A Molecular Study with A Comparison of the Odds of Diagnostic Methods For *Burkholderia Cepacia* Bacteria Isolated from Patients with Diabetic Foot Ulcer

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Abstract

The study aim to isolation and identification of Gram negative aerobic bacteria from clinical samples from various pathological conditions, investigation the virulence factors genes of bacteria. The present study included 250 specimens collected from patients suffering from diabetic foot ulcer , cystic fibrosis , people exposed to burns to different degrees , People with eye infections, in addition to those with fistulas , who attends to Alsader medical city, Al-Hakim General Hospital during the period extended from September 2020 to February 2021 for both sexes with an age ranged between)1 -75) years. The results revealed that 20 specimens of the total number of samples are *Burkholderia cepacia* . The results revealed that the frequency among males 198 (79.2%) more than female 52 (20.8%). The sample distribution according age, it appears high 7.6% with group 1-15 years in female , and high 22% with group 31-45 years in male, while high 26.8% with group 31-45 years male and female .

Keywords *: B. cepacia , API 20E , Vitek , PCR, diagnostic methods*

Introduction

Burkholderia cepacia is detection as a group of highly virulent organisms known as the Burkholderia cepacia complex (Bcc). Bcc is ubiquitous in nature 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 [(1)] .

B. cepacia is a Gram-negative bacilli, rod-shaped, non-sporeforming, motile, catalase-positive and lactose-non fermenting bacteria [(2)]. 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 fungal [(3-4)] . 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 [(5)]. 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 [(6)].

The species *B. cepacia* is a complex of organisms consisting of nine different genomovars [(7)]. Genomovers are similar in phenotype but different in genotype. Some of these have already been given their own species designations. In 2002, Genomover III, which contains some of the more infectious strains of *B. cenocepacia* was reassigned to the species designation [(8)].

This bacterium was relatively unknown as a human pathogen until the mid-1980s, when it surfaced as a nosocomial infection at cystic fibrosis clinics [(9)]. Like many opportunistic pathogens, *B. cepacia* can establish

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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* [10] . Also, 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 [11-12].

Methods

Samples Collection

The study included 280 specimens (Of which 180 swab specimens) collected from patients suffering from (diabetic foot ulcers, urine , bourn , wound , sputum and discharge from the eye),who attending to Alsader medical city, Al-Hakim General Hospital in ALNajaf / Iraq , during the period extended from septmper 2020 to February 2021 for both sexes with an age ranged between)1 -75) years. The specimens were transported by sterile transport swabs and inoculated using 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 [13].

All the suspected isolates obtained were examined under the microscope after staining with gram stain, they appeared as Gram- negative single short bacilli [14-15]. *B. cepacia* isolates grown on MacConkey agar medium appeared as non- lactose fermenters (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).

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-haemolytic [15,17] . *B. cepacia* non lactose fermenting colonies on MacConkey's agar and produced pigment on other media .

Results and Discussion

The biochemical test results recorded in Table (1) . 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 like odour , but negative result for production urease, Voges Proskauer and methyle red test and ,this is consistent with [15 , 18], while the indole production and H2S production test were positive .

Table 1 . Biochemical tests of *B. cepacia* isolates.

No.	Biochemical test	Result	No. of total –ve samples (80)	%	No. samples according PCR (30)	%
1	Triple sugar iron (TSI)	K/A	50	62.5%	30	100%
2	H2S production	+	40	50%	24	80%
		_	40	50%	6	20%
3	Catalase	+	60	75%	30	+
4	Oxidase	+	55	68.7%	19	63.3%
		_	25	31.3%	11	36.6%

Cont... **Table 1 . Biochemical tests of *B. cepacia* isolates.**

5	Indole production	+	42	52.5%	23	76.6%
		_	38	47.5%	7	25%
6	Citrate utilization	+	35	43.7%	30	100%
7	(voges proskauer) VP	_	62	77.5%	30	100%
8	Growth at 42 oC	+	20	25%	5	16.6%
		_	60	75%	25	83.3%
9	Motility	+	62	77.5%	30	100%
10	Smell	Dirt like odour	46	57.5%	30	100%
11	Growth on Cetrimide agar medium	+	33	41.2%	15	50%
		_	47	58.7%	15	50%

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 were (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. 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 (2) .

Table 2. 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, 41,44,45,49,51,58,59,60,62,63,66,69,70,7 2,74,78,79
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)	5,7,8,9,16,20,21,24,25,28,29,32,37,38,40,4 3,47,54,55,57,61,65,67, ,76,77

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 (1).

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 *recA*-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 [19-20].

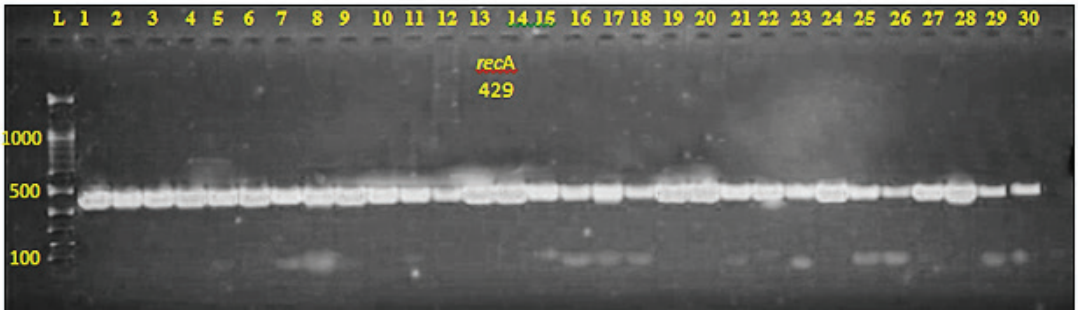

Figure 1. PCR amplification products of *B. cepacia* isolates that amplified with *recA* gene primers with product 429 bp. Lane (L), DNA molecular size marker (100-bp ladder), Lanes (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18 , 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30) show positive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

The result showed that the *16S rDNA* identification gene was detected in all isolates of *B. cepacia* (100%) as in figure (2).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 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, 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 [21-22] .

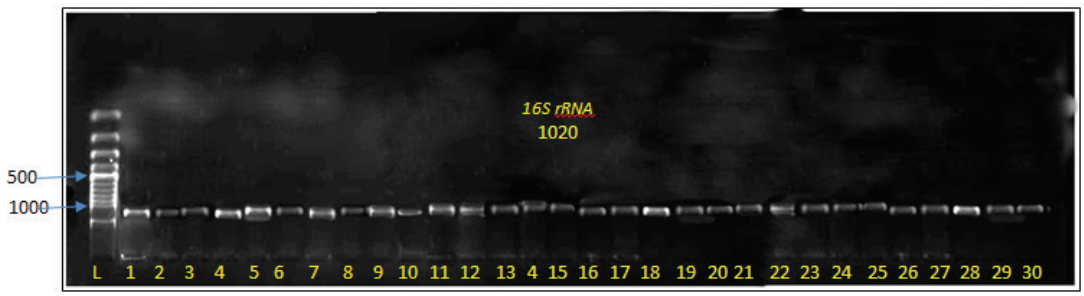

Figure 2. PCR amplification products of *B. cepacia* isolates that amplified with *16S rDNA* gene primers with product 1020 bp. Lane (L), DNA molecular size marker (100-bp ladder), all isolates show positive results with *16S rDNA* gene.

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 (3) .

Table 3 . Preparing diagnostic samples according to the methods used and according to the type of sample source.

Position * Method Cross tabulation							
Result				Method			Total
				Api 20	vitek	PCR	
B. cepaia isolates (+)	Position	Burn	Count	46	43	8	97
			%within POSITION	47.4%	44.3%	8.2%	100.0%
			% within Method	20.5%	19.9%	19.5%	20.2%
		sputum	Count	20	34	3	57
			%within POSITION	35.1%	59.6%	5.3%	100.0%
			% within Method	8.9%	15.7%	7.3%	11.9%
		Urine	Count	23	38	5	66
			% within POSITION	34.8%	57.6%	7.6%	100.0%
			% within Method	10.3%	17.6%	12.2%	13.7%
		wounds	Count	61	44	8	113
			% within POSITION	54.0%	38.9%	7.1%	100.0%
			% within Method	27.2%	20.4%	19.5%	23.5%
		Ulcer	Count	61	47	12	120
			% within POSITION	50.8%	39.2%	10.0%	100.0%
			% within Method	27.2%	21.8%	29.3%	24.9%
		control	Count	13	10	5	28
			% within POSITION	46.4%	35.7%	17.9%	100.0%
			% within Method	5.8%	4.6%	12.2%	5.8%

Through the results obtained from the three methods used in the current study, there were differences in the numbers of diagnosed samples, and the result of the Bissier test was considered the most accurate due to our use of the *B. cepacia* diagnostic gene, as well as the accuracy of the examination was statistically confirmed in the statistical program SPSS according to the appearance of the odds ratio [Exp(B)] .

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.

Ethical Clearance : Taken from University of Kufa ethical committee

Source of Funding : Self

Conflict of Interest : Nil

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