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Original Article

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

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الملخص

أهداف البحث: سلطت الدراسة الضوء على انتشار أنواع العصيات (بما في ذلك المقاومة للأدوية) في بيئات المستشفيات العامة وتدعو إلى تصميم استراتيجيات مثالية للحد من انتشارها وهو أمر بالغ الأهمية في جميع أنظمة الرعاية الصحية مثل الرعاية لعدد متزايد من المرضى الذين يعانون من ضعف المناعة.

طريقة البحث: تم جمع ما مجموعه 528 عينة مسحة من بيئة المستشفيات السعودية المختلفة. تم جمع عينات المسحات عن طريق مسح ما يقرب من 5 سم مربع من السطح في كل موقع باستخدام مسحات قطنية مبللة مسبقا مع محلول معادل بسعة 1 مل (ديفكو ، الولايات المتحدة الأمريكية). تم نقل المسحات في صناديق تبريد مع أكياس ثلج في غضون ساعتين من التجميع. تم إجراء العزل والتعريف وفقا لطرق التوصيف البكتريولوجية التقليدية ، والسمية الآلية ، والجزيئية. تم إجراء تصنيف المضادات الحيوية ضد مجموعات مختلفة من العوامل المضادة للميكروبات.

النتائج: أما الأنواع المعزولة فكانت العصوية الشمعية (46,6%) تليها العصوية الرقيقة (38,1%) بينما كانت العصوية القزمة الأقل انتشارا (1.1٪). غالبية عزلات العصيات (25,6%) كانت من قسم الطب الباطني يليه قسم الطوارئ (18.8٪) بينما أظهرت غرف العمليات أقل معدل انتشار (4.5٪). أظهر اختبار الحساسية لمضادات الميكروبات مقاومة عالية لعزلات العصيات للبيتا-لاكتام و تتراسيكلين هيدروكلوريد. العزلات المقاومة للأدوية المتعددة والتي كانت مقاومة ثلاثة أو أكثر من المضادات الحيوية (21.6٪). أظهر تصنيف المضادات الحيوية لـ 176 عزلة وجود 45 نوعا من المضادات وأكثرها شيوعا كانت من النمط 31، والتي اشتملت على 32 عزلة (18.2٪) مقاومة للبنسلين والسيفوكسيتين.

الاستنتاجات: كشفت هذه الدراسة عن الانتشار الواسع لأنواع العصيات في بيئات المستشفيات التي في الدراسة ذات المقاومة العالية لمضادات بيتا-لاكتام والتتراسيكلين. أظهر التحليل الجزيئي وجود تنوع وراثي بين عزلات العصيات المدروسة. وبالتالي، فإن مراقبة بيئة المستشفى هي أداة مهمة في الوقاية من العدوى المرتبطة بالمستشفيات بأنواع العصيات.

الكلمات المفتاحية: العصيات؛ التوصيف الجزيئي؛ بيئات المستشفى؛ مقاومة المضادات الحيوية؛ 16إس الرنا الريباسي

Abstract

Objective: This study highlighted the dissemination of *Bacillus* species (including drug-resistant species) in public hospital environments and calls for the design of optimal strategies to curb their spread. This a critical consideration for all health care systems such as caring for the increasing number of immune-compromised patient.

Methods: A total of 528 swab samples were collected from the environments of different Saudi hospitals. Swab samples were collected by swabbing approximately 5 cm^2 of different surfaces at each site using pre-moisturized cotton swabs with 1 mL of neutralizing buffer. The swabs were transported in cool boxes with ice packs within 2 h of collection. Isolation and identification were performed according to conventional bacteriological, semi-automated and molecular characterization methods. Antibiogram typing was carried against different groups of antimicrobial agents.

Results: The most prevalent of the isolated *Bacillus* species were *Bacillus cereus* (46.6%) followed by *Bacillus subtilis* (38.1%); the least prevalent was *Bacillus pumilus* (1.1%). Most *Bacillus* isolates (25.6%) were isolated from the Department of Internal Medicine followed by the Emergency Department (18.8%), while the operating rooms had the lowest prevalence (4.5%). Antimicrobial susceptibility testing revealed high levels of resistance in *Bacillus* isolates to β-lactams and tetracycline. Overall,

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21.6% of isolates showed multi-drug resistance to three or more antibiotics (21.6%). Antibiogram typing of the 176 isolates revealed 45 antibiotypes; the most common was antibiotype 31, which included 32 isolates (18.2%); this particular antibiotype was resistant to both penicillin and cefoxitin.

Conclusions: Analyses identified the high dissemination of *Bacillus* species in several hospital environments with high resistance to β-lactams and tetracycline antibiotics. Molecular analysis also revealed the existence of genetic diversity among the *Bacillus* isolates investigated. Thus, monitoring the hospital environment is an important tool in the prevention of hospital-associated infection by *Bacillus* species.

Keywords: 16S rRNA; Antibiotic resistance; *Bacillus*; Hospital environments; Molecular characterization

Introduction

Bacillus genus is a Gram-positive spore-forming bacteria that 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, but particularly in immuno-compromised individuals.[2] Infections acquired in a hospital setting significantly increase morbidity and mortality rates, lengthen hospital stays and raise health care expenses.[3]

Previous studies have investigated bacterial load on environmental surfaces, the contamination of handheld or touched surfaces and the ability of bacteria to proliferate and resist environmental conditions.[4] 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.[5] This study aimed to determine the prevalence and molecular characterization of genetic diversity among *Bacillus* species and their antibiotic susceptibility by establishing a resistance typing profile. In addition, the study highlighted the dissemination of *Bacillus* species (including drug-resistance) in public hospital environments and calls for the design of optimal strategies to curb their spread. This is a vital consideration for all health care systems such as caring for the increasing number of immune-compromised patients.

Materials and Methods

Samples collection

A total of 528 swab samples were collected by swabbing surfaces at each site from different departments including, the 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 different Saudi hospitals between April 2020 and August 2021.

Isolation and identification of bacterial isolates

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.[6] The identification of isolates was performed by Gram staining, catalase test, oxidase test, citrate utilization test, methyl red test, Voges–Proskauer test, deoxyribonuclease (DNase) test, gelatin liquefaction test, and growth in 6.5% NaCl. In addition, we used a semi-automated system Hi*Bacillus* identification kit (Himedia, India) in accordance with the manufacturer's instructions.

Antimicrobial susceptibility testing

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

Antibiogram typing of Bacillus *isolates*

Antibiogram typing of *Bacillus* isolates was performed based on antimicrobial resistant profiles 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 extraction 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, 0.4 mM each, and 4 mM $MgCl_2$, 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 min, followed by 30 cycles of denaturing at 95 °C for 1 min, primer annealing at 54 °C for 1 min, and elongation at 72 °C for 90 s. Finally, there was a 10-min extension step at 72 °C. The amplification products were checked on 1% w/v agarose gels (Promega, USA) and stained with ethidium bromide (0.5 mg/l).[7] Purified PCR products were obtained using the GeneJET PCR purification kit (Thermo Scientific, USA). The Macrogen Sequencing Facilities ABI PRISM® 3100 Genetic Analyzer was used to sequence the DNA of PCR products (Macrogen, Korea).

Sequence analysis and phylogenetic relationships between strains

Sequence analysis was performed using 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 Neighbor-Joining method with MEGA 11.0 software using the alignment of sequences from the 26 *Bacillus* isolates sequenced in this study.

Statistical analysis

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

Results

Prevalence of Bacillus *species*

Of the 528 collected samples in this study, 407 bacterial isolates were identified. The most frequently isolated were *Bacillus* spp. (176; 4.2%) followed by coagulase-negative staphylococci (CoNS; 78; 19.2%), *Staphylococcus aureus* (62; 15.2%), *Enterococcus* spp. (41; 10.1%), Gram-negative rods (36; 8.9%), *Micrococcus* spp. (14; 3.4%). With regards to the isolated *Bacillus* species, *B. cereus* (82; 46.6%) was the most prevalent species followed by *B. subtilis* (67; 38.1%), while *B. pumilus* (2; 1.1%) was the least prevalent, as showed in Table 1. The distribution of *Bacillus* isolates varied in different wards and showed that the internal medicine department had a higher prevalence of 45 (25.6%) followed by the emergency department (33; 18.8%); the operating rooms had the lowest least prevalence (8; 4.5%), as shown in Table 1.

Antibiotic susceptibility testing

Antimicrobial susceptibility testing was performed against 12 antimicrobial agents. Antimicrobial susceptibility tests revealed 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%), sulfamethoxazole-trimethoprim (96.6%), erythromycin (95.5%) and finally clindamycin (93.2%).

Antibiogram typing of Bacillus *species*

In the present study, different resistotypes (45 resistotypes) were detected, as shown in Table 2. In addition, 22 distinct resistotypes were MDR. This shows the wide diversity of *Bacillus* dissemination in hospitals and provides data relating to the dissemination of resistance patterns among isolates.

Antibiogram typing of the 176 isolates investigated in this study revealed 45 antibiotypes as shown in Table 2. The most common was designated antibiotype 31, 32 (18.2%) isolates, which showed resistance to both penicillin and cefoxitin; followed by antibiotype 3, 28 (15.9%) isolates, which showed complete susceptibility to all tested antimicrobial agents; followed by antibiotype 21, 23 (13.1%) isolates, which showed resistance to penicillin only. Finally, antibiotype 4, 16 (9.1%) isolates, which showed resistance to tetracycline only.

Prevalence of MDR isolates

According to antimicrobial susceptibility profiles, we found that of the 176 *Bacillus* isolates, 38 (21.6%) isolates were MDR. The highest rate of MDR in *Bacillus* isolates was detected among isolates recovered from the emergency room

Table 1: Frequency of *Bacillus* isolates by sampling site.

Isolates	Sampling site								Number (%)[a]
	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 licheniformis	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 polymyxa	–	1	1	1	1	2	–	–	**6 (3.4%)**
Total	9	19	16	8	33	45	18	28	**176 (100%)**

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.

[a] Percentage of the total number of *Bacillus* isolates (176).

Table 2: Antibiogram patterns of all *Bacillus* isolates.

Antibiotype	Antibiogram pattern	No. of isolates (%)*
1	Resistant to erythromycin, penicillin and cefoxitin	2 (1.1%)
2	Resistant to rifampicin, erythromycin, penicillin and cefoxitin	2 (1.1%)
3	Susceptible to all tested antimicrobial agents	28 (15.9%)
4	Resistant to tetracycline	16 (9.1%)
5	Resistant to rifampicin, erythromycin, penicillin and clindamycin	1 (0.6%)
6	Resistant to rifampicin, cefoxitin and clindamycin	3 (1.7%)
7	Resistant to rifampicin and tetracycline	3 (1.7%)
8	Resistant to rifampicin, penicillin and tetracycline	7 (4%)
9	Resistant to cefoxitin and tetracycline	1 (0.6%)
10	Resistant to cefoxitin	9 (5.1%)
11	Resistant to rifampicin, penicillin and tetracycline	6 (3.4%)
12	Resistant to sulfamethoxazole-trimethoprim, rifampicin, erythromycin and tetracycline	1 (0.6%)
13	Resistant to gentamycin and penicillin	1 (0.6%)
14	Resistant to erythromycin and penicillin	1 (0.6%)
15	Resistant to rifampicin and penicillin	4 (2.3%)
16	Resistant to sulfamethoxazole-trimethoprim, chloramphenicol, ciprofloxacin and cefoxitin	1 (0.6%)
17	Resistant to chloramphenicol and penicillin	1 (0.6%)
18	Resistant to erythromycin, penicillin, cefoxitin and clindamycin	1 (0.6%)
19	Resistant to penicillin, cefoxitin and tetracycline	1 (0.6%)
20	Resistant to sulfamethoxazole-trimethoprim, rifampicin, clindamycin and cefoxitin	1 (0.6%)
21	Resistant to penicillin	23 (13.1%)
22	Resistant to clindamycin and cefoxitin	1 (0.6%)
23	Resistant to sulfamethoxazole-trimethoprim and penicillin	1 (0.6%)
24	Resistant to sulfamethoxazole-trimethoprim, rifampicin, clindamycin and gentamycin	1 (0.6%)
25	Resistant to sulfamethoxazole-trimethoprim, cefoxitin and penicillin	1 (0.6%)
26	Resistant to rifampicin, cefoxitin, tetracycline and penicillin	2 (1.1%)
27	Resistant to rifampicin, erythromycin, tetracycline and penicillin	1 (0.6%)
28	Resistant to gentamycin, cefoxitin and penicillin	1 (0.6%)
29	Resistant to rifampicin, cefoxitin and penicillin	6 (3.4%)
30	Resistant to rifampicin and linezolid	1 (0.6%)
31	Resistant to penicillin and cefoxitin	32 (18.2%)
32	Resistant to rifampicin, penicillin, tetracycline and linezolid	1 (0.6%)
33	Resistant to rifampicin	2 (1.1%)
34	Resistant to linezolid	1 (0.6%)
35	Resistant to ceftaroline, cefoxitin and penicillin	1 (0.6%)
36	Resistant to penicillin and linezolid	1 (0.6%)
37	Resistant to rifampicin, ceftaroline, cefoxitin and penicillin	1 (0.6%)
38	Resistant to penicillin and clindamycin	1 (0.6%)
39	Resistant to rifampicin, gentamycin and clindamycin	2 (1.1%)
40	Resistant to rifampicin and cefoxitin	1 (0.6%)
41	Resistant to ciprofloxacin and cefoxitin	1 (0.6%)
42	Resistant to ciprofloxacin and sulfamethoxazole-trimethoprim	1 (0.6%)
43	Resistant to penicillin, cefoxitin and clindamycin	1 (0.6%)
44	Resistant to penicillin and ciprofloxacin	1 (0.6%)
45	Resistant to rifampicin, tetracycline and cefoxitin	1 (0.6%)

(23.7%) followed by the internal medicine department (21.1%), obstetrics and gynecology (13.2%), surgery department (10.5%), renal unit (7.9%), new born nursery (5.3%), operating room (5.3%) and intensive care unit (2.6%), as shown in Figure 1.

Antibiogram typing of MDR Bacillus *spp.*

Antibiogram typing of the selected 38 MDR *Bacillus* isolates investigated in this study revealed 22 antibiotypes. The most common was antibiotype 11, including 6 (15.8%) MDR *Bacillus* isolates that 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 shown in Figure 2.

Molecular characterization of Bacillus *isolates*

Amplification of the 16S rRNA gene

In the present study, the 16S rRNA gene of 26 *Bacillus* isolates were amplified using universal primers. The amplicon products of the tested isolates showed expected bands at approximately 1500 bp, as shown in Figure 3.

Sequencing of the 16S rRNA gene

The species identity of isolates was further confirmed by sequencing of the 16S rRNA gene products followed by detecting the degree of similarity using the BLAST tool in GenBank database which suggests the relatedness of the isolates and their identity within the genus *Bacillus*, as shown in Table 2. All 26 partial 16S rRNA gene sequences

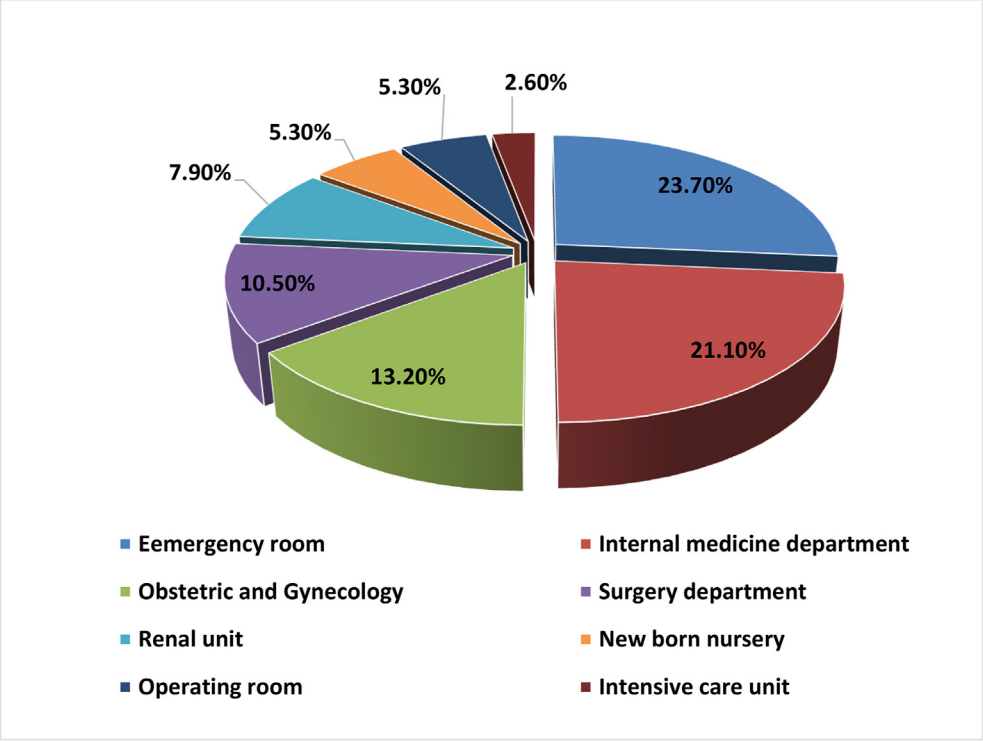

Figure 1: Prevalence of MDR in *Bacillus* isolates by departmental sampling site.

were deposited in the 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 licheniformis*, (OM279798) for *Bacillus mycoides*, (OM280059, ON306915, ON306928) for *Bacillus pumilus* and (OM280058) for Bacillus poly*myxa*, as shown in Table 3.

Phylogenetic diversity among Bacillus isolates

Next, a phylogenetic tree was mapped using the neighbor joining method with the MEGA 11 program and an alignment of the sequences of the 26 *Bacillus* isolates sequenced in this study. The constructed phylogenetic tree showed a close relationship of both *B. licheniformis* and *B. pumilus* to the *B. subtilis* group, whereas *B. mycoides* was more related to the *B. cereus* group, as shown in Figure 4.

Discussion

Health care environments play a crucial role in the transmission of environmental contamination infections. These pathogens can be transmitted from person to person or by touching inanimate items, particularly articles that come into direct contact with patients.[8] 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 infections associated with health care.[9] *Bacillus* isolates, especially *B. cereus*, are associated with food poisoning and infections such as eye infections, sepsis and fatal CNS infections.[10]

However, there has been little investigation into the distribution of *Bacillus* isolates in the environments of Saudi hospitals. This paucity of research highlights the need to monitor *Bacillus* isolates in health care settings. In the current study, the genus *Bacillus* was found in different sampling sites through all wards of the Saudi hospitals studied with an overall prevalence rate of 176 (43.4%). This finding was lower than the higher rate of 50% reported previously in a Sudanese hospital survey.[9] However, our prevalence was higher than the rate of 17% reported previously in KwaZulu-Natal province, South Africa.[2] In this study, the prevalence of *B. cereus* (46.6%) was higher than prevalence rate (16%) in a hospital setting reported previously in the St. Azzhria University Hospital in Isfahan, Malaysia.[11]

In the current study there were fluctuations in the prevalence rate of *Bacillus* species across the wards of public hospitals; the internal medicine department (25.6%) and emergency department (18.8%) showed the highest prevalence while the operating rooms (4.5%) showed the least prevalence rate. From an epidemiological point of view, hospital sites become connected through their shared patients or the exchange of articles.[12] The internal medicine department and emergency department are both key referral points within hospitals with a constant exchange of patients entering and exiting from other points within the hospital such as clinics. This may increase community exposure and thus cause a higher prevalence of bacteria if stringent infection and prevention control measures are not put in place.

Bacillus isolates are often susceptible to broad-spectrum antibiotics such as tetracycline, ciprofloxacin, and erythromycin, which are used to treat gastroenteritis caused by these

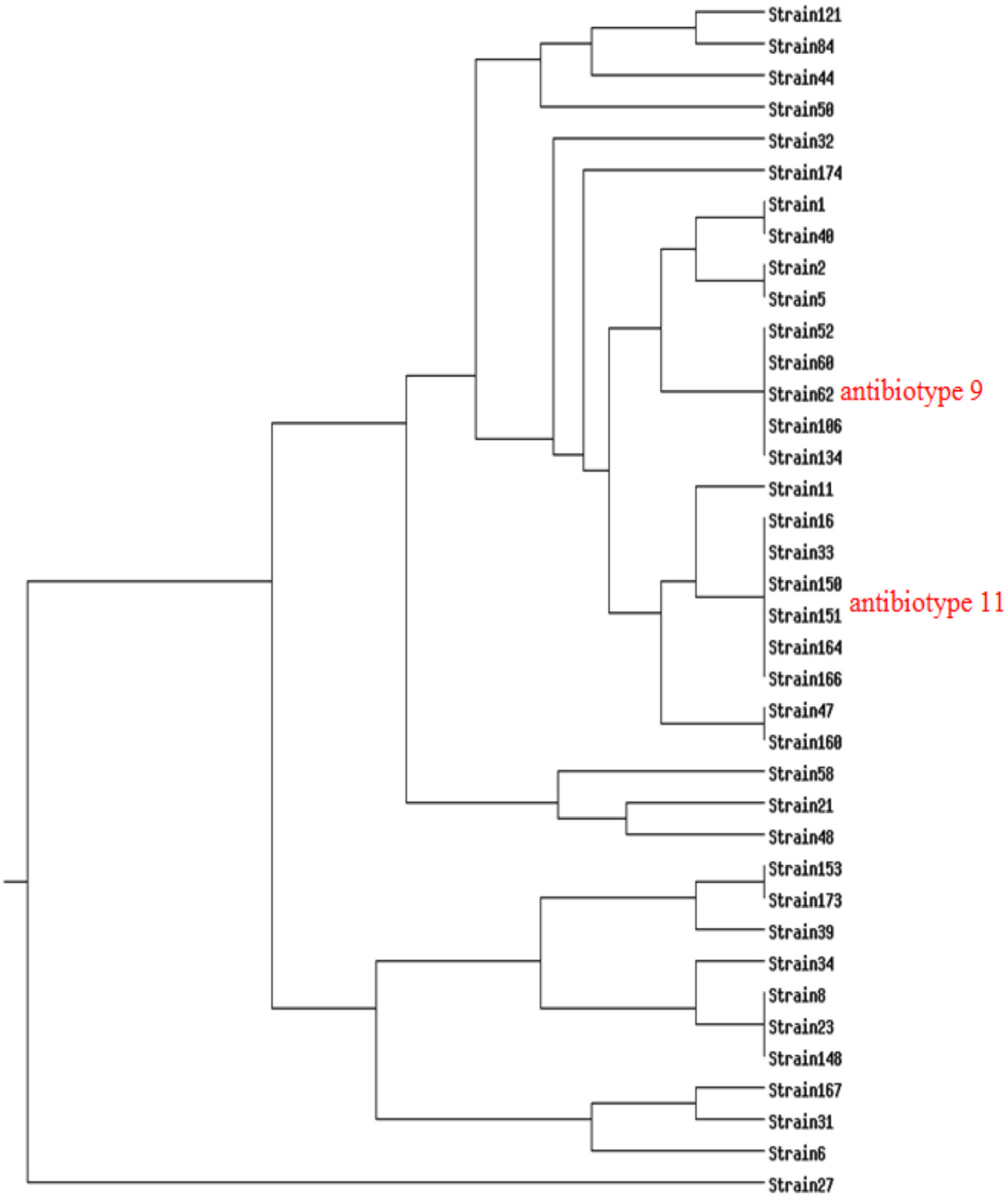

Figure 2: Dendrogram of antibiogram typing for MDR *Bacillus* isolates. Cluster analysis was generated with the Dice similarity coefficient and UPGAMA clustering method.

bacteria.[13] Certain *Bacillus* species, such as *B. cereus*, are intrinsically resistant to β-lactams, except carbapenems[14] and can acquire resistance to antibiotics that are commonly used for infection therapy, such as ciprofloxacin, cloxacillin, erythromycin, tetracycline and streptomycin.[15]

In the current research, a high resistance rate of penicillin G (56.8%) was detected; this was inconsistent with the findings of other studies.[13,16] These findings may be explained by the fact that most *B. cereus* isolates produce β-lactamases; this renders them resistant to penicillin and cephalosporin.[18] With regards to tetracycline, this study showed a 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.[13,16]

Figure 3: Full length 16S rRNA gene (1500bp) amplicons of *Bacillus* isolates amplified with universal primers. The amplicons were resolved on a 1% agarose gel. M, DNA ladder lanes 1−26: amplified products of full-length 16S r RNA gene.

The high resistance to penicillin and tetracyclines is not in agreement with a previous report,[13] which showed that *Bacillus* species are usually susceptible to broad-spectrum antibiotics such as tetracycline, ciprofloxacin and erythromycin, which are used in the treatment of gastroenteritis disease caused by these bacteria. However, certain species within the *Bacillus* genus, such as *B. cereus*, are intrinsically resistant to β-lactams, excluding carbapenems,[14] and can furthermore acquire resistance to commonly used antibiotics for the treatment of infections, such as ciprofloxacin, cloxacillin, erythromycin, tetracycline and streptomycin.[15] Most *B. cereus* isolates produce β-lactamases[16] and are resistant to penicillin and cephalosporins. This explains the resistance rate to penicillin G in our study which was similar to the results of a previous study.[17] However, resistance to tetracycline and erythromycin has previously been observed in these bacteria in the United States and Europe.[18] Erythromycin was associated with high susceptibility (95.5%) in this study; thus was consistent with a previous study[19] in which 81% of isolates were susceptible to erythromycin. Clindamycin revealed high susceptibility (93.2%) in this study; this was not consistent with a previous study[20] which reported the complete susceptibility (100%) of all *Bacillus* isolates to clindamycin.

In the current study, high susceptibility was detected for both ciprofloxacin (97.9%) and gentamicin (97.2%). This finding agrees with a previous study[21] which reported a susceptibility rate of 74.5% for ciprofloxacin and 82.3% for gentamicin. With regards to sulfamethoxazole-trimethoprim, this study revealed a high rate of susceptibility among *Bacillus* isolates (96.6%). This finding is not in

Table 3: 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 licheniformis*	1496	*Bacillus licheniformis* 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 polymyxa*	1460	*Paenibacillus polymyxa* strain DSM 36	97.7%

agreement with a previous study[21] which reported an intermediate susceptibility rate of 80.4% for sulfamethoxazole-trimethoprim In the present study, 45 different resistotypes were detected. In addition, 21.6% of the total *Bacillus* isolates were MDR and belonged to 22 distinct resistotypes. This supports the idea of diverse *Bacillus* dissemination in hospitals; our data are in accordance with a previous study in this respect.[22] Antibiotyping (resistotyping) is a phenotypic method that consists of testing bacterial strains against a set of arbitrarily chosen antibiotics, whereby a resistance pattern that is characteristic of a strain is generated and is used to describe the isolates for epidemiological purposes.[23]

These findings support the ubiquitous nature of *Bacillus* isolates, which allows them to colonize easily. In addition, research has shown the ability of spores to withstand environmental changes, dry heat, and certain chemical disinfectants for long durations.[24] This finding is in agreement with a previous study[25] which used cluster analysis based on the antimicrobial susceptibility profiles of *Bacillus* isolates collected at different periods to confirm the ubiquitous nature of *Bacillus*.

Horizontal gene transfer in prokaryotes has discouraged the phenomenon of phenotypic characters for phylogenetic analysis and the identification of bacteria. Hence a robust tool is DNA sequence analysis of evolutionary conserved genes; this technique is now widely applied for the phylogenetic classification and identification of *Bacillus* species.[26] The 16S rRNA gene shows high levels of conservation and hypervariable regions; the sequence homology of this gene can be used for categorization and the phylogenetic analysis of prokaryotes.[27] In this study, resistant isolates were characterized morphologically, physiologically, biochemically and genetically. On the basis of biochemical characterization, isolates were identified and their identification was rechecked by 16S rRNA gene sequencing analysis. Several reference databases are available that feature the abundance and quality of 16S rRNA gene sequences.[27] The 16S rRNA gene sequence is approximately 1550 bp in length and contains both variable and conserved regions. The gene is large and there are sufficient 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 a specific 540 bp region or at the end of the sequence (approximately 1550 bp); the variable region in between these regions is often used for comparative taxonomy.[28]

In this study, PCR fragments of the 16S RNA gene were used. A gene fragment (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, thus revealing considerable diversity among isolates. Molecular typing of bacteria is the standard method of determining the source and thereby understanding the epidemiology of the bacteria.[29] This method separates strains below the species or subspecies level and may be used to

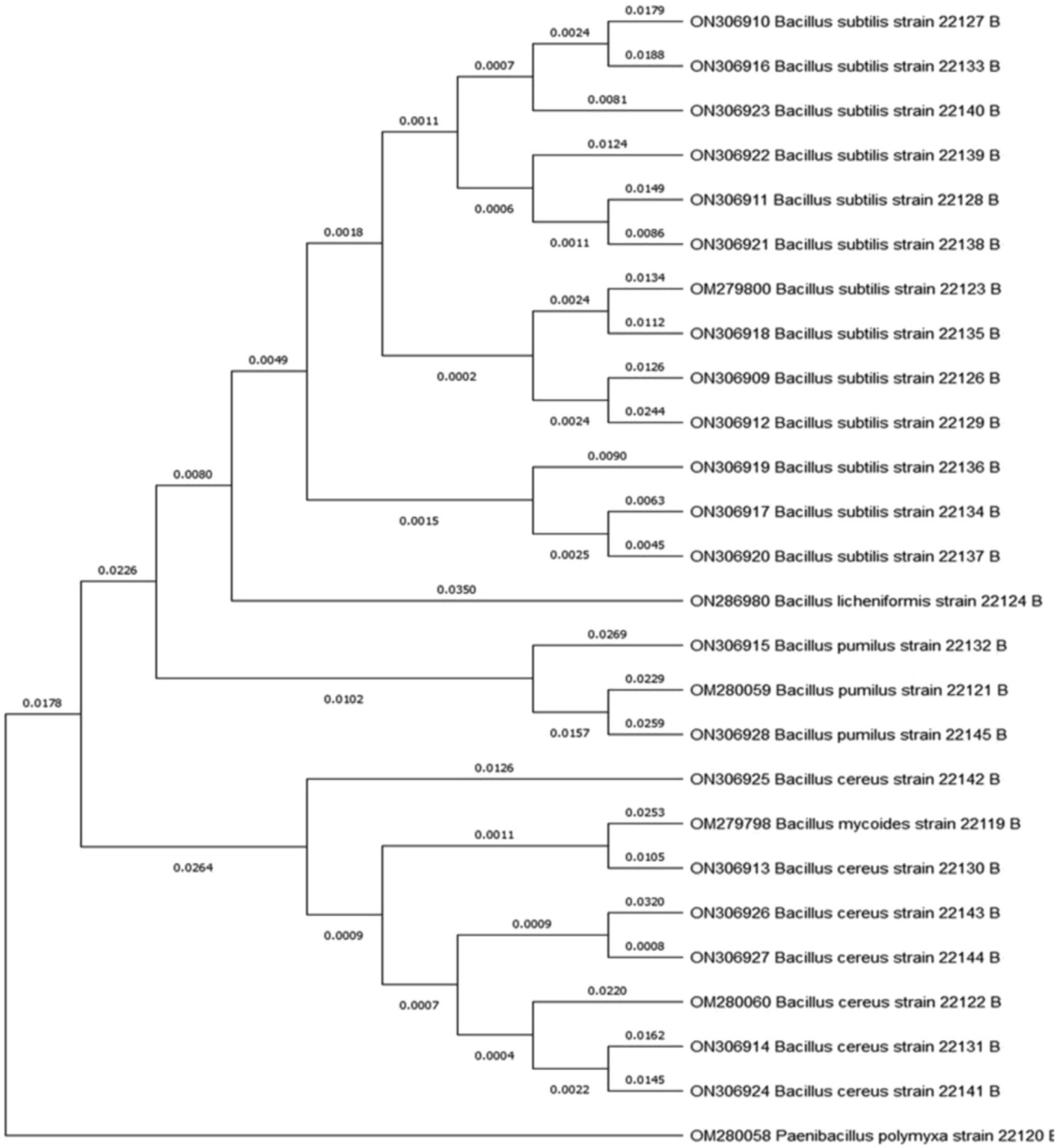

Figure 4: Phylogenetic tree showing the distance between sequences of 16S rDNA of *Bacillus* isolates constructed by the neighbor-joining method.

identify the cause of an outbreak, thus allowing the study of microbial population dynamics; this method may also be useful in the epidemiological surveillance of microbial illnesses.[30]

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. This might be explained by their ability to undergo sporulation.[31] Therefore, this study was conducted to reveal the prevalence, molecular characterization and antibiotic susceptibility of *Bacillus* species isolated from different environments in public hospitals. The prevalence, molecular characterization of genetic diversity and antibiotic susceptibility of the isolates were analyzed. Forty-five different resistotypes were detected and 22 distinct resistotypes were MDR. This supports the idea of diverse *Bacillus* dissemination in hospitals. Antimicrobial susceptibility testing revealed high resistance of *Bacillus*

isolates to β-lactams and tetracycline. In total, 21.6% of multi-drug resistance isolates were resistant to three or more antibiotics. Therefore, this study revealed the prevalence and characteristics of *Bacillus* species isolated from different environments in Saudi hospitals, indicating the potential risk and will help to develop effective prevention and control measures for *Bacillus* species.

Conclusion

This study revealed the high prevalence of *Bacillus* species in hospital environments with high resistance rates to β-lactam and tetracycline antibiotics. The high rates of resistance reported in this study suggest that treating people infected with these strains may be problematic. Molecular analysis revealed the existence of genetic diversity among *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 author have no conflict of interest to declare.

Ethical approval

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

Author contribution

Supervision, conceptualization, administration, methodology, data curation, original draft preparation, virtual screening, scrutinization, software/tools, correspondence: Marwah Bakri. All authors have critically reviewed and approved the final draft and are responsible for the content and similarity index of the manuscript.

Data availability

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

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