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Conventional Versus Molecular Methods for Diagnosis of *Burkholderia cepacia* from Different Clinical Samples of Iraqi Patients

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ABSTRACT

The study aims at isolating and identification of *Burkholderia cepacia* bacteria from clinical samples from various pathological conditions such as diabetic foot ulcers, burn, wound, sputum and urine. The present study includes 280 samples collected from patients suffering from diabetic foot ulcer, cystic fibrosis, burns, sputum, and wounds who attend Alsader Medical City and Al-Hakim General Hospital during the period extended from September 2020 to February 2021 (men and women) with age groups between (1 -75) years. The identification of bacterial isolates were detected by classical and molecular technique (PCR), where the frequency among males 213 (79.2%) was more than that in female 67 (20.8%). The samples distribution is made according to age group; it appears high for the following high 26.8% with group (31-45) years male and female; 22% with group (31-45) years in male and 7.6% with the group (1-15) years in female. The results revealed that 42/80 specimens of the total number of samples are *Burkholderia cepacia* by using *16SrRNA* gene to differentiate the *B. cepacia* from other (G-) bacteria, and 30/80 *recA* gene to differentiated *B. cepacia* from *B. cepacia* complex.

1. Introduction

Burkholderia cepacia is detected as a group of highly virulent organisms known as the *Burkholderia cepacia* complex (Bcc). Bcc is ubiquitous and is most commonly found in moist environments, plant roots, and soils. Due to its high inherent antibiotic resistance, Bcc is a major cause of morbidity and mortality in inpatients. It is most commonly reported in immunocompromised patients, especially those with cystic fibrosis (Ranjan, et al., 2017).

B. cepacia is Gram-negative bacilli, rod-shaped, non-spore-forming, motile, catalase-positive, and lactose-non fermenting bacteria (Tavares, et al., 2020). It is considered a common environmental species that have been isolated as free-living microorganisms, they live in close interaction with many animals, plants, ameobozoon hosts, or fungi (Stopnisek, et al., 2016; Xu, et al., 2016). The evolution of microbial genes involved in the biodegradation of foreign body molecules can be a powerful and positive development in the fight against environmental pollution (Verma, et al., 2019). Many strains of these bacteria have often been reported to be isolated from different plants capable of promoting host plant growth, producing antifungal metabolites, and degrading organic pollutants (Jung, et al., 2018).

The species *B. cepacia* is a complex of organisms consisting of nine different genomovars (Safdar, 2015). Genomovers are similar in phenotype but different in genotype. Some of these have already been given their species designations. In 2002, Genomover III, which contains some of the more infectious strains of *B. cenocepacia* was reassigned to the species designation (Al-Sadi, et al., 2015).

This bacteria causes respiratory disease in patients with cystic fibrosis and various nosocomial infections including bloodstream infections, pneumonia, surgical wound

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infections, and genitourinary tract infections. One of the common features of *Burkholderia* species is their ability to resist multiple antibiotics and a disinfectant, which makes the infection, caused by them hard to treat and makes them emerge as an important cause of morbidity and mortality in patients (Farzana, et al., 2020).

This bacterium was relatively unknown as a human pathogen until the mid-1980s when it surfaced as a nosocomial infection at cystic fibrosis clinics. Like many opportunistic pathogens, B. cepacia can establish an infection in any favorable environment. However, for currently unknown reasons, the organism "prefers" the lungs of patients with cystic fibrosis. A 2018 study conducted through the Cystic Fibrosis Foundation's National Patient Registry found that 2.6% of all patients with cystic fibrosis in the United States were infected with *B. cepacia* (Bernier, et al., 2003). In addition, these more serious infections appear to be caused primarily by the *B. cepacia* strain or *B. cenosepacia* from Genomovar III. Genomovar III is responsible for about 50% of all cystic fibrosis infections in the United States and 80% in Canada, although other genomovars can cause infections in patients with cystic fibrosis (Martina, et al., 2020; Cheesbrough, 2005).

1.1. Aims of the Study

- The study aimed to diagnose *Burkholderia cepacia* from different clinical cases.
- Draw attention to the pathological importance of these bacteria and their effect on infected people.
- Giving the difference in diagnosis between the different methods of diagnosis by conventional examination and molecular diagnosis .
- Recommending the adoption of molecular diagnostics for its accuracy and importance.

2. Materials and Methods

2.1. Samples Collection

The study included 280 specimens

- The 180 swab specimens were collected from patients suffering from (diabetic foot ulcers, urine, bourn, wounds, sputum, and discharge from the eye).
- The specimens were transported by sterile transport swabs and inoculated using a direct method of inoculation on culture media, in addition to a specialized medium only for growth *Burkholderia cepacia*, then inoculated at 37°C for 18-24 hours (Miller, et al., 2002).
- All the suspected isolates obtained were examined under the microscope after staining with gram stain; they appeared as Gram- negative single short bacilli.
- *B. cepacia* isolates grown on MacConkey agar medium appeared as non-lactose fermented (NLF), small, pale pink color colonies, after 4-7 days, colonies became dark pink to red due to oxidation of lactose (16, 2), with clinical specimens at patient age (1-75 years).
- It was shown that 280 samples were distributed accordingly as shown in Table (1), the lowest incidence was in the age groups over (61-75) 7 %, and the highest incidence was the (31-45) age group (26.4 %).

Table 1
The distribution of patients according to age groups and sex is shown

Sex	Age group No. (%)					
	01-15	16-30	31-45	46-60	61-75	total
Female	24	12	16	12	5	69
	-8.5	-4.2	-5.7	-4.2	-1.7	-24.6
Male	44	48	58	46	15	211
	-15.7	-17.1	-20.7	-16.4	-5.3	-75.3
Total	68	60	74	58	20	280
	-24.2	-21.9	-26.4	-20.6	-7	-100%

2.2. Morphologically Characterization

The bacterial isolates obtained from clinical samples were identified initially according to cultural morphology, microscopic characteristics, and biochemical tests. Microscopically *B. cepacia* appeared gram-negative bacilli; the cultural identification of *B. cepacia* was depended on the colonial morphology. Since the colonies of *B. cepacia* were grown on, blood agar appears diffuse-hemolytic, (Hasan, 2019). B. cepacia non-lactose fermenting colonies on MacConkey's agar and produced pigment on other media as in figure (1).

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

2.3. The Biochemical Tests

The biochemical test results recorded in Table (2) are it is considered to complement the initial identification of the *B. cepacia* isolate. The isolates confirm to general characteristics, isolates were positive for oxidase , catalase test, motility, citrate utilization, gelatinize and smell dirty, but negative result for production urease, Voges Proskauer and methyl red test and, this is consistent reviewed studies, while the indole production and H2S production test were positive (Figure 2).

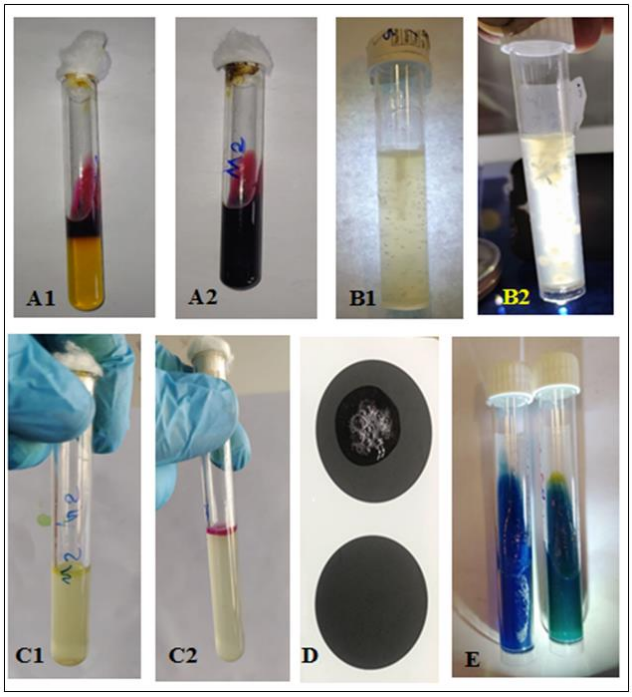

Fig. 2. The biochemical tests : A- TSI test (Alkaline, Acid, H2S) B- Motility test (1- negative , 2- positive) C- Indole production (1-negative , 2- positive), D- Catalase test, E- Citrate utilization { green (-), blue (+)}

Table 2
Biochemical tests of *B. cepacia* isolates

%	No. samples according PCR (30)	%	No. of total –ve samples (80)	Result	Biochemical test	No.
100%	30	62.50%	50	K/A	Triple sugar iron (TSI)	1
80%	24	50%	40	+	H2S production	2
20%	6	50%	40	_		
+	30	75%	60	+	Catalase	3
63.30%	19	68.70%	55	+	Oxidase	4
36.60%	11	31.30%	25	_		
76.60%	23	52.50%	42	+	Indole production	5
25%	7	47.50%	38	_		
100%	30	43.70%	35	+	Citrate utilization	6
100%	30	77.50%	62	_	(voges proskauer) VP	7
16.60%	5	25%	20	+	Growth at 42 oC	8
83.30%	25	75%	60	_		
100%	30	77.50%	62	+	Motility	9
100%	30	57.50%	46	Dirt like odour	Smell	10
50%	15	41.20%	33	+	Growth on Cetrimide agar medium	11
50%	15	58.70%	47	_		

3. Results & Discussion

As shown in table (1), of the 280 patients 211 (75.3 %) were male and 69 (24.6 %) were female. Increasing infected male more than female frequency similar to the result of ALmuhana and Alammar (2020), they observed that male more frequency than female (61.7 % vs 38.3%) from the total 120 clinical samples (180 male and 90 female). Shekhar *et al.*, (2014) showed that men recorded 72.2% as a male-female ratio is 2: 1 and the age range is 36-75 years. Harness-Brumley, *et al.,* (2014), observed that all pathogens increased the risk of female to male-based mortality from the original hazard ratio of 1.25. As the study shows, the predominance of men over female patients can be explained by the fact that men are exposed to external environmental factors. The wounds heal in females better than in males, maybe that due to differences in hormones and explain that increased estrogen receptors in females increase healing wound which acts as endogenous enhancers of the healing process while in the male the increase in the level of androgen; where was considered harmful for wound healing since androgenic species decrease repair of the dermis (ALmuhana & Al-Ammar, 2020).

Regarding the *B. cepacia* detection which the biochemical test results recorded in Table (2) are considered to complement the initial identification of the *B. cepacia* isolate. The isolates confirm to general characteristics, isolates were positive for oxidase, catalase test, motility, citrate utilization, and smell dirty, but the negative result with Voges Proskauer test, this is consistent with Raheem and Said (2018), and ALmuhana and Alammar (2020), and it appeared in our study that indole positive and production H2S during an examination of the triple sugar iron (TSI) test were positive results (Figure 2).

An oxidase test was performed to distinguish between isolates that produce the enzyme cytochrome C oxidase

producers, which contains a chromogenic reducing compound, which turns blue or purple color within 15 seconds (Raheem and Said, 2018). Another study was done by Abbas, (2017) performed that some of B. cepacia isolate showed a negative result in the oxidase test.

Concerning enzyme catalase, which is produced by bacteria that respire using oxygen, this enzyme is responsible for mediating the breaking down of H2O2 into H2O and O2, so it neutralizes the bactericidal effects of H2O2 and protects the bacterial cells, this finding goes under the approved by Al-Dahash *et al.*, (2012) and ALmuhana and Alammar (2020), when they showed that all their B. cepacia isolates are catalase positive.

The *B. cepacia* isolates in this study show variable growth at 42 C and this result is in simile with Ragupathi, and Veeraraghavan, (2019) while Raheem and Said (2018) were mentioned that *B. cepacia* isolates differ in their ability to grow at 42 0C. All *B. cepacia* isolates were grown on the triple sugar iron (TSI) medium was (K/A + -) which indicates oxidation of glucose and produces acid from it, as well as produce H2S appears black color, These results resemble the information mentioned by (26; 27). In our study, most of the isolates produce the Indole, as well as the *B. cepacia*, have the ability to motile by Polystichum flagella. B. *cepacia* was growing on a cetrimide medium and tolerates the high concentration of cetrimide in this medium. All isolates (100%) grew on this medium Table (2). However, this is consistent with ALmuhana and Alammar (2020).

3.1. Results obtained by ID GNB cards and VITEK 2 System

The results were out of the 80 gram-negative samples according to the initial examination of the gram stain, the results obtained from the VITEK 2 System by ID GNB cards showed that identification of (45) samples was (16) isolate (20%) showed confidence value 99-96% (excellent identification), (9) isolate (11.2%) showed confidence values 96-95% (very good identification) and only (20) isolate (25%) showed confidence value (89%) acceptable identification value, while 35(43.7%) samples not diagnosed. The isolates of *B. cepacia* were distributed among 11 isolates, designated biopatterns based on the results obtained, according to the results obtained from the SPSS these presences compare with the results obtained from the PCR these results accuracy more than.

3.2. API (Analytical Profile Index) Microsystems

Based on 80 samples as a total number the results of API 20E test showed the 33 (41.3%) isolates, 22(27.5%) nearest identity, and 25(31.2%) no identity, as show in table (3). In this technique, the results obtained les accuracy than VITEK 2 System and PCR.

Table 3
Results obtained from APi 20E Kit

Description of group	Codes of results test	Numbers of isolates
First group (Exact identity)	(5 300 004)	1,4,6,10,13,15,18,19,11,2,30,31,33,36,39,41,44,45,49,51,58,59,60,62 ,63,66,69,70,72,74,78,79
Second group (nearest identity)	(5 302 004)	3,14,17,22,12,23,26,27,34,35,42,46,48,50,52,53,56,64,68,71,73,75
Third group (no identity)	(5 302 000)	5,7,8,9,16,20,21,24,25,28,29,32,37,38,40,43,47,54,55,57,61,65,67, ,76,77

3.3. Extraction of Genomic DNA

DNA of The isolated *B. cepatia* was prepared by boiling method. Briefly, colonies were suspended in 100 microliters of sterile distilled water and boiled at 1000C in the water bath for 15 minutes then rapidly cooled at -200C for one hour, then centrifugation and the supernatant were preserved for use in the amplification processes (Payne, et al., 2005).

3.4. Estimation of DNA Concentration

By reading the optical density of a sample at 260 and 280 nm in spectrophotometry, the concentration and purity of DNA can be calculated, with 5 µL of each DNA sample being applied to 995 µL. From well combined purified water. The 260 nm reading. It allows the nucleic acid concentration in the sample to be measured. 1 OD260 = 50 ug/ mL For pure double-stranded DNA (20). An estimation of the purity of the nucleic acid is given by the ratio between the readings at 260 and 280 nm (OD 260/OD 280). Pure DNAA preparation has an OD 260/OD 280 value of 1.8 and the DNA concentration has been determined using the formula:

DNA concentration (µg/ml) = O.D 260nm × 50 × dilution factor

3.5. Preparing the Primers Suspension

The DNA primers (from Macrogen company, Korea) were resuspended by dissolving the lyophilized product after spinning down briefly with TE buffer molecular grad depending on manufacturer instruction as stock suspension.

Working primer tube was prepared by diluted with TE buffer molecular grad. The final picomoles depended on the procedure of each primer.

3.6. Polymerase Chain Reaction Protocol

The DNA extract of bacterial isolates was subjected to primers genes listed in Table (3-7) by using PCR. The protocol was used depending on the Promega Biosystem manufacturer's instruction. As shown in table (3-7), a single reaction (final reaction volume of 20 µl) is used. All PCR components were assembled in a PCR tube and mixed by refrigerated microcentrifuge at 50 rcf for 10 seconds.

3.7. Identification of *B. cepacia* by rec gene

The gene was used to identify bacteria *B. cepacia recA* gene, wer the study evidenced that the recA gene was observed in 30 samples out of 45 samples were diagnosed with a device VITEK 2 System as in the figure (3).

In bacterial systematics, the *recA* gene has been widely used and has been very useful for the identification of *B. cepacia* complex species, with phylogenetic study of sequence variance within the gene allowing all nine current species to be discriminated against within the *B. cepacia* complex. However, the original *rec*A-based PCR primers,

BCR1 and BCR2, are unique only to the members of the *B. cepacia* complex and do not amplify this gene from other species of *B. cepacia* . Although this can be used as a constructive way of verifying the location of an isolate within the complex, it restricts the use of the technique to classify other species of *Burkholderia* in different natural environments (Payne, et al., 2005; Al-Sadi, et al., 2015). The results obtained about gene techneque (PCR) that's very accuracy and we can depended it.

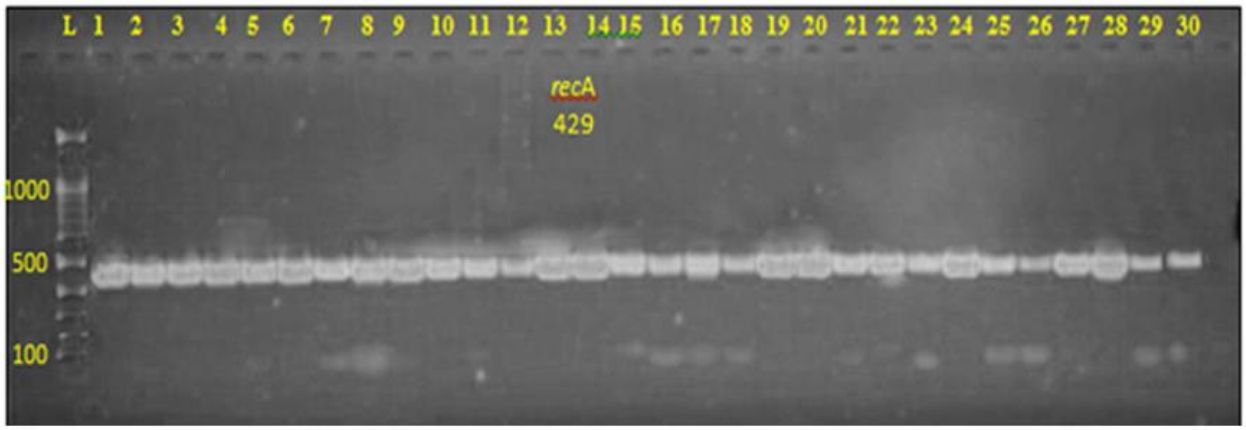

Fig. 3. PCR amplification products of *B. cepacia* isolate that amplified with *recA* gene primers with product 429 bp. Lane (L), DNA molecular size marker (100-bp ladder), Lanes (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18 , 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30) show positive results with the *recA* gene

3.8. *16S rDNA* gene

The result showed that the *16S rDNA* identification gene was detected in all isolates of *B. cepacia* (100%) as in figure (4). The 16S *rDNA* gene consists of closely conserved sequences of nucleotides, interspersed with genus- or species-specific variable regions. Nucleotide sequence analysis of the PCR product, followed by a comparison of this sequence with known sequences stored in a database, may classify bacteria. In the last decade, 16S *rDNA* sequencing has played a crucial role in the accurate detection of bacterial isolates and the discovery of new bacteria in clinical microbiology laboratories due to the extensive use of PCR and DNA sequencing. 16S *rDNA* sequencing is particularly important for bacterial detection in the case of bacteria with unique phenotypic profiles (Woo, et al., 2008), rare bacteria, slow-growing bacteria, uncultivable bacteria, and culture-negative infections. It has not only offered research into infectious disease etiologies, yet also helps physicians select antibiotics and assess the length of therapy and infection management procedures. Through the use of *rDNA* 16S, in the 21st century (2001-2007) sequencing, 215 new bacterial species, 29 of which belong to novel genera, were discovered from human specimens (Sapkota, et al., 2018).

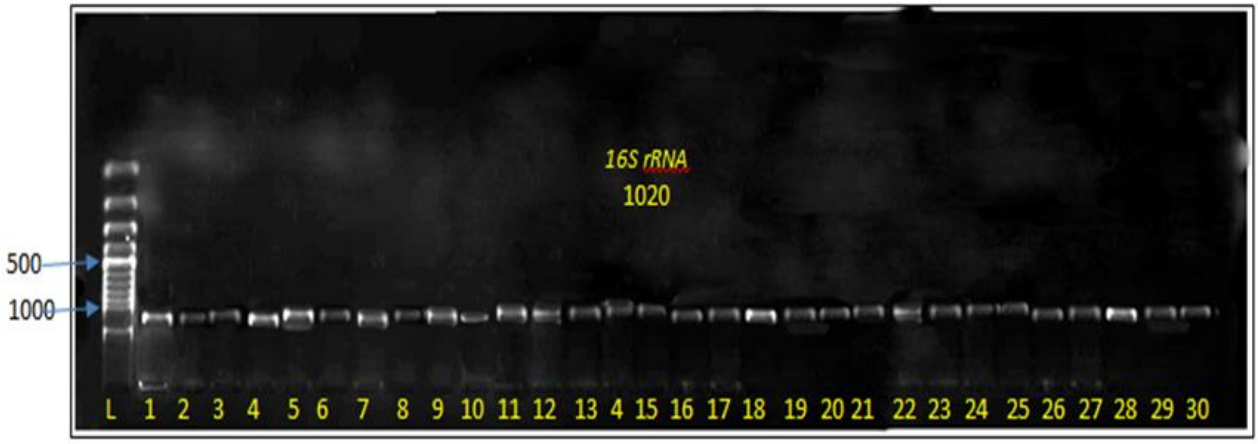

Fig.4. PCR amplification products of *B. cepacia* isolates that amplified with *16S rDNA* gene primers with product 1020 bp. Lane (L), DNA molecular size marker (100-bp ladder), all isolates show positive results with 16S rDNA gene

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3.9. Statistical Analysis

Depending on the results obtained from the diagnosis of bacteria in three ways (API 20E , Vitek 2 system and PCR), there were significant differences between the methods, and

the likelihood of testing PCR in terms of accuracy in diagnosis depending on the presence of the diagnostic gene of *B. cepacia* isolates, as in tables (4,5,6) .

Table 4
Preparing diagnostic samples according to the methods used and the type of sample source

POSITION * Method Crosstabulation

	RESULT			Method		
				Api 20	vitek	PCR	Total
B. cepaia isolates (+)	POSITION	Burn	Count	46	43	8	97
			%within POSITION	47.40%	44.30%	8.20%	100.00%
			% within Method	20.50%	19.90%	19.50%	20.20%
		sputum	Count	20	34	3	57
			%within POSITION	35.10%	59.60%	5.30%	100.00%
			% within Method	8.90%	15.70%	7.30%	11.90%
		Urine	Count	23	38	5	66
			% within POSITION	34.80%	57.60%	7.60%	100.00%
			% within Method	10.30%	17.60%	12.20%	13.70%
		wounds	Count	61	44	8	113
			% within POSITION	54.00%	38.90%	7.10%	100.00%
			% within Method	27.20%	20.40%	19.50%	23.50%
		Ulcer	Count	61	47	12	120
			% within POSITION	50.80%	39.20%	10.00%	100.00%
			% within Method	27.20%	21.80%	29.30%	24.90%
		control	Count	13	10	5	28
			% within POSITION	46.40%	35.70%	17.90%	100.00%
			% within Method	5.80%	4.60%	12.20%	5.80%

Table 5
The difference between the efficiency of the methods used in the diagnosis

GROUPS	RESULT	N	Mean	Std. Deviation	Std. Error	95%Confidence Interval for Mean		Min.	Max.	LSD Sig.
						Lower Bound	Upper Bound			
PATIENTS	-	Api 20	5	37.80 a	19.892	8.896	13.10	62.50	19	60	16.576
		vitek	5	38.80 a	5.167	2.311	32.38	45.22	33	46	0.001
		PCR	5	72.80 b	3.421	1.530	68.55	77.05	68	77	
	+	Api 20	5	42.20 a	19.892	8.896	17.50	66.90	20	61	16.576
		vitek	5	41.20 a	5.167	2.311	34.78	47.62	34	47	0.001
		PCR	5	7.20 b	3.421	1.530	2.95	11.45	3	12	

Table 6
The likelihood between the methods used to diagnose between disease cases and control

Parameter Estimates

GROUPS	RESULTa,b		B	Std. Error	Wald	df	Sig.	Exp(B)	95% Confidence Interval for Exp (B)	
									Lower Bound	Upper Bound
PATIENTS	-ve	[Method=1]	-0.110-	0.100	1.209	1	0.272	0.896	0.736	1.090
		[Method=2]	-0.060-	0.100	0.360	1	0.549	0.942	0.774	1.146
		[Method=3]	2.314	0.175	175.361	1	0.000	10.111	7.179	14.240
CONTROL	-ve	[Method=1]	0.268	0.368	0.530	1	0.467	1.308	0.635	2.692
		[Method=2]	0.693	0.387	3.203	1	0.074	2.000	0.936	4.273
		[Method=3]	1.609	0.490	10.793	1	0.001	5.000	1.914	13.061

a. The reference category is: +ve. b. The reference category is: +ve

Exp (B) means(OR) , the odds ratio explained by method 3 PCR, its likelihood in the shoulder compared to the Boztve is 10.111 times the odds of the first and second methods in patients, while the odds of 3 PCR in control is equivalent to 5.000.

4. Conclusion

- The use of GNB ID cards of VITEK 2 System has less error potential than the APi 20 technique, and both techniques are less accurate than the PCR technology for diagnosis of *B. cepacia* bacterial isolates.
- The molecular identification using 16s *rRNA* gene followed by sequencing the product and analysis and *recA* gene is very accurate and low cost method compared to the previous two techniques method to confirm the identification of *B. cepacia* isolates.
- Statistical analysis by the method of likelihood proved the accuracy of the PCR technique in diagnosis of *B. cepacia* isolates.

4.1. Acknowledgments

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Ethical clearance: The research ethical committee oversees scientific research in Iraq with the ethical approval of the ministries of the environment, health, and the higher education scientific research.

4.2. Recommendations

- B. cepacia bacteria is one of the most important causes of complications associated with the injury, which leads to exacerbation of infections and ineffectiveness of treatment, and compared with previous studies, we found that there is a clear increase in the spread of this bacteria in hospitals, noting that it is neglected by doctors when diagnosing and treating.

- The Oxidation-Fermentation Base and OFPBL agar medium is advised to be used for rapid primary identification of Bcc isolates.
- The PCR is a very important system that is used for the identification of different bacterial species, and is advisable to be used for fast and correct diagnosis of bacterial samples, especially in hospitals.
- Imipenem and meropenem are the drug choice to be used to treat infections caused by *B. cepacia.*

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