Archives of Razi Institute, Vol. 77, No. 2 (2022) 717-725

DOI: 10.22092/ARI.2022.357464.2041

Original Article

Molecular Research Comparing the Probabilities of *Burkholderia* Cepacia Bacterium Diagnosis Procedures

Maki Al-Nasrawy, L[1]*, Abdali, S. A[2], Mohammed Jawad, S[3]

1. Department of Physiology and Pharmacology, College of Nursing, Altoosi University College, Najaf, Iraq
2. Department of Biology, Faculty of Sciences, University of Kufa, Kufa, Iraq
3. Department of food production, College of Agriculture, University of Kufa, Kufa, Iraq

Received 1 January 2022; Accepted 16 January 2022
Corresponding Author: alnasrawylaila@gmail.com

Abstract

Burkholderia cepacia is found as part of the *B. cepacia* complex (Bcc), a collection of highly pathogenic organisms. The Bcc is present almost everywhere in nature; however, it is most prevalent in damp settings, plant roots, and soils. Moreover, Bcc is a major source of morbidity and death in patients due to its high intrinsic antibiotic resistance. The present study aims to isolate and identify gram-negative aerobic bacteria from clinical samples derived from a variety of pathological diseases and investigate the bacterium's virulence factors and genes. The current study included 250 specimens collected from patients suffering from diabetic foot ulcers, urine, burn, wound, sputum, and discharge from the eyes. The samples were collected from both sexes with the age range of 1-75 years. The recorded data showed that males had a higher frequency of infection (79.2%) than females (52%). The results revealed that 7.6% of infected females were between 1-15 years old, while 22% of infected males were aged between 31-45 years. In addition, 26.8% of infected patients (both males and females) were aged between 31-45 years.

Keywords: *Burkholderia cepacia*, G- bacteria, Foot ulcers, Cystic fibrosis

1. Introduction

Burkholderia cepacia is found as part of the *B. cepacia* complex (Bcc), a collection of highly pathogenic organisms. The Bcc is present almost everywhere in nature; however, it is most prevalent in damp settings, plant roots, and soils. Moreover, Bcc is a major source of morbidity and death in patients due to its high intrinsic antibiotic resistance. Immunocompromised people, particularly those with cystic fibrosis, are the most typically affected (1).

B. cepacia is a gram-negative bacteria that is rod-shaped, non-sporeforming, motile, catalase-positive, and lactose-intolerant. It is a common environmental species that has been isolated as free-living microorganisms, and they dwell in close proximity to a variety of animals, plants, ameobozoon hosts, and fungal spores. The emergence of microbial genes involved in the biodegradation of foreign body molecules has the potential to be a significant and beneficial advance in the battle against pollution. Many strains of these bacteria have been isolated from various plants and have been documented to promote host plant growth, produce antifungal metabolites, and degrade organic contaminants (2).

B. cepacia is a complex of organisms that includes nine distinct genomovars. While the phenotype of genomovars is similar, the genotype is distinct. Some of them have already been given their own genus and

species names. Genomovar III, which comprises some of *B. cenocepacia's* most infectious strains, was transferred to the species designation in 2002.

Until the mid-1980s, when it was discovered as a nosocomial infection in cystic fibrosis clinics, this bacteria remained virtually unknown as a human disease (3). *B. cepacia*, like many other opportunistic infections, may infect anyone in any situation. However, for reasons that are now unknown, the organism "prefers" the lungs of cystic fibrosis patients. According to a 2016 research conducted by the Cystic Fibrosis Foundation's National Patient Registry, *B. cepacia* was discovered in 2.6 percent of all cystic fibrosis patients in the United States (4). Furthermore, the *B. cepacia* strain or *B. cenosepacia* from genomovar III appears to be the primary source of these more severe illnesses. Although different genomovars can cause infections in cystic fibrosis patients, genomovar III is responsible for nearly half of all cystic fibrosis infections in the United States and 80% in Canada.

2. Materials and Methods

2.1. Samples Collection and Inoculation

Samples were collected from patients suffering from diabetic foot ulcers, urine, burn, wound, sputum, and discharge from the eyes, who attended Al-Sader Medical City, Al-Hakim General Hospital in AL-Najaf, Iraq, between September 2020 and February 2021. The samples were obtained from both sexes with the age range of 1-75 years. The specimens were carried by sterile transport swabs and injected onto culture media using a direct technique of inoculation, as well as a specific medium solely for *B. cepacia* development was used. The inoculation was performed at 37°C for 18-24 h, as previously described by Cheesbrough (5). After staining with Gram stain, all probable isolates were examined under the microscope and revealed to be gram-negative single short bacilli. The *B. cepacia* isolates cultured on MacConkey agar medium emerged as non-lactose fermenters, tiny, light-pink color colonies that became dark-pink to red after 4-7 days, due to lactose oxidation. Table 1 shows the distribution of 280 samples: the lowest prevalence was in the age groups above (61-75) 7%, and the highest incidence was in the age group of (31-45) 26.4 %.

2.2. Morphologically Characterization

Initial identification of bacterial isolates acquired from clinical samples was based on culture morphology, microscopic features, and biochemical assays. The *B. cepacia* seemed to be a gram-negative bacilli under the microscope; however cultural identification of *B. cepacia* was based on colonial morphology. Since *B. cepacia* colonies were cultivated on blood agar, they appear to be diffuse-haemolytic (6, 7).

Non-lactose fermenting colonies of *B. cepacia* grew on MacConkey agar and generated pigment on other medium, as shown in figure 1. According to the previously described method (8).

Table 1. The distribution of patients according to age group and sex

Sex	Age group No. (%)					
	1-15	**16-30**	**31-45**	**46-60**	**61-75**	**total**
Female	21 (8.4)	9 (3.6)	13 (5.2)	9 (3.6)	2 (0.8)	54 (21.6)
Male	41 (16.4)	45 (18)	55 (22)	43 (17.2)	12 (4.8)	196 (78.4)
Total	62 (24.8)	54 (21.6)	68 (27.2)	52 (20.8)	14 (5.6)	250 (100%)

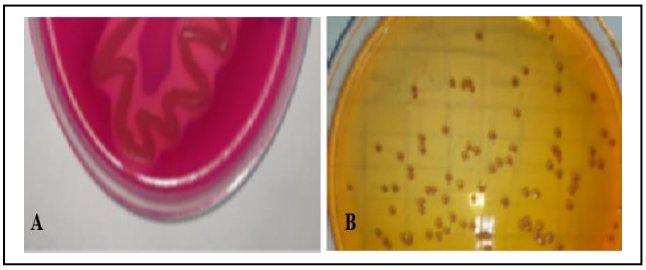

Figure 1. Growth of *B. cepacia* on (A) β-hemolytic on blood agar medium, (B) MacConkey agar medium

3. Results and Discussion

3.1. Biochemical Testing

The results of biochemical tests are shown in table 2. The biochemical tests are regarded as a useful addition to the initial identification of the *B. cepacia* isolate. Isolates were positive for oxidase, catalase, motility, citrate utilization, gelatinize, and dirt-like odor. The isolates were negative for urease production, Voges-Proskauer, and methyl red test, which is consistent with Alnasrawy and AL-Aammar (7), while indole production and H_2S production tests were positive.

3.2. ID-GNB Cards and the VITEK 2 System

The results showed that from 45 identified samples, 16 (20%) isolates were with confidence values of 99-96% (excellent identification), 9 (11.2%) isolates showed confidence values of 96-95% (very good identification), and only 20 (25%) isolates manifested confidence values of 89% according to the initial examination of the Gram stain. The *B. cepacia* isolates were divided into 11 groups, each assigned to a bio-pattern based on the obtained results.

3.3. Analytical Profile Index (API) Microsystems

The API 20E test revealed that from 80 samples, 33 (41.3%) isolates were exactly identified, 22 (27.5%) showed the nearest identification, and 25 (31.2%) had no identity (Table 3).

Table 2. *B. cepacia* isolates subjected to biochemical assays

No.	Biochemical test	Result	No. of the total samples (80)	Percentage	No. samples based on PCR (20)	Percentage
1	Catalase	+	60	75%	20	100%
2	H_2S production	+	40	50%	16	80%
		-	40	50%	4	20%
3	Triple sugar iron (TSI)	K/A	50	62.5%	20	100%
4	Oxidase	+	55	68.7%	13	65%
		-	25	31.3%	7	35%
5	Indole production	+	42	52.5%	15	75%
		-	38	47.5%	5	25%
6	Citrate utilization	+	35	43.7%	20	100%
7	Motility	+	62	77.5%	20	100%
8	Growth at 42ºC	+	20	25%	3	15%
		-	60	75%	16	80%
9	Voges-Proskauer (VP)	-	62	77.5%	20	100%
10	Smell	Dirt-like odor	46	57.5%	20	100%

Table 3. The API 20E test results

Description of groups	Codes of test results	Numbers of isolates
First group (Exact identity)	(6 300 009)	1,4,6,10,13,8,9,16,20,21,30,31,33,36,39,41,64,68,71,73,75,59,60,62,63,66,69,70,72,74,78,79
Second group (nearest identity)	(6 301 007)	3,14,17,22,12,23,26,27,34,35,42,46,48,50,52,53,56, 44,45,49,51,58
Third group (no identity)	(6 301 000)	5,7,24,25,28,29,32,37,38,40,43,47,54,55,57,61,65,67,76,77,15,18,19,11,2

3.4. *RecA* Gene Identification of *B. cepacia*

The *recA* gene was used to detect the bacterium *B. cepacia*. The present study found that the *recA* gene was found in 30 of 45 samples tested using the VITEK 2 System, as shown in figure 2.

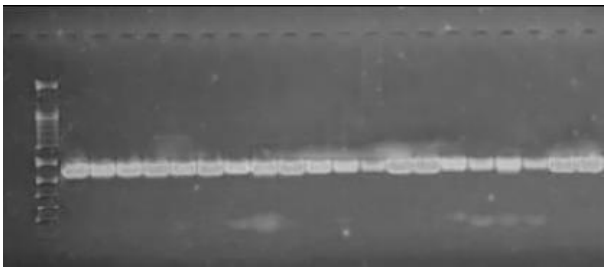

Figure 2. PCR results of *B. cepacia* isolates amplified with *recA* gene primers, having a product size of 429 bp. DNA molecular size marker Lane (L) (100-bp ladder).

The *recA* gene has been widely utilized in bacterial systematics. It has proven to be particularly beneficial for identifying Bcc species. The *recA* gene with a phylogenetic analysis of sequence variation within the gene allows all nine current species within the Bcc to be distinguished.

However, the original *recA*-based PCR primers, *BCR1* and *BCR2*, are specific to members of the Bcc and do not amplify this gene in other *B. cepacia* species. While this can be a useful way of confirming an isolate's location within the complex, it limits the technique's application to classifying other *B.* species in different natural environments (9).

3.5. *16S rDNA* Gene

As shown in figure 3, the *16S rDNA* was found in all *B. cepacia* isolates. The *16S rDNA* gene comprises highly conserved nucleotide sequences interspersed with genus- or species-specific variable sections. Bacteria can be classified by analyzing the nucleotide sequence of the PCR result and comparing it to known sequences in a database (10). Due to the widespread use of PCR and DNA sequencing in clinical microbiology laboratories over the last decade, 16S rDNA sequencing has played a critical role in the correct identification of bacterial isolates and the discovery of novel bacteria.

In the case of bacteria with unusual phenotypic profiles, uncommon bacteria, slow-growing bacteria, uncultivable bacteria, and culture-negative illnesses, 16S rDNA sequencing is very significant for bacterial identification. It has not only provided study into the etiologies of infectious diseases, but it has also assisted clinicians in selecting medicines, determining the length of therapy, and managing infection measures. Of 215 new bacterial species, 29 belonged to unique genera and were found in human specimens using 16S rDNA sequencing in the twenty-first century (2001-2007) (11).

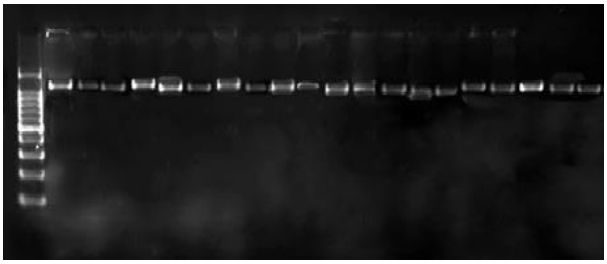

Figure 3. PCR results of *B. cepacia* isolates amplified with *16S rDNA* gene primers, having a product size of 1020 bp. Lane (L): DNA molecular size marker (100-bp ladder); all isolates had positive *16S rDNA* gene.

3.6. Numerical Taxonomy and Cluster Analysis

Many studies used numerical taxonomy to classify and identify problems of bacteria and other microorganisms. Cluster analysis refers to a class of methods of data reduction used to sort events, results, or variables from a given data set into homogeneous classes that differ from each other (7). The numerical review focused on the results of the research on the general characteristics, bio-patterns, anti-bio patterns, and virulence patterns of the *B. cepacia* isolates under study:

Depending on Sj and Unweighted Pair Group Method with Arithmetic Mean (UPGMA) clustering from numerical analysis of the biochemical tests (Table 4), the dendrogram (Figure 4) consists of three major clusters, A, B, and C, which could be distinguished at $Sj \geq 33.3\%$.

Table 4. The percentage of positive tests shown by 20 isolates of *B. cepacia* based on biochemical tests

biochemical tests	Percentage of positive tests		
	Cluster A	**Cluster B**	**Cluster C**
TSI	100%	100%	100%
H_2S production	62.5%	85.7%	100%
VP	0	0	0
Catalase	100%	100%	100%
Oxidase	75%	57.1%	60%
Motility	100%	100%	100%
Indole production	100%	57.1%	60%
Citrate utilization	100%	100%	100%
Growth at 42°C	25%	14.2%	0
Smell	100%	100%	100%
Growth on Cetrimide agar medium	25%	57.1%	80%

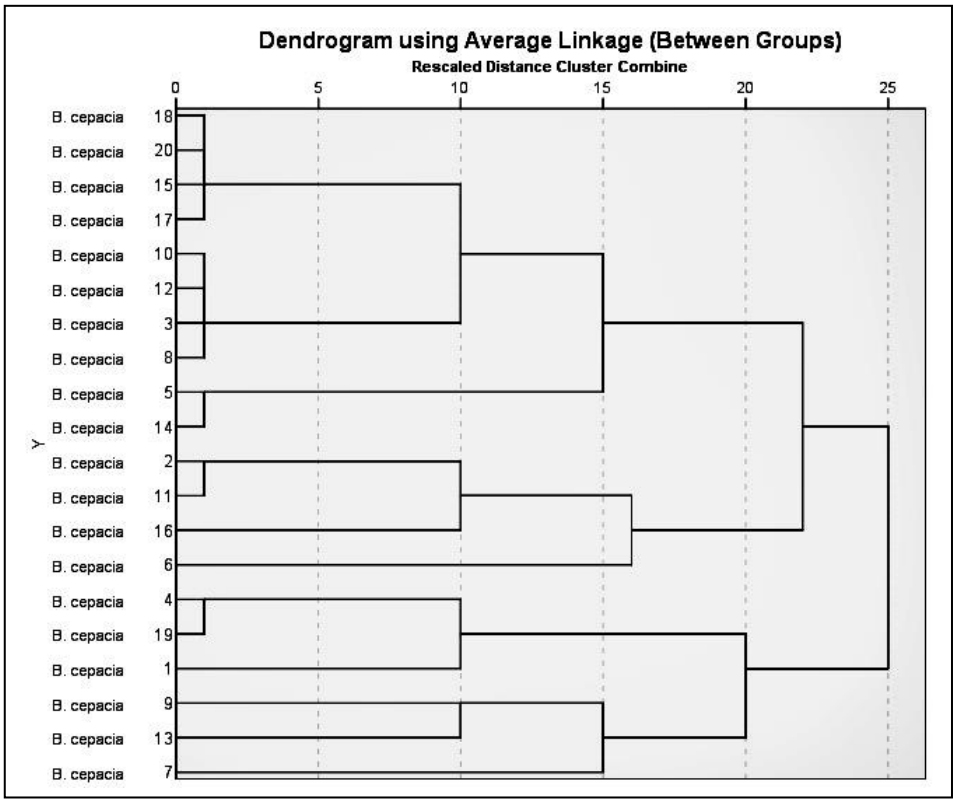

Figure 4. Hierarchical tree chart for *B. cepacia* 20 isolates obtained from cluster analysis using Jaccard's Coefficient depending on biochemical tests patterns. Cluster A included 10 (50%) *B. cepacia* isolates and was divided into several sub-clusters, namely (18, 15, 17, 10, 12, 3, 8, 5, 14). Cluster B isolates included 4 (20%) *B. cepacia* isolates and was divided into sub-clusters (2, 11, 16, 6). Cluster C isolates included 6 (30%) of *B. cepacia* isolates and were divided into several sub-clusters, namely (4, 19, 1, 9, 13, 7).

Cluster A (Sj≥33.3%) included 10 (50%) *B. cepacia* isolates which were divided into several sub-clusters. Cluster A isolates belong to (9) bio-patterns, namely (18, 15, 17, 10, 12, 3, 8, 5, 14), to (9) anti-bio patterns, namely (20, 15, 17, 10, 12, 3, 8, 5, 14), and also belongs to (7) virulence patterns, namely (20, 15, 17, 10, 12, 8, 5).

Cluster B isolates (Sj ≥ 32.6%) included 4 (20%) *B. cepacia* isolates and were divided into some sub-clusters, namely (2, 11, 16, 6). Cluster B isolates belong to (2) anti-bio patterns, namely (11, 6), and to (1) virulence pattern, namely (2).

Cluster C isolates (Sj≥33.9%) included 6 (30%) *B. cepacia* isolates and were divided into several sub-clusters, namely (4, 19, 1, 9, 13, 7).

From the numerical analysis of the virulence factor tests (Table 5) and based on Sj and UPGMA clustering, there are two major clusters, A and B, as shown in the dendrogram (Figure 5), which could be distinguished at Sj ≥ 67.9%.

Cluster A (Sj≥67.9%) included 15 (75%) *B. cepacia* isolates and were divided into several sub-clusters. Cluster A isolates belong to (12) bio-patterns, namely (10, 17, 6, 11, 16, 8, 19, 4, 5, 13, 12, 15) and to (12) anti-bio patterns, and also belong to (10) biochemical tests patterns, namely (20, 10, 17, 6, 11, 16, 8, 5, 12, 15). Cluster B isolates (Sj≥67.9%) included 4 (20%) *B. cepacia* isolates and were divided into some sub-clusters, namely (3, 9, 2, 14). Cluster B isolates belong to (1) biochemical pattern, namely (9).

Table 5. The percentage of positive tests shown by 20 isolates of *B. cepacia* based on virulence factor tests results

Virulence factors	Percentage of positive tests	
	Cluster A	Cluster B
Phospholipase C	100%	100%
Urease	68.7%	50%
Siderophore production	100%	100%
Capsule Detection	100%	100%
Gelatinase	68.7%	100%
Hemolysis on blood agar	50%	75%
Biofilm Formation	100%	0

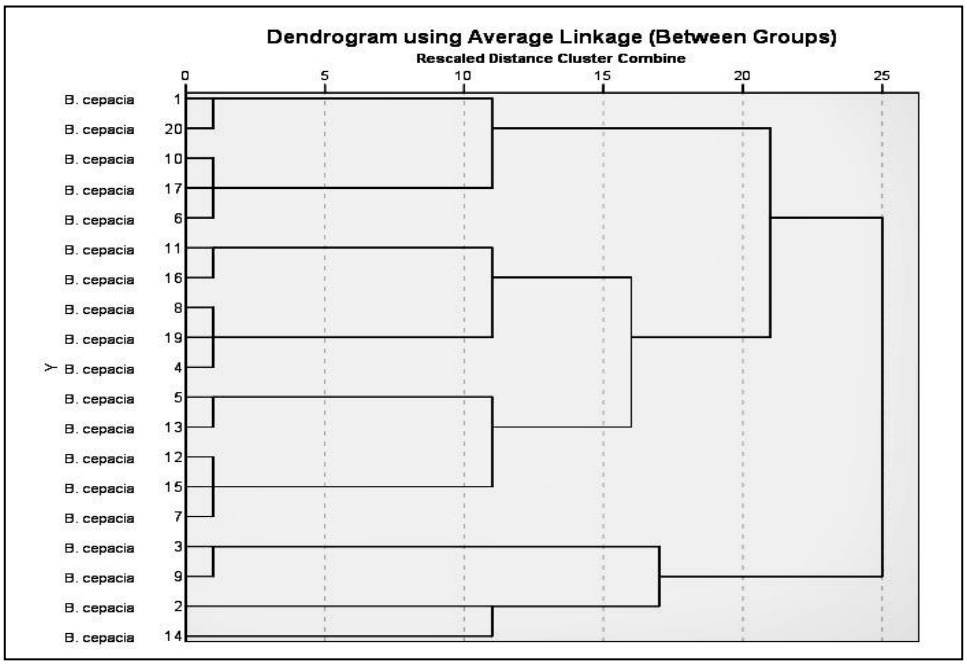

Figure 5. Hierarchical tree chart for *B. cepacia* 40 isolate obtained from cluster analysis using Jaccard's Coefficient depending on virulence patterns. Cluster A included 15 (75%) *B. cepacia* isolates and were divided into several sub-clusters, namely (10, 17, 6, 11, 16, 8, 19, 4, 5, 13, 12, 15). Cluster B isolates included 4 (20%) *B. cepacia* isolates and were divided into some sub-clusters, namely (3, 9, 2, 14).

From the numerical analysis of the bio-patterns (Table 6), depending on Sj and UPGMA clustering, there are two major clusters, A and B, as shown in the dendrogram (Figure 6) which could be distinguished at Sj ≥ 7.16%.

Cluster A (Sj≥7.16%) included 17 (85%) *B. cepacia* isolates which were divided into several sub clusters. Cluster A isolates belong to (13) virulence patterns, namely (17, 19, 12, 8, 13, 10, 16, 18, 11, 5, 15, 4) and belong to (14) anti-bio patterns, namely (9, 17, 19, 12, 8, 13, 2, 10, 3, 16, 14, 5, 15, 4).

Cluster B (Sj≥7.16%) included 3 (15%) *B. cepacia* isolates that were divided into one sub-cluster and a single isolate number (1). Cluster B isolates belong to (1) anti-bio patterns, namely (1).

Table 6. The percentage of positive tests shown by 40 isolates of *B. cepacia* based on tests results of VITEk 2 System

Tests	Percentage of positive tests		Tests	percentage of positive tests		Tests	Percentage of positive tests	
	Cluster A	Cluster B		Cluster A	Cluster B		Cluster A	Cluster B
APPA	35.7%	50%	PLE	0	83.3%	dMAN	0	33.3%
ADO	35.7%	16.6%	TyrA	14.2%	83.3%	BXYL	0	16.6%
PyrA	0	0	URE	14.2%	16.6%	BAIap	14.2%	33.3%
IARL	85.7%	50%	dSOR	7.1%	50%	ProA	16.6%	50%
dCEL	71.4%	33.3%	SAC	7.1%	33.3%	LIP	7.1%	16.6%
BGAL	0	33.3%	dTAG	7.1%	33.3%	CMT	50%	83.3%
H_2S	42.8%	50%	dTRE	14.2%	16.6%	BGUR	16.6%	0
BNAG	14.2%	16.6%	CIT	42.8%	66.6%	O129R	35.7%	83.3%
AGLTp	78.5%	50%	MNT	35.7%	50%	GGAA	0	16.6%
dGLU	85.7%	100%	5KG	0	0	ODC	7.1%	0
GGT	14.2%	50%	ILATK	28.5%	83.3%	LDC	0	33.3%
OFF	14.2%	0	AGLU	14.2%	0	IHISa	0	0
BGLU	0	50%	SUCT	7.1%	66.6%	IMLTa	14.2%	66.6%
dMAL	25%	33.3%	NAGA	0	16.6%	GIyA	7.1%	0
ELLM	0	33.3%	AGAL	0	50%			
ILATa	0	16.6%	PHOS	7.1%	16.6%			

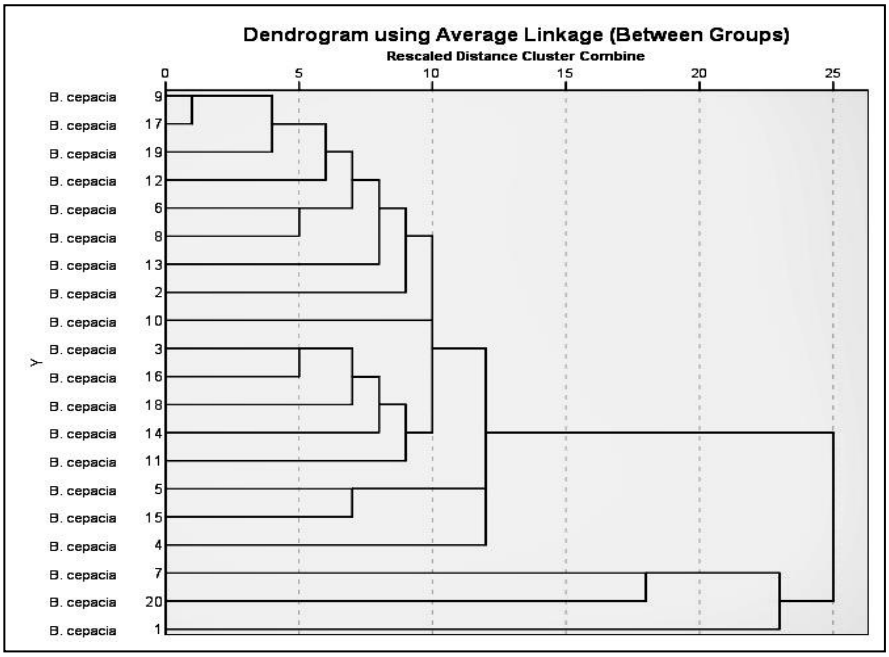

Figure 6. Hierarchical tree chart for *B. cepacia* 40 isolates obtained from cluster analysis using Jaccard's Coefficient depending on VITEK 2 System. Cluster A included 17 (85%) *B. cepacia* isolates divided into several sub-clusters. Cluster B included 3 (15%) *B. cepacia* isolates were divided into one sub-cluster (7, 20, 1) and a single isolate number (1).

Figure 7 shows the numerical analysis of the anti-bio patterns (Table 7), depending on Sj and UPGMA clustering. As shown by the dendrogram, there are two major clusters, A and B, which could be distinguished at Sj ≥ 37.2%.

Cluster A (Sj≥37.2%) included 16 (80%) *B. cepacia* isolates which were divided into several sub-clusters and five single isolates, namely (3, 10, 4, 14, 2, 20).

Cluster A isolates belong to (12) virulence patterns, namely (17, 19, 5, 12, 10, 4, 8, 16, 13, 15, 7, 20), belong to (9) biochemical patterns, namely (17, 3, 5, 12, 10, 8, 15, 14, 20), and also belong to (11) bio-patterns, namely (9, 17, 19, 5, 12, 4, 8, 16, 13, 15, 14).

Cluster B (Sj≥37.2%) included 4 (20%) *B. cepacia* isolates that were divided into two sub-clusters isolates, namely (11, 18, 1, 6). Cluster B isolates belong to (1) biochemical patterns, namely (1), and belong to (1) bio-patterns, namely (1).

As shown in Figure 8, the dendrogram consists of two major clusters, A and B, based on all bio-patterns, anti-bio patterns, virulence patterns, and biochemical tests.

From these variations, we also achieved the numerical taxonomy of these dendrograms and the similarities between *B. cepacia* isolates in this study showed that different strains of *cepacia* bacteria were present in certain hospitals in Al-Najaf Al-Ashraf city, Iraq.

Table 7. The percentage of positive tests shown by 20 isolates of *B.cepacia* based on tests results of antibiotic

Antibiotic pattern	Percentage of positive tests	
	Cluster A	Cluster B
GM	25%	25%
CIP	12.5%	25%
AK	18.7%	50%
IMP	50%	0
MEM	50%	0
AZM	56.2%	75%
B	18.7%	100%
TOB	12.5%	0
CAZ	68.7%	100%
OFX	0	0
LEV	31.2%	0
PRL	12.5%	0
TIM	6.2%	25%
FEP	6.2%	0

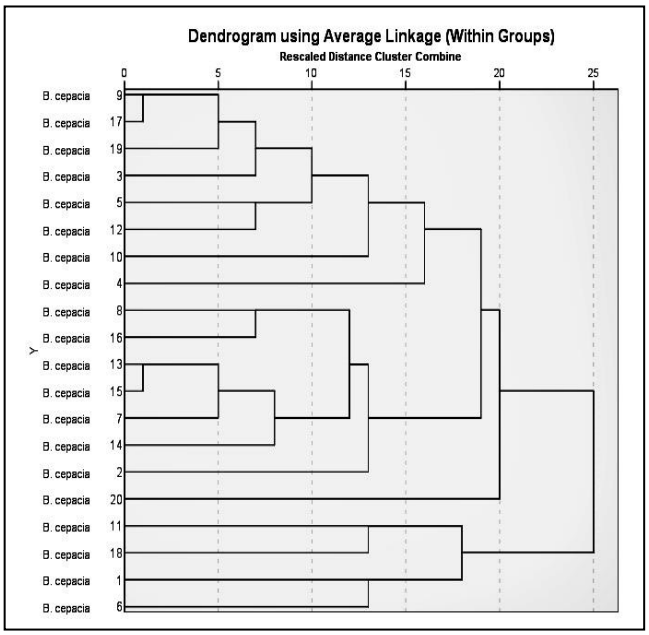

Figure 7. The hierarchical tree chart for B. cepacia 20 isolates was obtained from cluster analysis using Jaccard's Coefficient depending on anti-bio patterns only. Cluster A included 16 (80%) *B. cepacia* isolates divided into several sub-clusters and five single isolates (3, 10, 4, 14, 2, 20). Cluster B included 4 (20%) *B. cepacia* isolates divided into two sub-clusters isolates (11, 18, 1, 6).

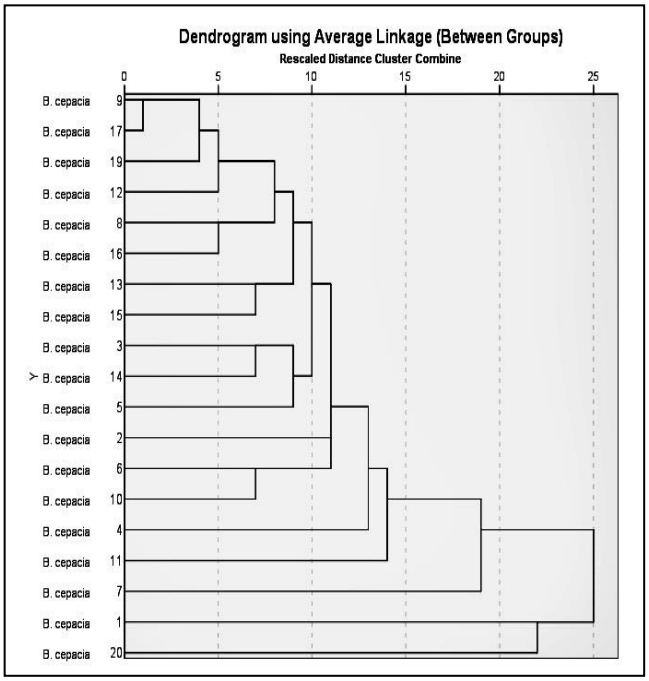

Figure 8. The hierarchical tree chart for *B.cepacia* 40 isolates was obtained from cluster analysis using Jaccard's Coefficient depending on all bio-, anti-bio, virulence patterns, and biochemical tests.

Authors' Contribution

Study concept and design: S. A. A.
Acquisition of data: S. A. A.
Analysis and interpretation of data: L. M. A.
Drafting of the manuscript: S. M. J.
Critical revision of the manuscript for important intellectual content: L. M. A.
Statistical analysis: S. A. A.
Administrative, technical, and material support: L. M. A.

Ethics

The human study was approved by Altoosi University College, Najaf, Iraq Review Board.

Conflict of Interest

The authors declare that they have no conflict of interest.

References

1. Ritesh Ranjan PC, Aman Kamra. Community Acquired Burkholderia cepacia Bacteraemia Presenting as MODS in an Immunocompetent Individual: An Unusual Case. J Clin Diagn Res. 2017;11(3):1-2.
2. El-Barrawy M. Existence of Burkholderia cepacia at Burn and Intensive Care Units in Alexandria Main University Hospital, Egypt. J Med Sci. Clin Res 2017;5.
3. Schwager S, Agnoli K, Köthe M, Feldmann F, Givskov M, Carlier A, et al. Identification of Burkholderia cenocepacia strain H111 virulence factors using nonmammalian infection hosts. Infect Immun. 2013;81(1):143-53.
4. Knapp EA, Fink AK, Goss CH, Sewall A, Ostrenga J, Dowd C, et al. The Cystic Fibrosis Foundation Patient Registry. Design and methods of a national observational disease registry. Ann Am Thorac Soc. 2016;13(7):1173-9.
5. Cheesbrough M. District Laboratory Practice in Tropical Countries. 2nd ed. Cambridge: Cambridge University Press; 2006.
6. Al.Fahadawi MA, Al.Obadi WI, Hasan AS. Antibiogram of Pseudomonas aeruginosa Isolated from Burn & Wound Infections Among Inpatients and Outpatients Attending to Ramadi Teaching Hospital in Ramadi, Iraq. Egypt Acad J Biol Sci. 2019;11(1):13-22.
7. Alnasrawy WD, AL-Aammar MH. A Molecular Study with A Comparison of the Odds of Diagnostic Methods For Burkholderia Cepacia Bacteria Isolated from Patients with Diabetic Foot Ulcer. Indian J Forensic Med Toxicol. 2021;15(3):4603.
8. Bharara T, Chakravarti A, Sharma M, Agarwal P. Investigation of Burkholderia cepacia complex bacteremia outbreak in a neonatal intensive care unit: a case series. J Med Case Rep. 2020;14(1):76.
9. Payne GW, Vandamme P, Morgan SH, LiPuma JJ, Coenye T, Weightman AJ, et al. Development of a recA gene-based identification approach for the entire Burkholderia genus. Appl Environ Microbiol. 2005;71(7):3917-27.
10. Johnson JS, Spakowicz DJ, Hong B-Y, Petersen LM, Demkowicz P, Chen L, et al. Evaluation of 16S rRNA gene sequencing for species and strain-level microbiome analysis. Nat Commun. 2019;10(1):5029.
11. Woo PCY, Lau SKP, Teng JLL, Tse H, Yuen KY. Then and now: use of 16S rDNA gene sequencing for bacterial identification and discovery of novel bacteria in clinical microbiology laboratories. Clin Microbiol Infect. 2008;14(10):908-34.