

Identification, characterization and antibiotic susceptibility testing for *Bacillus* species

Noha Alaa Eldin Fahim¹, Ghada Abd El-Wahed Ismail¹, Moataz Bellah Abdelaleem^{1*}, Mervat Elanany^{2,3}, Sally Mohamed Saber¹, Marwa Abd EL-Rasoul EL-Ashry¹

¹Department of Clinical Pathology, Faculty of Medicine, Ain Shams University, Cairo, Egypt

²Department of Clinical Pathology, Faculty of Medicine, Cairo University, Giza, Egypt

³Department of Microbiology, Children's Cancer Hospital Egypt, Cairo, Egypt

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ABSTRACT

Background and Objectives: With the increase in immunosuppressed patients and antimicrobial misuse, *Bacillus* species have risen as opportunistic pathogens in hospitalized patients. The present study aimed at comparing chromogenic media, automated identification cards versus MALDI-TOF as the gold standard method for identification of different *Bacillus* species and determining minimal inhibitory concentration (MIC) of different antibiotics by broth microdilution method (BMD) to suggest recommendations for *Bacillus* treatment.

Materials and Methods: The study included 30 *Bacillus* species isolates recovered from normally sterile sites of the human body and were subjected to identification by MALDI-TOF, Vitek-2c, and HiCrome Bacillus Agar. BMD test was performed to determine the MIC of vancomycin, gentamicin, and ciprofloxacin.

Results: Our study showed *B. cereus* was the most commonly isolated species (76.66%) followed by *B. subtilis* (23.33%). Regarding the different methods of identification, the highest agreement with MALDI-TOF was exhibited by HiCrome agar without polymyxin B (93.3%) followed by Vitek-2C and HiCrome agar with polymyxin with an agreement of 83.3%. Concerning the antibiogram, the tested isolates showed a susceptibility of 93.3%, 86.6%, and 83.3% towards vancomycin, gentamicin, and ciprofloxacin respectively.

Conclusion: In conclusion, we spotlight that *Bacillus* species should no longer be considered contaminant bacteria in cultures, particularly in immunosuppressed patients. HiCrome Bacillus Agar without polymyxin B displayed the highest agreement with MALDI-TOF. Hence, it represents a good option for identification for routine laboratories where expensive instruments are unavailable. The high susceptibility towards the tested antibiotics can suggest the possibility of empirical use of vancomycin, gentamicin, and ciprofloxacin.

Keywords: *Bacillus cereus*; *Bacillus subtilis*; Identification; Antibiotic resistance

INTRODUCTION

Bacillus species are Gram-positive, spore-forming, rod-shaped, aerobic or facultative anaerobic bacteria (1). Predisposing factors responsible for hu-

man infections with *Bacillus* spp. other than *Bacillus anthracis* include chronic alcoholism, presence of intravascular devices, intravenous drug abuse, and trauma (2).

Although *Bacillus* species are uncommonly re-

*Corresponding author: Moataz Bellah Abdelaleem, Ph.D, Department of Clinical Pathology, Faculty of Medicine, Ain Shams University, Cairo, Egypt. Tel: +2-01009197632 Fax: +20224346753 Email: Drmoatazbellah@hotmail.com

ported as major human pathogens, they have been frequently reported among debilitated patients including hospitalized patients. The major drawback of the inadequate reporting of *Bacillus* spp. infections by clinical microbiology laboratories are the fact that most of these bacteria are saprophytic and their isolation in human clinical specimens is neglected as laboratory contaminants (3). The clinical range of infections caused by *Bacillus* spp. include bacteremia, endocarditis, wound infections, eye infection, respiratory tract infections, urinary tract infections and gastrointestinal tract, food poisoning, and meningitis (3, 4).

Laboratory identification of *Bacillus* spp. includes certain biochemical reactions and physiological properties such as anaerobically growth, motility, catalase production, citrate utilization, nitrate reduction, and glucose fermentation (3).

To overcome the limitations of conventional plating methods, selective chromogenic media are now widely used for the identification and differentiation of *B. cereus* from other members of the group. These media contain synthetic fluorogenic and chromogenic substrates that are cleaved by specific enzymatic activities (5).

Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS), which can be used to analyze the protein composition of a bacterial cell, has arisen as a new technology for species identification. MALDI-TOF MS is suitable for high-throughput and rapid diagnostics at low costs and can be considered an alternative for conventional biochemical and molecular identification systems in a conventional microbiological laboratory (6).

The present study aimed at comparing chromogenic media, automated identification cards versus MALDI-TOF as the gold standard method for identification of different *Bacillus* species and determining minimal inhibitory concentration of different antibiotics by broth microdilution method (BMD) to suggest recommendations for *Bacillus* treatment.

MATERIALS AND METHODS

Study design. This study is a cross-sectional study, which was conducted at the Central Microbiology Laboratory of Ain Shams University Hospitals in collaboration with Children's Cancer Hospital Egypt 57357 hospitals during the period between December

2018-December 2019. This research was approved by the Ethical Research Committee, Faculty of Medicine, Ain Shams University. (Ethical approval number: FWA 000017585), December 2018.

Study population and inclusion criteria. Thirty (30) non-duplicate pure *Bacillus* species isolates were recovered from sterile sites of the human body e.g. blood cultures, body fluids, and infected deep site surgical wounds. We collected isolates suggested to be pathogenic from ICU patients with a history of immunosuppression such as the use of immunosuppressive drugs and those with debilitating diseases. Also, we selected patients to be enrolled in our study based on their clinical data such as symptoms suggesting infections like bacteremia and deep-seated wound infections. Isolates were preliminarily identified as *Bacillus* spp. by Gram stain and biochemical tests that included positive Oxidase test, positive Catalase test, and positive Motility test in accordance with the identification procedures followed at the microbiology laboratory (3).

All the 30 isolates of *Bacillus* spp. were identified by Matrix-Assisted Laser Desorption Ionisation - Time of Flight (MALDI-TOF MS) (BioMérieux SA, Marcy l'Etoile, France).

Isolates were subcultured on 5% sheep blood agar and incubated for 24 h at 35°C in 5 to 10% CO₂. Then, a thin film of fresh colony growth was picked by VITEK PICKME™ pen and NIBS and smeared directly onto a well of the VITEK MS-DS™48-welled target slides plate, then 1 µL of ready-to-use α-cyano-4-hydroxycinnamic acid (VITEK MS-CHCA™ matrix) was added directly to the organism on the target slide and allowed to dry for 1-2 minutes to absorb energy from the VITEK MS laser and transferred it to the microorganisms to enable ionization (Fig. 1). As recommended by the manufacturer's instructions, the *Escherichia coli* ATCC 8739 strain, used as a calibrator and internal ID control, was inoculated on the calibration spots of each acquisition group (a small spot in the middle of each acquisition group) (7).

2. Vitek@2C BCL ID card for *Bacillus* species (bioMérieux SA, Marcy l'Etoile, France):

The bacterial suspensions were prepared from fresh colonies in 5 ml of sterile saline to be adjusted using the VITEK2 DensiChek (bioMérieux SA, Marcy l'Etoile, France) to a McFarland standard 2.0 with accepted range of 1.8-2.2. Then, each suspension was

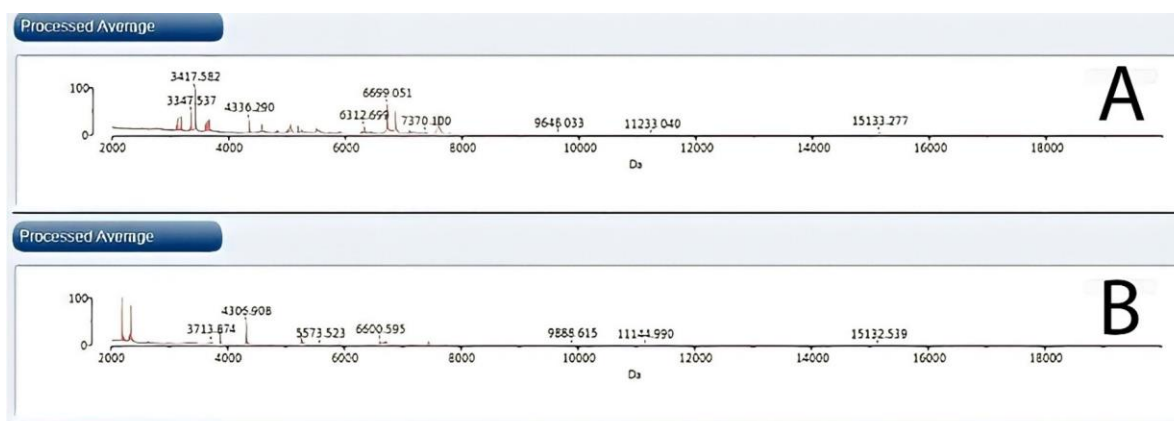


Fig. 1. Mass spectra for *Bacillus* spp. (a) *B. cereus* (b) *B. subtilis*.

inoculated to the corresponding ID card and then cards were filled automatically in the VITEK vacuum chamber, sealed, incubated at 35.5°C and read automatically every 15 min for 14 h (Fig. 2) (8).

3. Chromogenic media for *Bacillus* spp. (Hi-Crome™ *Bacillus* Agar, India). The media was prepared and dispensed in screw-capped flasks. Briefly, flasks were boiled and sterilized by autoclaving for 15 minutes. After boiling, the medium was homogenized under continuous stirring and cooled to 50°C. In the case of preparing media supplemented with polymyxin, aseptically rehydrated content of 1 vial of polymyxin B was added to one liter. Then, the fresh 24 hours colonies were sub-cultured on two culture plates, one chromogenic media supplemented with polymyxin B (10 gm per liter) and another without polymyxin B (Fig. 3).

As recommended by the manufacturer's instructions, polymyxin B was not added to improve the recovery of *Bacillus* species beyond the *B. cereus* group (9). Also, the *Enterococcus faecalis* ATCC 29212 strain can be used for quality control (Fig. 4).

Broth microdilution (BMD). We performed BMD test to determine the minimum inhibitory concentration of vancomycin, gentamicin, and ciprofloxacin (Sigma-Aldrich Chemical Co., Cairo, Egypt). The antibiotics were selected, and MICs were interpreted according to (CLSI M45, 2016) (10).

Fifty microliters (50 µl) of Mueller Hinton broth (MHB) were added to the wells in 96-well plates, and then each antibiotic was added in serial dilution in a row, with starting drug concentration of 64 µg/ml for vancomycin, gentamicin and 32 µg/ml for cipro-

floxacin. Then were serially diluted, so that the starting concentration and final dilution covered fourfold above the resistant breakpoint and fourfold below the sensitive one, finally, five microliters (5 µl) of bacterial suspension adjusted to a 0.5 McFarland turbidity standard was inoculated into each antibiotic containing well. *Staphylococcus aureus* ATCC® 29213 was used as a quality control strain as recommended by CLSI M45 (2016) (10).

Data management and atatistical analysis. The collected data was revised, coded, tabulated and introduced to a PC using the Statistical Package for Social Science (IBM Corp. Released 2019. IBM SPSS Statistics for Windows, Version 25.0 Armonk, NY: IBM Corp). Data were expressed as both numbers and percentages for categorized data.

The following tests were done:

1. Chi-square test to study the association between each 2 variables or comparison between 2 independent groups as regards the categorized data.
2. Cohen's Kappa statistics was used to measure the agreement between every two techniques. The agreement was interpreted as follows: Kappa's 0.80-1.00 is excellent, 0.61 to < 0.80 is good, 0.40-0.60 is moderate, Fair if 0.21- < 0.40, and poor if below 0.20.

The probability of error at 0.05 was considered significant, while at 0.01 and 0.001 are highly significant.

3. Diagnostic validity test was assessed by calculating the following:

- a. The diagnostic sensitivity: It is the percentage of diseased cases truly diagnosed (TP) among total diseased cases (TP+FN).
- b. The diagnostic specificity: It is the percentage of

bioMérieux Customer: **Microbiology Chart Report** Printed April 9,2022 2:30:52 PM CST
 Patient Name: moataz doctor Patient ID:dr
 Location: Physician:
 Lab ID: M6 Isolate Number:1

Organism Quantity:
 Selected Organism :**Bacillus cereus**
 Source : BCL Collected:

Comments:	

Identification Information	Analysis Time: 13.80 hours	Status: Final
Selected Organism	99% Probability Bacillus cereus	
ID Analysis Messages	Bionumber: 1227001144446621	

Biochemical Details																	
1	BXYL	(+)	3	LysA	-	4	AspA	-	5	LeuA	-	7	PheA	+	8	ProA	-
9	BGAL	-	10	PyrA	+	11	AGAL	-	12	AlaA	(+)	13	TyrA	+	14	BNAG	+
15	APPA	-	18	CDEX	-	19	dGAL	-	21	GLYG	-	22	INO	-	24	MdG	-
25	ELLM	+	26	MdX	-	27	AMAN	-	29	MTE	(+)	30	GlyA	(-)	31	dMAN	-
32	dMNE	-	34	dMLZ	-	36	NAG	+	37	PLE	-	39	IRHA	-	41	BGLU	+
43	BMAN	-	44	PHC	-	45	PVATE	(+)	46	AGLU	(-)	47	dTAG	-	48	dTRE	+
50	INU	-	53	dGLU	+	54	dRIB	+	56	PSCNa	-	58	NaCl 6.5%	+	59	KAN	+
60	OLD	-	61	ESC	+	62	TTZ	(-)	63	POLYB I	+						

Fig. 2. Vitek2 report showing *Bacillus cereus* with probability 99%



Fig. 3. HiCrome™ *Bacillus* Agar showing Blue large flat colonies with blue center of *Bacillus cereus* (Left) and yellowish green colonies of *Bacillus subtilis* (Right).

non-diseased truly excluded by the test (TN) among total non-diseased cases (TN+FP).

c. The predictive value for a positive test: It is the percentage of cases truly diagnosed among total positive cases.

d. The predictive value for a negative test: It is the percentage of cases truly negative among total negative cases.



Fig. 4. HiCrome™ *Bacillus* Agar showing green colonies of *Enterococcus faecalis* ATCC 29212

RESULTS

The present study was conducted on 30 *Bacillus* species isolates that were recovered from different clinical specimens submitted to the Central Microbiology Laboratory, Ain Shams University Hospitals for culture and antimicrobial susceptibility testing. Isolates were identified biochemically following the microbiology laboratory procedures and were tested for further identification including MALDI-TOF as the reference method, VITEK2-C and chromogenic agar media and also tested for their susceptibility to different antimicrobial agents by the MIC method.

As regards types of samples in our study, 80% (24/30) were isolated from blood culture and 20% (6/30) were isolated from a deep wound, also 71.4% (5/7) of *B. subtilis* were isolated from wound culture and 28.6% (2/7) of *B. subtilis* were isolated from blood culture, while 91.4% (21/23) of *B. cereus* were isolated from blood culture and 8.6% (2/23) of *B. cereus* were isolated from wound culture. The types of samples enrolled in our study are summarized in Fig. 5.

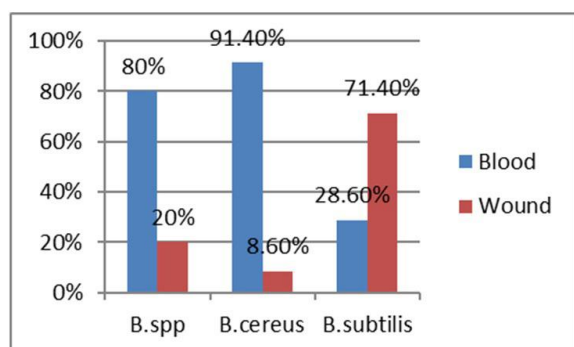


Fig. 5. Type of sample from which *Bacillus* spp. were isolated.

The results of the identification of 30 isolates of *Bacillus* spp. by Vitek-MS revealed that 23/30 (76.66%) were *Bacillus cereus* and 7/30 (23.33%) were *Bacillus subtilis*.

Regarding identification by the Vitek2 Compact system, it correctly identified 76.66% (23/30) of *Bacillus cereus* and 6.6% (2/30) of *Bacillus subtilis*. However, it failed to successfully identify the rest of the *Bacillus subtilis* isolates, where 3.3% (1/30) was *Geobacillus caldxylosilyticus*, 3.3% (1/30) was *Bacillus coagulans*, 3.3% (1/30) was *BerriBacillus Laterosporus* and 6.6% (2/30) were unidentified (Table 1).

Using Hicrome™ *Bacillus* Agar supplemented with polymyxin B supplement showed that 76.66%

(23/30) were *Bacillus cereus*, 6.6% (2/30) were *Bacillus subtilis*, and 16.6% (5/30) were inhibited showing no growth. While with using Hicrome™ *Bacillus* Agar without using polymyxin B supplement, 76.66% (23/30) were *Bacillus cereus*, 16.6% (5/30) were *Bacillus subtilis*, and 6.6% (2/30) were inhibited showing no growth. The data are summarized in Table (2).

As for the diagnostic performance for each identification method performed in the current study, we found that the VITEK 2c system and chromogenic media supplemented with polymyxin B for *B. cereus* had specificity, sensitivity, and negative predictive value, and a positive predictive value of 100% each. While for *B. subtilis*, the method had specificity, sensitivity, negative predictive value and positive predictive value of 100%, 28.6%, 82.1%, and 100% respectively. While the chromogenic media without polymyxin B for *B. cereus* had specificity, sensitivity, negative predictive value and positive predictive value of 100% for each. *B. subtilis* had specificity, and a positive predictive value of 100% with better sensitivity and negative predictive values of 71.4% and 92% respectively.

Regarding the agreement in the identification results of the *Bacillus* species among Vitek MS and Vitek2c system methods, data in Table 3 showed a statistically fair/good agreement (Kappa = 0.58). Both methods showed 100% agreement in the identification of *B. cereus* and 83.3% agreement in *B. subtilis* isolates.

Also, a statistically fairly good agreement was observed between Vitek MS and Hicrome Agar with supplement method regarding the identification results of the *Bacillus* spp. (Kappa = 0.58) is shown in Table 4. Both methods showed 100% agreement in the identification of *B. cereus* and 83.3% agreement in *B. subtilis* isolates.

Table 5 shows that a statistically excellent agreement was observed between Vitek MS and HiCrome Agar without supplement method regarding the identification results of the *Bacillus* spp. (Kappa = 0.821). Both methods showed 100% agreement in the identification of *B. cereus* and 93.3% agreement in *B. subtilis* isolates.

Regarding the antibiogram using the BMD method to detect the minimal inhibitory concentration of the 30 studied *Bacillus* species isolates, our results showed that 93.3% (28/30), 86.6% (26/30), and 83.3% (25/30) were susceptible to vancomycin, gentami-

Table 1. Identification of *Bacillus* spp. by Vitek2compact system

Organism retrieved by Vitek2 Compact system BCL cards	Percent	Number =30
<i>B. cereus</i>	76.66%	23
<i>B. subtilis</i>	6.6%	2
Misidentified		
<i>GeoBacillus caldxylosilyticus</i>	1%	1
<i>Bacillus coagulans</i>	1% 3%	1
<i>Bervibacillus Laterosporus</i>	1%	1
Unidentified	6.6%	2

Table 2. Identification of *Bacillus* spp. by Hicrome™ *Bacillus* Agar

Organisms retrieved by Hicrome <i>Bacillus</i> Agar	With Polymyxin B	Without Polymyxin B
<i>Bacillus cereus</i>	76.66% (23/30)	76.66% (23/30)
<i>Bacillus subtilis</i>	6.6% (2/30)	16.6% (5/30)
Inhibited growth	16.6% (5/30)	6.6% (2/30)

Table 3. Agreement between the Vitek MS and the Vitek2-C

		Vitek MS		Total	Kappa agreement	P value	
		<i>Bacillus cereus</i>	<i>Bacillus subtilis</i>				
Vitek 2C	<i>Bacillus cereus</i>	Count	23	0	23	0.58	0.000
		%	100.0%	0.0%	76.7%		
	<i>Bacillus Coagulans</i>	Count	0	1	1		
		%	0.0%	14.3%	3.3%		
	<i>Bacillus subtilis</i>	Count	0	2	2		
		%	0.0%	28.6%	6.7%		
	<i>Bervibacillus Laterosporus</i>	Count	0	1	1		
		%	0.0%	14.3%	3.3%		
	<i>Geobacillus caldxylosilyticus</i>	Count	0	1	1		
		%	0.0%	14.3%	3.3%		
	Unidentified Organism	Count	0	2	2		
		%	0.0%	28.6%	6.7%		
Total	Count	23	7	30			
	%	100.0%	100.0%	100.0%			

*HS= highly significant

cin and ciprofloxacin respectively, and 6.7% (2/30), 13.4% (4/30) and 16.7% (5/30) of isolates were resistant respectively.

Concerning the MIC results of the three tested antibiotics, the MIC of the two resistant isolates for vancomycin were 16 and 64 ug/ml. Whilst the MIC of the four resistant isolates for gentamicin ranged from 32 to 64 ug/ml. As for ciprofloxacin, the MIC of the five resistant isolates ranged from 8 to 32 ug/ml. Table 6 summarizes

the results of the MIC of the thirty studied isolates.

DISCUSSION

Although *Bacillus* spp. have not been recognized a major human pathogen, but with recent advances in medical technology and increased number of immunosuppressed patients, they have been increasingly recognized as opportunistic pathogens in hospital-

Table 4. Agreement between the Vitek MS and HiCrome Agar with supplement.

			Vitek MS		Total	Kappa agreement	P value
			<i>Bacillus cereus</i>	<i>Bacillus subtilis</i>			
HiCrome agar with supplement	<i>Bacillus cereus</i>	Count	23	0.000 S*	23	0.58	0.000 HS*
		%	100.0%	0.0%	76.7%		
	<i>Bacillus subtilis</i>	Count	0	2	2		
		%	0.0%	28.6%	6.7%		
	Inhibited	Count	0	5	5		
		%	0.0%	71.4%	16.7%		
	Total	Count	23	7	30		
		%	100.0%	100.0%	100.0%		

*HS= highly significant

Table 5. Agreement between the Vitek MS and HiCrome Agar without supplement

			Vitek MS		Total	Kappa agreement	P value
			<i>Bacillus cereus</i>	<i>Bacillus subtilis</i>			
HiCrome agar without Supplement	<i>Bacillus cereus</i>	Count	23	0.000 S*	23	0.821	0.000 HS*
		%	100.0%	0.0%	76.7%		
	<i>Bacillus subtilis</i>	Count	0	5	5		
		%	0.0%	71.4%	16.7%		
	Inhibited	Count	0	2	2		
		%	0.0%	28.6%	6.7%		
	Total	Count	23	7	30		
		%	100.0%	100.0%	100.0%		

*HS= highly significant

ized patients that can cause a wide range of infections (3, 11).

The identification of species in the genus *Bacillus* by classical methods is often difficult, despite still prevailing in many microbiology laboratories, due to similarities among closely related species that share a pattern of morphological, biochemical, and genetic characteristics. The use of matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) as a diagnostic technique for *Bacillus* spp. identification has been applied for addressing the challenges associated with the identification of these organisms (12).

We conducted the present study in Ain Shams University Hospitals in collaboration with 57357 hospitals during the period between December 2018 and December 2019. A total of 30 *Bacillus* isolates were recovered from different clinical specimens (blood

and deep wound swab) from the clinically diagnosed ICU bacteremic patients and those with infected deep-seated wounds respectively. Isolates were preliminarily identified phenotypically by Gram stain, oxidase, catalase, and motility test. They were further subjected to subspecies identification by MALDI-TOF MS, Vitek@2C ID cards, and chromogenic agar media. We performed minimal inhibitory concentration measurement of the following antibiotics (vancomycin, gentamicin, ciprofloxacin) by BMD to suggest recommendations for *Bacillus* treatment.

Regarding the results of MALDI-TOF, the present study showed that 23/30 (76.66%) were *Bacillus cereus* and 7/30 (23.33%) were *Bacillus subtilis*. Eighty percent of the isolates were recovered from blood culture while twenty percent were recovered from deep wounds. In a similar fashion, Fujita and Nishimura (2018) from Japan who did their research during

Table 6. MIC values of the thirty *Bacillus* isolates enrolled in the current study

Isolates	Antibiotic result by MIC		
	Vancomycin*	Gentamicin**	Ciprofloxacin***
	ug/ml	ug/ml	ug/ml
<i>Bacillus cereus</i>	0.5	0.5	1
<i>Bacillus cereus</i>	0.5	0.5	1
<i>Bacillus cereus</i>	1	0.5	1
<i>Bacillus subtilis</i>	0.5	1	1
<i>Bacillus cereus</i>	0.5	0.5	1
<i>Bacillus cereus</i>	2	2	0.5
<i>Bacillus cereus</i>	1	32	0.5
<i>Bacillus cereus</i>	2	2	1
<i>Bacillus cereus</i>	0.5	1	8
<i>Bacillus cereus</i>	1	0.5	0.5
<i>Bacillus cereus</i>	0.5	2	0.5
<i>Bacillus cereus</i>	2	1	32
<i>Bacillus subtilis</i>	2	0.5	1
<i>Bacillus subtilis</i>	0.5	0.5	1
<i>Bacillus subtilis</i>	2	1	1
<i>Bacillus cereus</i>	0.5	32	32
<i>Bacillus subtilis</i>	1	1	0.5
<i>Bacillus subtilis</i>	2	2	1
<i>Bacillus subtilis</i>	0.5	32	0.5
<i>Bacillus cereus</i>	1	1	1
<i>Bacillus cereus</i>	0.5	1	16
<i>Bacillus cereus</i>	2	0.5	32
<i>Bacillus cereus</i>	1	1	1
<i>Bacillus cereus</i>	1	4	0.5
<i>Bacillus cereus</i>	16	0.5	1
<i>Bacillus cereus</i>	4	64	0.5
<i>Bacillus cereus</i>	0.5	1	0.5
<i>Bacillus cereus</i>	64	0.5	1
<i>Bacillus cereus</i>	0.5	1	1
<i>Bacillus cereus</i>	4	1	0.5

* Vancomycin: sensitive ≤ 4 ug/ml

** Gentamicin: sensitive ≤ 4 ug/ml; intermediate 8 ug/ml; resistant ≥ 16 ug/ml

*** Ciprofloxacin: sensitive ≤ 1 ug/ml; intermediate 2 ug/ml; resistant ≥ 4 ug/ml

the period from 2011 to 2016 in a cancer hospital, found that the majority of *Bacillus* species isolated from blood cultures were *Bacillus cereus* (42/52, 80.7%) followed by *Bacillus subtilis* (4/52, 7.7%) (13). Also, Celandroni and coworkers (2016) from Italy worked on seventy-five *Bacillus* spp. clinical isolates that were collected from sterile body sites over two years period and noted that one-third of them were *B. cereus* species. They isolated *B. pumilus* and *B. subtilis* at a high rate as well (14).

We noticed that *B. cereus* displayed several group-specific peaks at 3417, 6699 Da. The intensity of which varied depending on the degree of sporulation. Comparable findings were reported by Lasch and colleagues (2009) in Germany who worked on veterinarian strains and they noted that the *B. cereus* group members *B. anthracis*, *B. cereus*, and *B. thuringiensis* displayed several group-specific peaks at 3,683, 4,334, 5,171, 5,886, and 7,368 Da respectively (15).

Concerning VITEK 2 Compact system, results were 23 (76.66%) *Bacillus cereus* and 2 (6.6%) *Bacillus subtilis* i.e. 83.3% of the total isolates were well identified. On the other hand, the Vitek 2 Compact system failed to identify the remaining *Bacillus subtilis* isolates where 3 (9.9%) were misidentified and 2 (6.6%) were unidentified. Similar findings were reported by Halket and coworkers (2010) from the UK. They worked on one hundred and nine *Bacillus* species isolates from industrial, environmental and clinical sources and found that 101/109 (93%) were identified correctly to the species level including *Bacillus cereus*, *B. licheniformis*, *Bacillus pumilus*, *Bacillus subtilis*, and *Geobacillus*. While, 7/109 (6%) were misidentified including *Bacillus flexus*, *Bacillus simplex*, *Lysinibacillus sphaericus*, *Paenibacillus lautus* and *Virgibacillus pantothenicus*. One strain (1%) of *Bacillus megaterium* remained unidentified (8).

Using HiCrome *Bacillus* Agar supplemented with polymyxin B, 83.3 % of total isolates were well identified with 76.66% (23/30) *Bacillus cereus* and 6.6% (2/30) *Bacillus subtilis*. It is worthy to note that the presence of polymyxin B supplement resulted in inhibition of 16.6 % (5/30) of total isolates showing no growth. While in absence of the polymyxin B supplement, the performance of HiCrome *Bacillus* Agar improved and 93.3 % of total isolates were successfully identified with 76.66% (23/30) were *Bacillus cereus* and 16.6% (5/30) were *Bacillus subtilis* with lowering the inhibition rate to 6.6% (2/30). In Argentina, a study done by Alippi and Abrahamovich (2019) on eighty-three isolates of *B. cereus* of honey coincided with our findings and reported that HiCrome media correctly identified all their isolates (9).

Among the tested methods, HiCrome *Bacillus* Agar without polymyxin B displayed the highest agreement with our reference method. Hence, it represents a good option for *Bacillus* identification in routine laboratories where expensive and sophisticated instruments are unavailable.

As for the results of antimicrobial testing using the BMD Method, out of the 30 studied isolates, 28 (93.3%), 26 (86.6%), and 25 (83.3%) were susceptible to vancomycin, gentamicin and ciprofloxacin respectively. Similarly, Celandroni and his team in their study stated that all *Bacillus* species were susceptible to vancomycin and ciprofloxacin by MIC using E-test (14). Based on our reported results, this high susceptibility towards the examined antibiotics can

propose their potential effect as empirical therapy. However, we must consider performing antimicrobial susceptibility to avoid the phenomenon of antimicrobial resistance.

On searching several databases, we found that similar studies were lacking and most of the available studies were not concerned about the medical aspect. Instead, almost all the researchers' interests were directed toward environmental, pharmaceutical and food studies. Hence, we faced difficulties to find counterparts with whom we could compare our results. Very few studies regarding the antimicrobial susceptibility of clinical *Bacillus* species were available. Several case reports had recorded successful clinical outcomes using various antibiotic combinations. A case report by Aygun et al. (2016) in Istanbul University, Turkey stated that *Bacillus cereus* was isolated in blood cultures obtained from a 16-month-old male patient and vancomycin, meropenem, and amikacin sulfate were started empirically and continued for 14 days till recovery (16).

Likewise, Hillard and colleagues (2003) from the USA stated that a case of bacteremia caused by *B. cereus* in a 19-day-old preterm neonate was successfully treated with vancomycin, aminoglycosides, meropenem, and clindamycin (17).

Also, Ozkocaman et al (2006) isolated three *B. cereus* from cases diagnosed with bacteremia in patients suffering from haematological malignancies. Three isolates appeared susceptible to aminoglycosides, aztreonam, cefepime, imipenem, meropenem, ciprofloxacin, levofloxacin, ofloxacin, tetracycline and vancomycin. Moreover, the isolates appeared resistant to clindamycin, penicillin and other β -lactam agents (18).

In a retrospective study between April 2003 and March 2012, Ikeda and his team (2015) from Japan investigated the antimicrobial susceptibility of *B. cereus* in bacteremic patients. Their 29 isolates expressed no resistance towards vancomycin, gentamicin, and imipenem. However, 65.5% and 10.3% were resistant to clindamycin and levofloxacin respectively (19).

In our study, we highlighted the value of *Bacillus* spp. as an opportunistic pathogen affecting those with immunocompromised status. To our knowledge, we are one among the few studies to compare all these methodologies for the identification of *Bacillus* species. Moreover, medical studies regarding *Bacillus* spp. in humans and scarce and most of the available

studies are concerned about those in the food industry as well as of the environmental source. Thus, we felt the urge to withdraw the attention to such a neglected organism that is not receiving enough attentiveness.

It is worthy to note that although the majority of our isolates were susceptible to almost all the tested antibiotics. However, this low rate of resistance can raise the awareness of the possibility of development of higher rate resistance especially in the era of multidrug resistance. Thus, further studies are recommended to follow up the resistance pattern of the *Bacillus* species. Molecular studies, as well, are highly encouraged for better understanding of the genetic basis of resistance.

CONCLUSION

Bacillus spp. should no longer be considered as contaminating bacteria in cultures especially in patients with a history of immunosuppression such as the use of immunosuppressive drugs and ICU patients with a debilitating disease. Hence testing of isolates from normally sterile sources (eg, deep tissue, CSF, multiple positive blood cultures) may be warranted.

B. cereus was the most frequently isolated *Bacillus* spp. (76.66%) followed by *B. subtilis* (23.33%). The majority of *B. cereus* isolates were from blood cultures from immunocompromised patients mostly on steroid therapy, while *B. subtilis* was isolated from surgical site infection.

As regards the different methods of identification in the present study, HiCrome agar without polymyxin B exhibited the highest agreement (93.3%) which was comparable with our gold standard method "MALDI-TOF" followed by Vitek-2C and HiCrome agar with polymyxin with an agreement of 83.3% each. We found that the addition of HiCrome polymyxin B selective supplement - according to the manufacturer's instruction - has no additional value in the identification of *Bacillus* species, especially with inhibition of growth of *B. subtilis*. Also, using the chromogenic media can aid in correctly identifying a large number of isolates using the same plate instead of using a large battery of biochemical reactions which can be tiresome. Thus, we recommend using HiCrome agar without polymyxin as a practical, easy and affordable method for the identification of *Bacillus* even in those laboratories with limited

resources. However, further studies using different types of *Bacillus* isolates should be conducted to test the capability of HiCrome media for the identification of other types.

Concerning the antibiogram of the studied isolates using BMD, the tested isolates showed a susceptibility of 93.3%, 86.6%, and 83.3% towards vancomycin, gentamicin, and ciprofloxacin respectively. Further clinical studies on a wider scale with a large number of patients are highly recommended to verify the effectiveness of the suggested empirical therapy reported in the current study.

It is worthy to note that although the majority of our isolates were susceptible to almost all the tested antibiotics. However, this low rate of resistance can raise the awareness of the possibility of developing a higher rate of resistance, especially in the current era of multidrug resistance. Thus, further studies are recommended to follow up on the resistance pattern of the *Bacillus* species. And molecular studies, as well, are highly encouraged for a better understanding of the genetic basis of resistance.

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