), which can cause high-risk complications in immunocompromised patients.

To our knowledge, this is the first report of the healthcare-linked *B. cepacia* complex in the south of Libya. Identifying Bcc is a big challenge, and misidentification may delay finding the proper antibiotic, resulting in extended hospitalization and increased mortality rates.

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This study showed that combining BCSA and biochemical tests using USP<60> as a guideline would help identify this bacteria more accurately.

The present study tried to develop a reliable and straightforward technique to help microbiologists identify these bacteria, especially in developing countries where advanced techniques are unavailable. Selective cultures can help in the early Bcc diagnosis [28]. Nevertheless, a combination of biochemical tests recommended by Bergys and the isolation technique according to the USP <60> chapter will give more reliable and accurate results, as we showed here. We used the BSCA medium to isolate *Burkholderia* species, and such findings are also seen in a recent report [29].

The present study focused on detecting the most virulence factors in Bcc, including siderophores production, lipase enzyme, capsules, and biofilm formation. Our study showed that 89% of the Bcc isolates could produce biofilms, a phenomenon that enhances antibiotic resistance and provides nutrients for the organism, and a possible cause of disease recurrence [30].

Our results showed that 91% of all 47 isolates had a capsule. BBC can produce capsular polysaccharides that enhance the colonization of bacteria and facilitate biofilm formation and intensify bacteria adherence to surfaces [31]. Our result revealed that all Bcc isolates could produce siderophores, which are vital in iron uptake and regulation. As with other pathogenic bacteria, *Burkholderia* species can capture iron from the host environment [32]. This chemical element is essential for many enzymes and metabolic processes in all living organisms.

Recently, in Sebha Medical Center, the infections by Gram-negative opportunistic bacilli that are extensively resistant to antibiotics have increased. Therefore, gram-negative Bcc might be overlooked or misidentified due to a lack of facilities and advanced techniques for accurate identification. Hence, we strongly recommend that the microbiologists use our simple methods combined with the USP <60> chapter to study these bacteria and help the clinicians control the associated nosocomial infection.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

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