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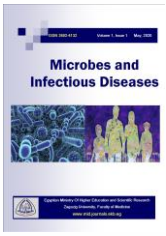

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Original article

Molecular characterization and prevalence of *Bacillus* species isolated from Egyptian hospitals

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Background: *Bacillus* species are widely distributed in all environments including health care settings and represent one of the highly resistant organisms. **Objective:** This study aimed to find out the prevalence, molecular characterization of genetic diversity among studied *Bacillus* species in Egyptian hospitals environment and their antibiotic susceptibility profile. **Methods:** A total 528 swab samples were collected from different hospitals environment. Isolation and identification were performed according to conventional bacteriological methods, semi-automated and molecular characterization methods. Antimicrobial susceptibility was carried against different groups of antimicrobial agents. **Results:** The most isolated microorganism was *Bacillus* spp. (43.2%), followed by coagulase-negative *Staphylococci* (19.2%), *Staphylococcus aureus* (15.2%), *Enterococus* spp. (10.1%), Gram-negative rods (8.9%), and *Micrococcus* spp. (3.4 %). The most prevalent species, were *Bacillus cereus* (46.6%) followed by *Bacillus subtilus* (38.1%) while, *Bacillus pumilus* was the least (1.1%). A majority of *Bacillus* isolates (25.6%) were isolated in Internal medicine department followed by Emergency department (18.8%) while operating rooms showed the lowest prevalence rate (4.5%). Antimicrobial susceptibility testing revealed high resistance of *Bacillus* isolates to β-lactams and tetracycline antibiotics. Multi-drug resistance (MDR) isolates which resistance to three or more antibiotics was (21.6%). Susceptibility reports of the 176 *Bacillus* isolates revealed 45 antibitypes and the most common was antibiotype 31, which included 32 isolates (18.2%), that is resistant to both penicillin and cefoxitin. **Conclusions:** This study revealed that, dissemination of *Bacillus* species in study hospital environments with high resistance to β-lactams and tetracycline antibiotics. The molecular analysis revealed the existence of genetic diversity among studied *Bacillus* isolates. Thus, monitoring the hospital environment is an important tool in the prevention of hospital-associated infection by *Bacillus* species.

Introduction

Bacillus genus is a Gram-positive spore-forming bacteria. It is found everywhere in nature and is widely spread [1]. *Bacillus* infections have been recorded sporadically in surgical wounds, eye infections, pneumonia, bacteremia, meningitis, sepsis and soft tissue infections, particularly in immuno- compromised persons [2]. This genus contains well-known food-poisoning organisms that produce diarrheal enterotoxins. As a result, consuming contaminated food may present a risk of triggering an outbreak [3]. Infections acquired in a hospital setting greatly increase morbidity and mortality rates, lengthen hospital stays and raise healthcare expenses [4]. Contaminated surfaces in

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hospitals can be a source of health care associated infection [5]. Bacterial load on environmental surfaces, frequency of contamination of handheld or touched surfaces, and bacterial ability to proliferate, resist environmental conditions, and divide have all been studied [6]. These bacteria are primarily transmitted through contaminated medical instruments such as stethoscopes, respiratory devices, gowns, doorknobs, bed rails, call buttons, masks, and gloves as well as the splashing of infected water on sterile equipment [7]. These bacteria can then be transferred from the environment to a health care worker, janitorial staff, or even a community member, and contact with a susceptible patient can result in infection [8]. There is little information available on the prevalence of *Bacillus* species in hospital settings as well as antibiotic resistance profiles. Furthermore, little is known about the source of this organism in the hospital setting. Thus, monitoring the hospital environment is an important tool in the prevention of hospital-acquired infections. This study, aimed to find out the prevalence, molecular characterization of genetic diversity and antibiotic susceptibility of *Bacillus* species in some Egyptian hospitals.

Material and Methods

Samples collection

A total of 528 swab samples were collected by swabbing surfaces with direct patient contact after routine cleaning at the from different departments including, Internal medicine department (95), Intensive care units (50), Surgery department (53), Obstetrics and Gynecology department (74), Operating rooms (45), Emergency department (89), Renal unit (42) and Newborn nursery (80), in Kasr El-Ainy university hospitals, Abo El-Resh hospital for children and El-Hussien university hospital during period from April 2017 to August 2018. The swab samples were collected by swabbing approximately $5cm^2$ of surface at each site using pre-moisturized cotton swabs with 1ml neutralizing buffer (Difco, USA). The swabs were transported in cooler boxes with ice packs and proceeded within two hours of collection.

Isolation and identification of bacterial isolates.

The swab samples were streaked on nutrient agar, MacConkey agar, blood agar and mannitol salt agar. The purified colonies were identified according to Berge's manual of determinative bacteriology [9]. Identification of isolates were done by Gram staining, catalase test, oxidase test, citrate utilization test, methyl red test, voges progeskaur test, deoxyribonuclase (DNase) test, gelatin liquefaction test, and growth at 6.5% NaCL. In addition to utilization of semi-automated system HiBacillus identification kit (Himedia, India) according to manufacturer's instructions.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing of 12 antimicrobial agents was carried out using Kirby-Bauer disk diffusion method according to (CLSI, 2018) guidelines. The used antimicrobial disks were, penicillin G 10 I.U, rifampicin 5 µg, linezolid 30 µg, chloramphenicol 30 µg, erythromycin 15 µg, clidamycin 2 µg, tetracycline 30 µg, ciprofloxacin 5 µg, gentamycin 10 µg, ceftaroline 30 µg, cefoxitin 30 µg, and sulphamethexazole-trimethoprim 30 µg. Isolates that showed resistance to at least three different classes of antimicrobial agents were considered as multidrug resistant (MDR)

Susceptibility reports of *Bacillus* isolates

Susceptibility reports of *Bacillus* isolates were done based on antimicrobial resistant profile against the tested 12 antimicrobial agents. Cluster analysis was generated with the Dice similarity coefficient and unweighted pair group method (UPGAMA) clustering method (http://insilico.ehu.es/dice_upgma /index.php).

DNA extraction

Total genomic DNA was extracted from selected isolates using the GeneJET genomic DNA purification kit (Thermo Scientific, USA) according to the manufacturer's instructions.

PCR amplification of bacterial 16S rRNA

Two oligonucleotide primers were used to amplify 16S rRNA, 27F forward: 5'-AGAGTTTGATCMTGGCTCAG-3' and 1492R reverse: 5'-CGG TTA CCT TGT TAC GAC TT-3'. The reaction mix (25 µL) included, 12.5 µL DreamTaq Green PCR Master Mix (2X) (Thermo Fisher, USA) containing (DreamTaq DNA polymerase, 2X DreamTaq green buffer, dATP, dCTP, dGTP, dTTP, 0.4mM each, and 4mM $MgCl_2$, 8.5 µL of purified water, 1 µL (10 mM) of each primer, and 2 µL(about 50 ng) of genomic DNA. The PCR conditions were designed with an initial denaturing step at 95 °C for 5 minutes, followed by 30 cycles of denaturing at 95 °C for 1 minute, primer annealing at 54 °C for 1 minute, and elongation at 72 °C for 90 seconds. Finally, a 10-minute extension step at 72 °C. The amplification products were checked on 1% w/v agarose gels (Promega, USA)

stained with ethidium bromide (0.5 mg/l) [10]. Purified PCR products were obtained using the GeneJET gel extraction kit (Thermo Scientific, USA). The Macrogen Sequencing Facilities ABI PRISM® 3100 Genetic Analyzer was used to sequence the DNA of the PCR product (Macrogen, Korea).

Sequence analysis and phylogenetic relationships between the strains

Sequence analysis was performed with the sequences in the national center for biotechnology information (NCBI) database (www.ncbi.nlm.nih.gov/blast) using the Basic Local Alignment Search Tool, (BLAST) and deposited in the GenBank with specific accession numbers. A phylogenetic tree was constructed by the method of Neighbor-Joining with the MEGA 11.0 program using the alignment of the sequences of the 26 *Bacillus* isolates sequenced in this study

Statistical analysis

Data statistical analysis was carried out using IBM SPSS software package version 26.0 (Armonk, IBM Corp, NY, US). Descriptive statistics were used to present the antimicrobial susceptibility patterns. Frequencies and percentages were used to summarize descriptive statistics.

Results

Prevalence of *Bacillus* species

Out of 528 collected samples in this study, 407 bacterial isolates were identified. A total 178 (43.7%) isolates were from Kasr El-Ainy teaching, 96 (23.6%) were from Abo El-Reesh and 133 (32.7%) from El-Hussein University Hospitals. The most isolated were *Bacillus* spp. 176 (43.2%) followed by coagulase-negative *Staphylococci* (CoNS) 78 (19.2%), *Staphylococcus aureus* 62 (15.2%), *Enterococus* spp. 41 (10.1%), Gram negative rods 36 (8.9%), *Micrococcus* spp. 14 (3.4 %). The frequency of *Bacillus* isolates among hospitals included in this study showed, the higher isolation rate from Kasr El-Ainy teaching 72 (40.9%) followed by El- Hussein University 56 (31.8%) and finally Abo El-Reesh hospitals 48 (27.3%). Regarding to isolated *Bacillus* species, *B. cereus* 82(46.6%) were the most prevalent species followed by *B. subtilus* 67 (38.1%) while *B. pumilus* 2 (1.1%) were the least as showed in table 1. The distribution of *Bacillus* isolates varies in different wards in this study showed that Internal medicine department revealed a higher prevalence of 45 (25.6%) followed by Emergency department 33 (18.8%) and operating rooms with the less prevalence of 8 (4.5%) as showed in **table (1)**.

Antibiotic susceptibility testing

The antimicrobial susceptibility testing was performed against 12 antimicrobial agent. The overall antimicrobial susceptibility reveled high resistance to penicillin G (56.8%) followed by cefoxitin (38%) and tetracycline (35.2%). High susceptibility was recorded to chloramphenicol (98.9%) followed by ceftaroline (98.9%), linezolid (97.7%), ciprofloxacin (97.7%), gentamycin (97.2%), sulphamethexazole -trimethoprim (96.6%), erythromycin (95.5%) and finally clindamycin (93.2%).

Susceptibility reports of *Bacillus* species.

Susceptibility reports of the 176 *Bacillus* isolates investigated in this study revealed to 45 antibiotypes as showed in **figure 1 (a, b and c)**. The most common was that showed resistant to both penicillin and cefoxitin which included 32 (18.2%), designed antibiotype 31 as showed in **figure (1c)**. Followed by that showed complete susceptibility to all tested antimicrobial agents which included 28 (15.9%), designed antibiotype 3 as showed in **figure (1a)**, then that showed resistance to penicillin only, included 23 (13.1%), designed antibiotype 21 as showed in **figure (1b)**. Finally, that showed resistance to tetracycline only, included 16 (9.1%), designed antibiotype 4 as showed in **figure (1a)**.

Prevalence of MDR isolates

According to antimicrobial susceptibility profiles It was found that out of 176 *Bacillus* isolates, 38 (21.6%) isolates were MDR, where 22 (57.9%) from Kasr Aliny, 7 (18.4 %) from Abo El-resh and 9 (23.7 %) from El-Hussien university hospital. MDR detected in four species: *Bacillus cereus* 29 (76.3%) followed by *Bacillus subtilis* 6 (15.8%) then *Bacillus mycoides* 2 (5.3%) followed by *Bacillus pumilus* 1 (2.6 %). The highest rate of MDR of *Bacillus* isolates were among isolates recovered from emergency department 9 (23.7%) followed by internal medicine department 8 (21.1%), obstetrics and gynecology department 7 (18.4%), surgery department 6 (15.7%), renal unit 3 (7.9%), new born nursery 2 (5.3%), operating rooms 2 (5.3%), intensive care unit 1(2.6%) as showed in **figure (2)**.

Susceptibility reports of MDR *Bacillus* isolates

Susceptibility reports of the selected 38 MDR *Bacillus* isolates investigated in this study revealed 22 antibitypes. The most common was antibiotype 11, including 6 (15.8%) MDR *Bacillus* isolates

that's were resistant to rifampicin, penicillin and tetracycline. The next was antibiotype 9, including 5 (13.2%) that were resistant to rifampicin, penicillin and cefoxitin as showed in **figure (3)**.

Molecular characterization of *Bacillus* isolates

Amplification of 16S rRNA gene

In the present study 16S rRNA gene of 26 *Bacillus* isolates were amplified using universal primers and amplicon products of tested isolates showed expected band at about 1500 bp. as showed in **figure (4)**.

Sequencing of 16S rRNA gene

Species identity of isolates was further confirmed by sequencing of 16S rRNA gene product followed by detecting degree of similarity using BLAST tool in GenBank database, which suggests the relatedness of the isolates with same and identity within the genus *Bacillus* as showed in **table (2)**. All 26 partial 16S rRNA gene were deposited in GenBank database under accession numbers (OM280060, ON306913, ON306914, ON306924 to ON306927) for *Bacillus cereus*, (OM279800, ON306909 to ON306912, ON306916 to ON306923) for *Bacillus subtilis* isolates, (ON286980) for *Bacillus lichniformis*, (OM279798) for *Bacillus mycoides*, (OM280059, ON306915, ON306928) for *Bacillus pumilus* and (OM280058) for *Bacillus polymyxia* as showed in **table (2)**.

Phylogenetic diversity among Bacillus isolates

As a result, a phylogenetic tree was mapped using the neighbor joining method with the MEGA 11 program using the alignment of the sequences of the 26 *Bacillus* isolates sequenced in this study. Constructed phylogentic tree showed close relation of both *B. lichniformis* and *B. pumilus* to *B. subtilis* group while *B. mycoides* was more related to *B. cereus* group showed in **figure (5)**.

Table 1. Frequency of *Bacillus* isolates regarding site of sampling.

Isolates	**Site of sampling**								**Number (Percentage)***
	ICU	**Su.**	**Ob.**	**Op.**	**Em.**	**In.**	**Re.**	**N. Nur.**	
Bacillus cereus	4	9	10	4	11	16	8	20	**82 (46.6 %)**
Bacillus subtilis	5	6	3	1	15	23	8	6	**67 (38.1%)**
Bacillus lichniformis	0	1	-	-	4	2	-	-	**7 (4%)**
Bacillus mycoides	0	2	2	2	1	2	1	2	**12(6.8%)**
Bacillus pumilus	-	-	-	-	1	-	1	-	**2 (1.1%)**
Bacillus polymyxia	-	1	1	1	1	2	-	-	**6 (3.4%)**
Total	9	19	16	8	33	45	18	28	**176 (100%)**

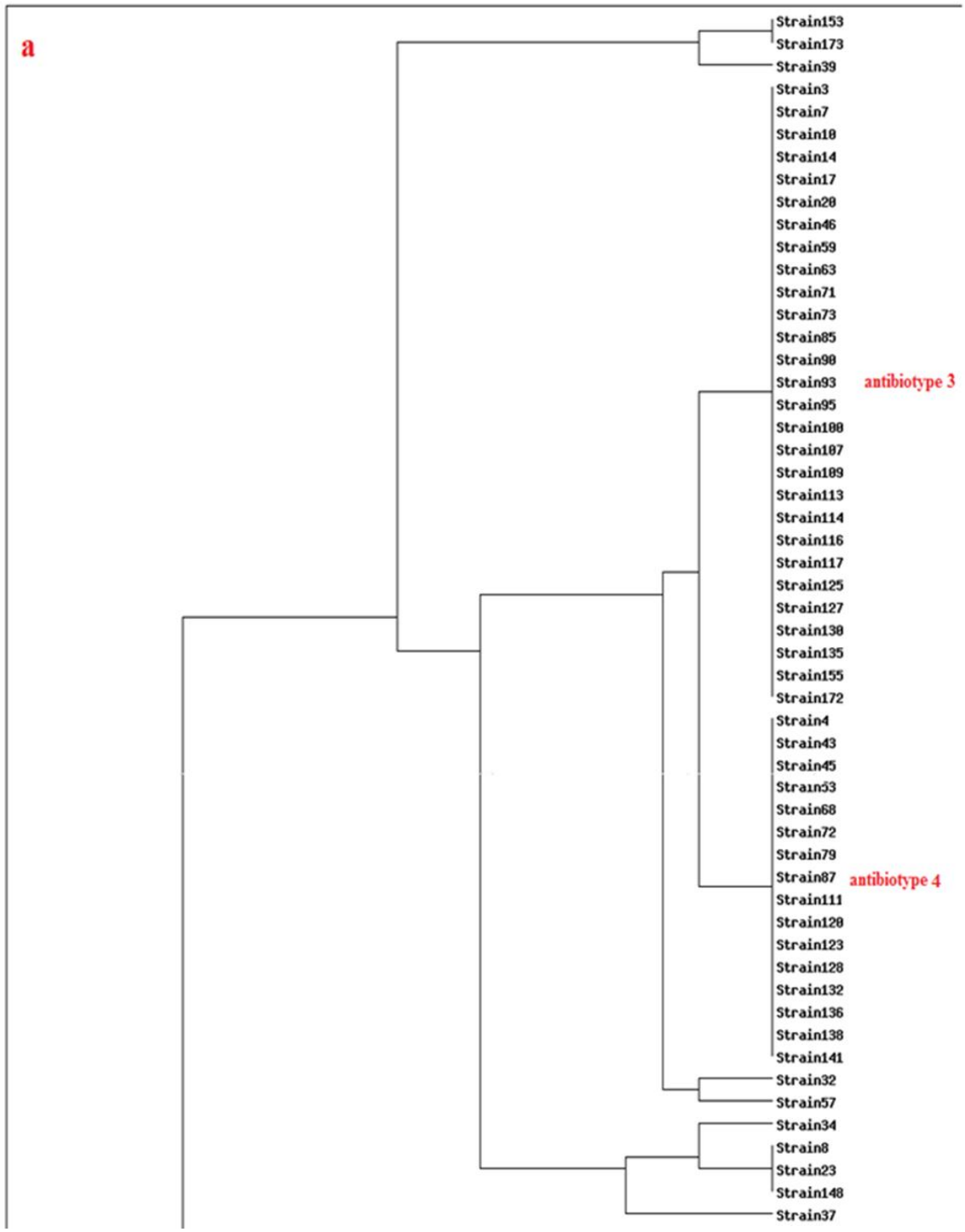
*Percentage correlated to the total number of *Bacillus* isolates (176).

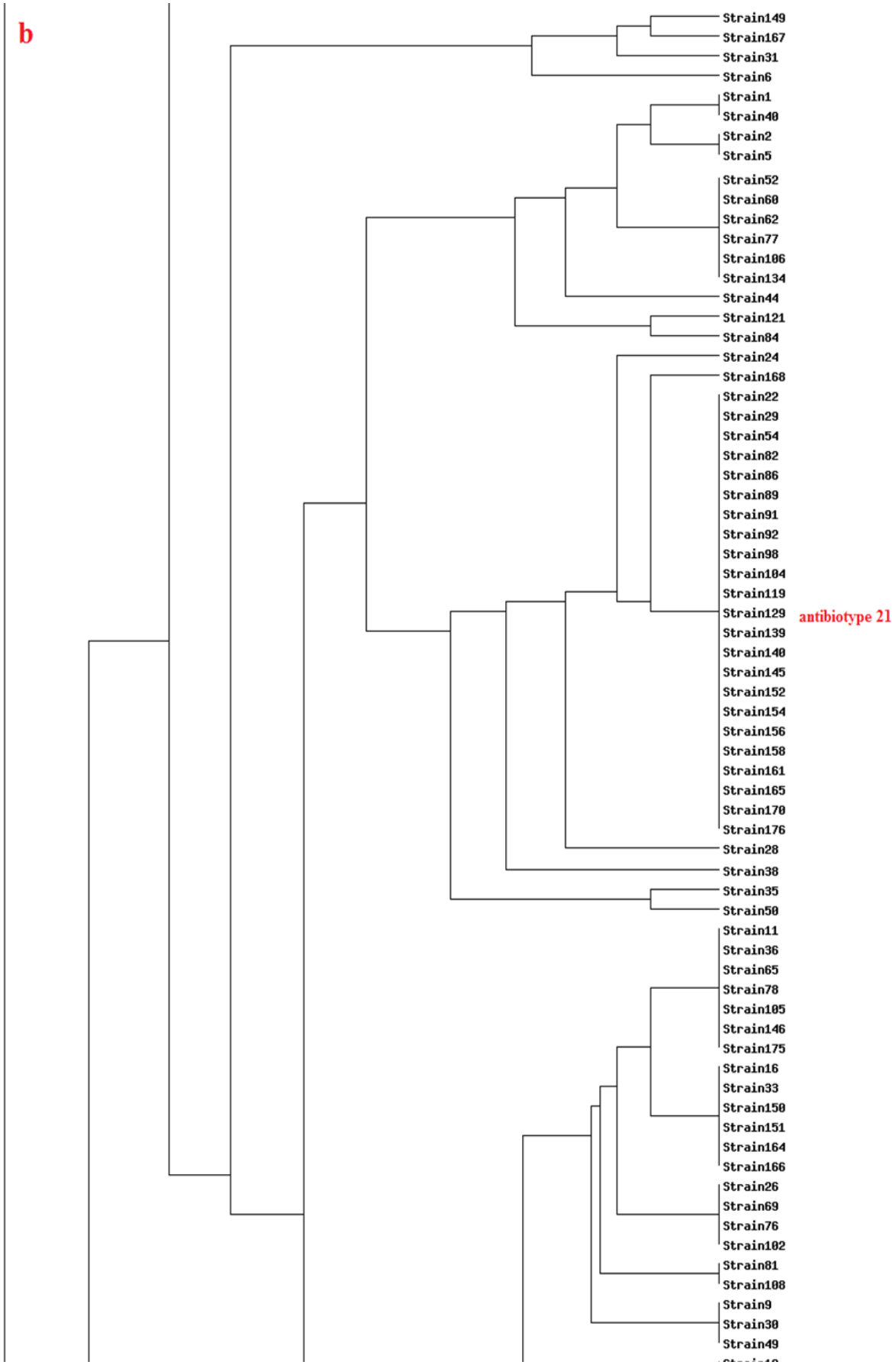
ICU = Intensive Care Unit; Su. = Surgery Department; Ob. = Obstetrics and Gynecology Department; Op. = Operating Rooms; Em. = Emergency Department; In. = Internal Medicine Department; Re. = Renal Unit; N Nur. = Newborn Nursery.

Table 2. Molecular characterization of selected *Bacillus* isolates.

N	Accession number on GenBank	Identification	Sequence length (bp)	Closest related bacterial accession number	Similarity
1	**ON306909**	***Bacillus subtilis***	1456	***Bacillus subtilis* strain DSM 10**	96.2 %
2	**ON306910**	***Bacillus subtilis***	1453	***Bacillus subtilis* strain JCM 1465**	95.8 %
3	**ON306911**	***Bacillus subtilis***	1456	***Bacillus subtilis* strain NBRC 13719**	95.8 %
4	**ON306912**	***Bacillus subtilis***	1470	***Bacillus subtilis* strain BCRC 10255**	95.3 %
5	**ON306913**	***Bacillus cereus***	1515	***Bacillus cereus* strain CCM 2010**	98.4%
6	**ON306914**	***Bacillus cereus***	1488	***Bacillus cereus* strain IAM 12605**	97.8%
7	**ON306915**	***Bacillus pumilus***	1415	***Bacillus pumilus* strain NBRC 12092**	97.4%
8	**ON306916**	***Bacillus subtilis***	1492	***Bacillus subtilis* strain SBMP4**	94.7%
9	**ON306917**	***Bacillus subtilis***	1545	***Bacillus subtilis* strain IAM 12118**	93.9%
10	**ON306918**	***Bacillus subtilis***	1422	***Bacillus subtilis* strain NCDO 1769**	95.8%
11	**OM280060**	***Bacillus cereus***	1471	**Bacillus cereus strain JCM 2152**	97.7%
12	**OM279800**	***Bacillus subtilis***	1410	***Bacillus subtilis* strain NRRL NRS-744**	98.7%
13	**ON286980**	***Bacillus lichniformis***	1496	***Bacillus lichniformis* strain DSM 13**	97.7%
14	**ON306919**	**Bacillus subtilis**	1494	***Bacillus subtilis* strain NCDO 1769**	94.6%
15	**ON306920**	***Bacillus subtilis***	1551	***Bacillus subtilis* strain IAM 12118**	96.9%
16	**ON306921**	***Bacillus subtilis***	1452	***Bacillus subtilis* strain NBRC 13719**	96.4%
17	**ON306922**	***Bacillus subtilis***	1455	***Bacillus subtilis* strain DSM 10**	95.3%
18	**ON306923**	***Bacillus subtilis***	1431	***Bacillus subtilis* strain JCM 1465**	96.1%
19	**ON306924**	***Bacillus cereus***	1480	***Bacillus cereus* strain NBRC 15305**	97.8%
20	**ON306925**	***Bacillus cereus***	1495	***Bacillus cereus* strain CCM 2010**	97.2%
21	**ON306926**	***Bacillus cereus***	1471	***Bacillus cereus* strain IAM 12605**	96.4%
22	**ON306927**	***Bacillus cereus***	1474	***Bacillus cereus* strain CCM 2010**	97.4%
23	**ON306928**	***Bacillus pumilus***	1412	***Bacillus pumilus* strain SBMP2**	97.9%
24	**OM279798**	***Bacillus mycoides***	1414	***Bacillus mycoides* strain 273**	97.8%
25	**OM280059**	***Bacillus pumilus***	1402	***Bacillus pumilus* strain NBRC 12092**	97.5%
26	**OM280058**	***Bacillus polymyxia***	1460	***Paenibacillus polymyxa* strain DSM 36**	97.7%

Figure 1(a, b and c). Dendrogram of susceptibility reports of all *Bacillus* isolates. Cluster analysis was generated with the Dice similarity coefficient and UPGAMA clustering method.

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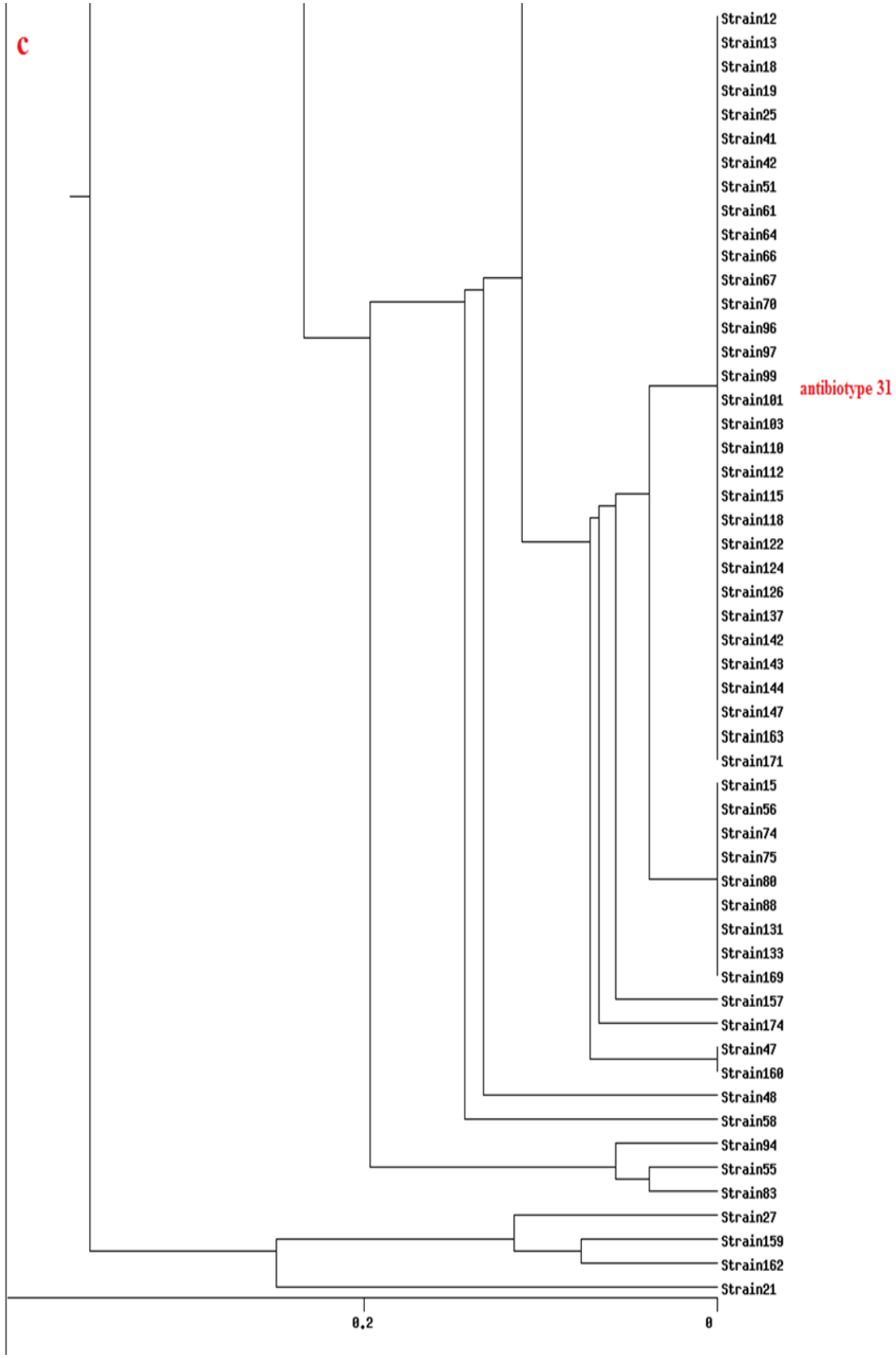
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Figure 2. Prevalence of MDR of *Bacillus* isolates: a, regarding study hospitals; b, regarding species and c, regarding sampling site.

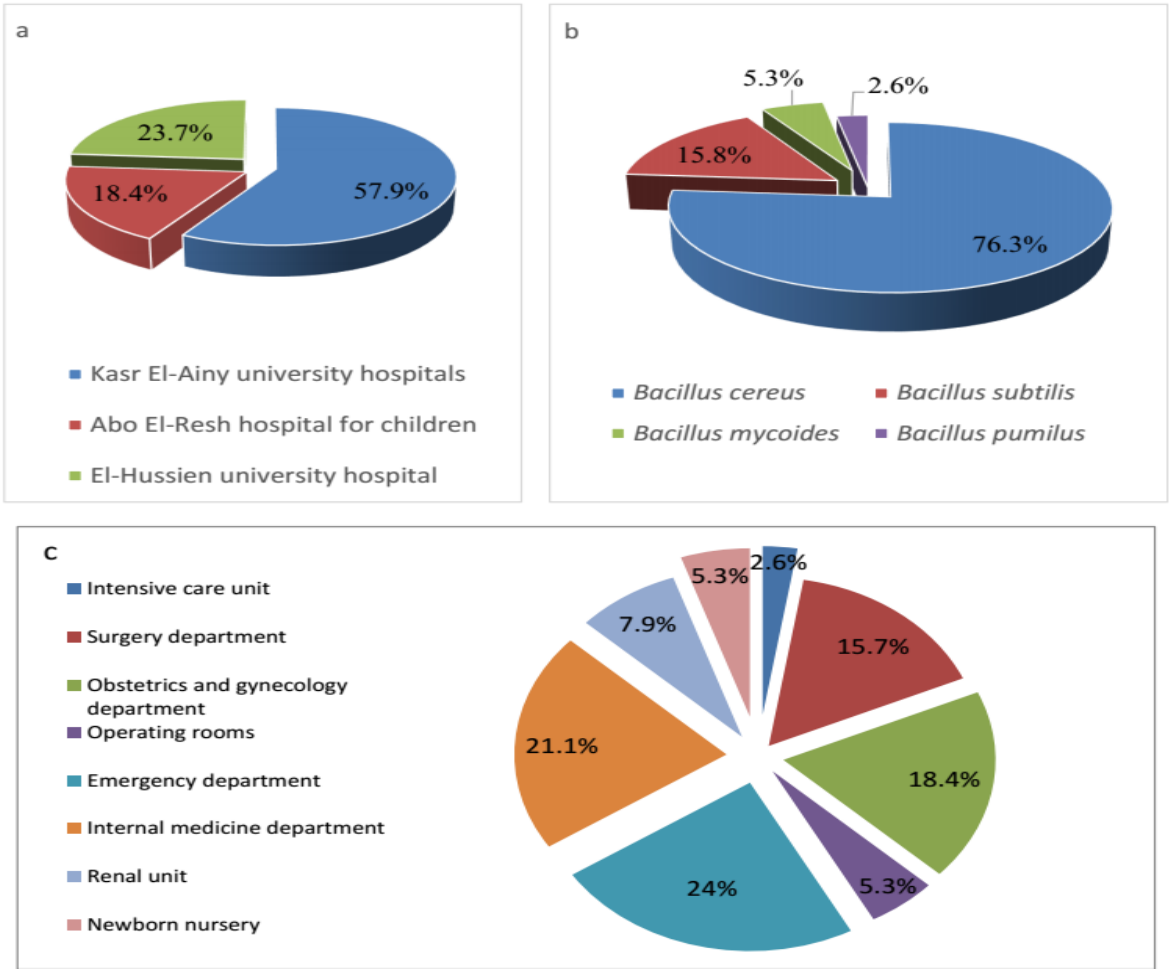


Figure 3. Dendrogram of susceptibility reports of MDR *Bacillus* isolates. Cluster analysis was generated with the Dice similarity coefficient and UPGAMA clustering method.

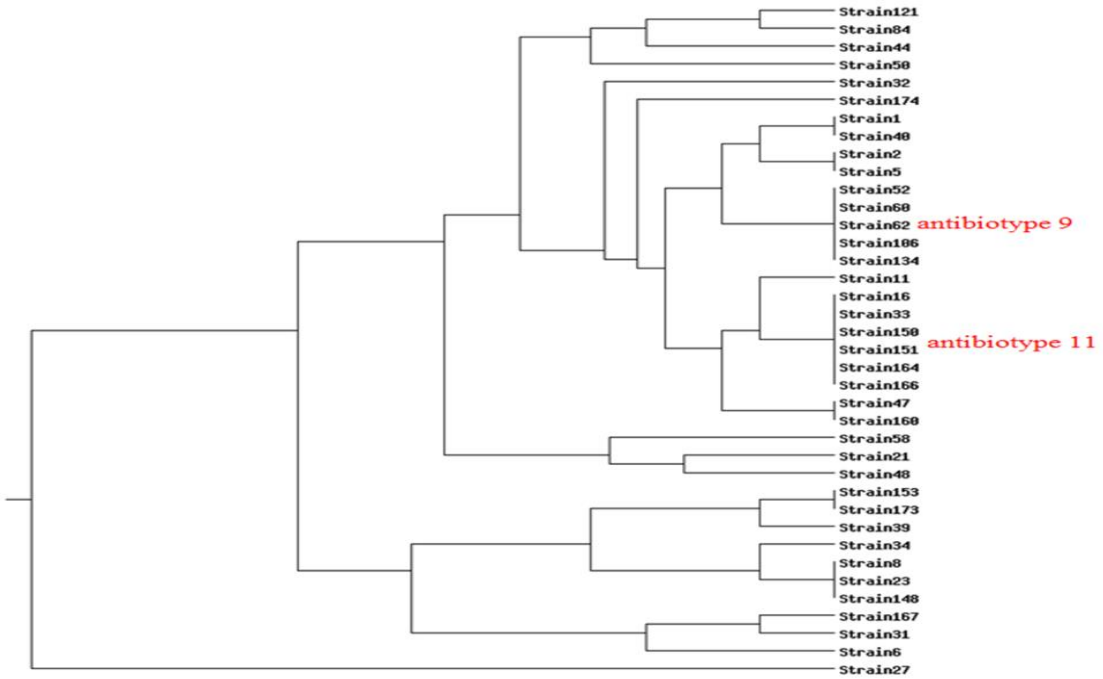


Figure 4. Full length16S rRNA gene (1500bp) of *Bacillus* isolates amplified with universal primers. The amplicon was resolved on 1% agrose gel. M, DNA ladder lanes 1-26: amplified products of full length 16S r RNA gene.

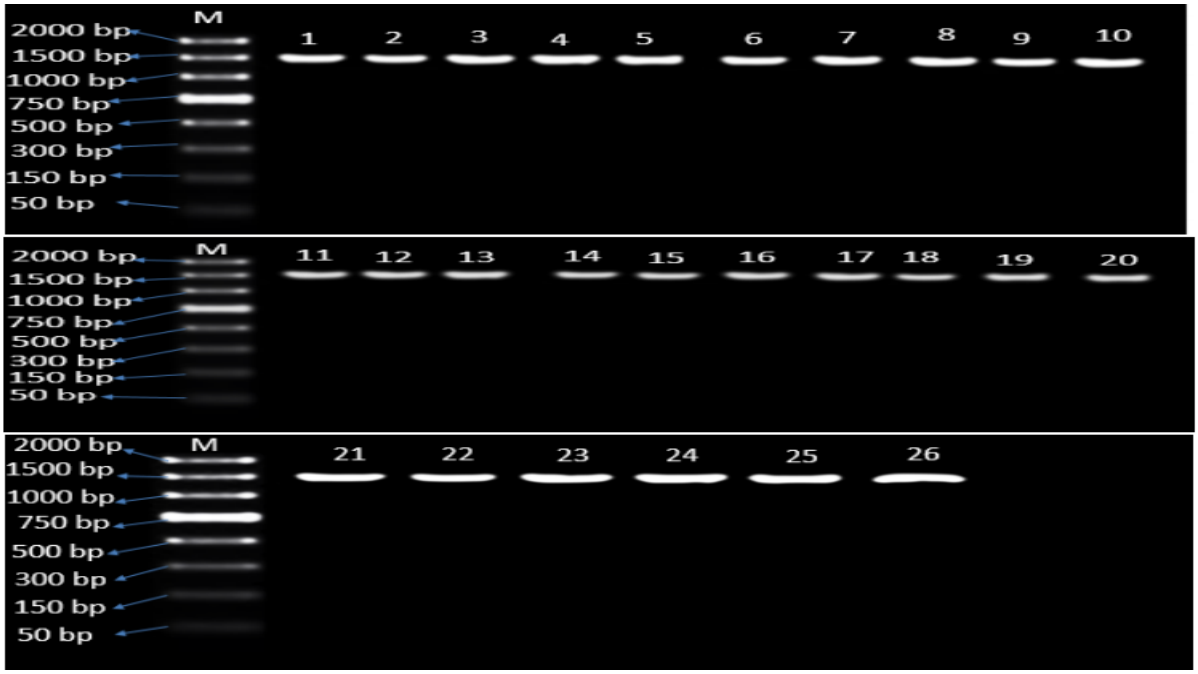
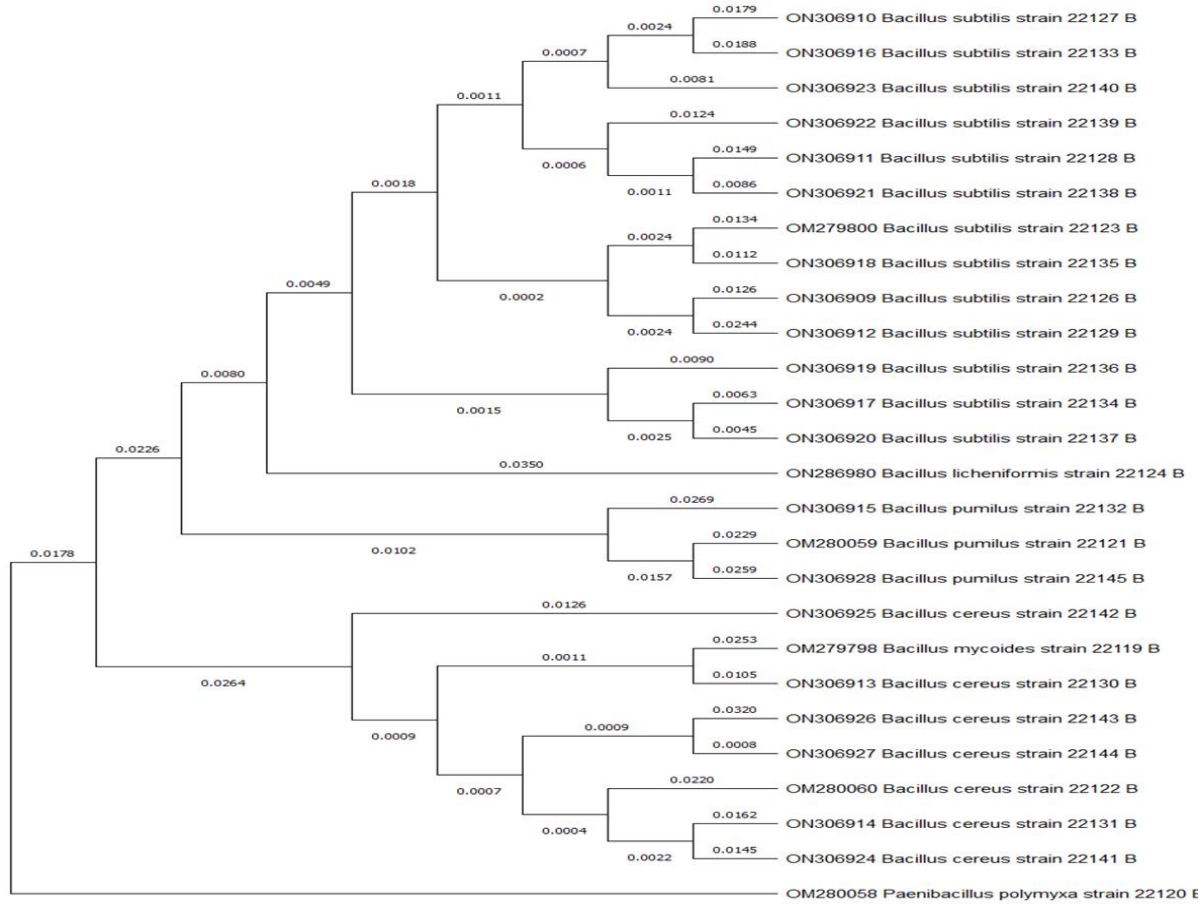


Figure 5. Phylogenetic tree showing distance between sequences of 16S rDNA of *Bacillus* isolates constructed by the method of Neighbor-joining.



Discussion

The environment in the health care plays a crucial role in the transmission of pathogens associated with nosocomial infections. These pathogens can be transmitted from person to person or by touching inanimate items, particularly articles that come into direct contact with patients [11]. Understanding the prevalence, antimicrobial resistance and relatedness of bacteria in hospital environments could provide a comprehensive picture of their spread and the risk of acquiring health care associated infections [12]. *Bacillus* isolates, especially *B. cereus* are associated with food poisoning and infections such as eye infections, sepsis and fatal CNS infections [13]. However, there has been little investigation into the distribution of *Bacillus* isolates in Egyptian hospitals. The paucity of research highlights the need of quality monitoring *Bacillus* isolates in healthcare settings.

In this study, an overall prevalence rate of (43.4%) for the *Bacillus* isolates recovered from all collected samples from three Egyptian hospitals. This finding was lower than rate of (50%) recorded in a Sudanese Hospital Survey [12]. However, it was higher than the rate of (17%) that reported in KwaZulu-Natal province, South Africa [2]. The prevalence of *B. cereus* in this study (46.6%) was greater than prevalence rate from the hospital setting which was (16%) in the St. Azzhria University Hospital in Isafan, Malaysia [14].

The prevalence rate of *Bacillus* species varied throughout public hospital wards, with internal medicine department (25.6%) Emergency department (18.8%) showed the highest prevalence rate while Operating rooms (4.5%) showed the lowest. From an epidemiological point of view, hospital facilities become linked through shared patients or the exchange of articles [15]. Internal medicine and Emergency departments are both referral points inside hospitals with patients entering and exiting from various points within the hospital, such as clinics. If effective infection and preventative control measures are not implemented, this may increase community exposure and consequently cause a higher prevalence of the bacteria.

Bacillus isolates are often susceptible to broad-spectrum antibiotics such as tetracycline, ciprofloxacin, and erythromycin, and are used to treat gastroenteritis caused by these bacteria [16]. Certain *Bacillus* species, such as *B. cereus*, are intrinsically resistant to -lactams, except carbapenems [17] and can acquire resistance to commonly used antibiotics for infection therapy, such as ciprofloxacin, cloxacillin, erythromycin, tetracycline, and streptomycin [18].

In the current work, high resistance rate of penicillin G (56.8%) was detected which inconsistent to results reported in other studies [16, 19]. These findings may be explained as most *B. cereus* isolates produce β-lactamases, which render them resistant to penicillin and cephalosporins [20]. Regarding tetracycline, this study showed high rate of resistance among Bacillus isolates (35.2%). This finding is not in agreement with other previous studies, as *Bacillus* isolates are usually thought to be susceptible to these classes of antibiotics [16, 19]. However, resistance to tetracycline and erythromycin in these bacteria has previously been observed in these bacteria in the United States and Europe [18,20]. Erythromycin revealed a high susceptibility (95.5%) in this study, which was in consistence with previous study done by [21] where 81% of the isolates were susceptible to erythromycin.

The present study revealed that different resistotypes (45 resistotypes) were detected. In addition (21.6%) of total *Bacillus*, isolates were MDR and belonged to 22 distinct resistotypes. These findings support *Bacillus* isolates' ubiquitous nature, which allows them to colonize, I addition, their spores ability to withstand environmental changes, dry heat, and certain chemical disinfectants for a longer duration [22].

The 16S rRNA gene sequence is approximately 1,550 bp long and contains both variable and conserved regions. The gene is large enough, and there are enough interspecific variations in the 16S rRNA gene to produce distinct and statistically meaningful measurements. Universal primers are often designed to be complementary to the conserved portions at the beginning of the gene and at either the 540 bp region or the end of the entire sequence (about the 1,550 bp region), and the variable region in between is used for comparative taxonomy [23, 24].

In this study PCR fragments of the 16S RNA were used, A gene (with a length of 1500 bp) from 26 *Bacillus* isolates was amplified and sequenced to confirm their taxonomic attribution to the genus *Bacillus* and to allow the development of a phylogenetic tree. A phylogenetic tree representing the evolution of the analyzed gene was

constructed based on the aligned sequences using the MEGA 11 program as a tool to study the relationship between isolates, revealing a considerable diversity among isolates.

This suggests that, despite careful cleaning efforts, *Bacillus* species can persist in the hospital environment and may continue to be a source of infection for patients. Their ability to sporulation could explain this [25].

Conclusion

This study highlights the prevalence of *Bacillus* species in hospital settings, as well as their spread within the same hospital but in different wards. The high rate of resistance to β-lactam and tetracycline antibiotics reported in this study suggests that treating people infected with these strains may be problematic. The molecular analysis revealed the existence of genetic diversity among studied *Bacillus* isolates. As a result, monitoring the hospital environment is a crucial strategy in the prevention of hospital associated infection.

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Conflict of interest

The authors declare that there is no conflict of interest.

Authors' contribution

All authors have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Data availability

All datasets generated or analyzed during this study are included in the manuscript.

Ethics statement

This article does not contain any studies with human participants or animals performed by any of the authors**.**

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