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Original Article

Drug resistance and phylogenetic grouping of bacteria isolated from visitors' hands in a secondary-care hospital

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Abstract

Introduction: Dynamic movement in the hospital environment promotes the transmission of nosocomial pathogens and multidrug resistance mechanisms through the dissemination of organisms that carry genetic determinants. Healthcare workers play an important role in the spread of pathogens; however, the role of visitors in this environment is poorly understood.

Objective: This study aimed to molecularly identify and examine the antibiotic resistance of the palmar microbiota of patients' companions in a hospital waiting room.

Methodology: Twenty-five palmar surface and interdigital space sample swabs were randomly collected and cultured on blood agar plates, and 19 colonies with different macro- and microscopic characteristics were isolated. The V4 and V6 hypervariable regions of the 16S rRNA gene from each isolate were amplified by PCR and sequenced. Maximum likelihood- and Bayesian inference-based phylogenetic analyses were performed to determine taxonomic relationships. Antibiotic resistance was evaluated by disk diffusion and broth microdilution.

Results: Among the isolates, 52.6% were related to *Bacillus*, 36.8% to *Staphylococcus*, 5.3% to *Enterococcus* and 5.3% to *Atlantibacter*. All of the isolates exhibited ampicillin and penicillin resistance, while 94.7% also exhibited dicloxacillin resistance. *Staphylococcus aureus* was resistant to penicillins but sensitive to the remaining drugs. Bacteria identified as *Bacillus subtilis* (MLM14B99), *Bacillus pumilus* (MLM23B07 and MLM25B06), *Staphylococcus epidermidis* (MLM24S31 and MLM29S04), and *Enterococcus* (MLM22E08) showed resistance to at least 46.7% of the antibiotics.

Conclusions: To decrease the transmission of pathogenic bacteria with an antibiotic resistance profile, re-evaluation of hand cleaning measures and their application by people who visit hospital centres is needed.

Key words: Skin microbiota; antibiotic resistance; 16S rRNA gene; pathogen dissemination; pathogen transmitter.

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Introduction

Nosocomial infections and antibiotic resistance affect millions of people around the world, as they are the cause of prolonged hospital stays, increased mortality and morbidity rates, and even increased socioeconomic costs [1,2]. Hospital-acquired infections are commonly caused by the patient's own microbiota; however, other determining factors also influence the occurrence of these infections. The clinical environment has developed into an important reservoir and site of transmission of nosocomial pathogens [3,4]. Poor hand hygiene and decontamination practices, as well as regular contact with surfaces, fomites or other individuals, lead to the mobilization of pathogens within the hospital area [4].

The transit of individuals has become the principal route of dissemination [5]. Healthcare workers perform many activities that increase hand contamination. Visiting different patients and direct or indirect contact with the environment, fomites, or fluids promote colonization by pathogens [6]. In addition, eventual exposure to antibiotics has been related to the appearance and maintenance of resistance genes [7,8]. Moreover, the transmission of antibiotic resistance mechanisms through the dissemination of bacteria that carry and share genetic determinants in various microbial communities through horizontal gene transfer promotes the acquisition of multi-drug resistant bacteria by dynamic movement in the hospital environment [8]. In this way, colonization by antibiotic-resistant bacteria from the hands of healthcare workers becomes the main route for the transmission and contamination of surfaces by resistant nosocomial bacteria [9,10].

Likewise, patients' companions represent another group of people who interact with the hospital environment. It has been estimated that actions

involving contact with the patient, as well as a prolonged stay in a healthcare environment, increase the probability of acquiring an infection associated with hospital centres. However, the means by which this phenomenon occurs is still unclear [11,12]. To date, no evidence of normal microbiota members predominating on surfaces frequented by visitors, except for nosocomial pathogens such as *Escherichia coli*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii* [13], has been presented. Nevertheless, information about antimicrobial resistance in this group is unavailable.

Antimicrobial resistance in the community has been shown to be important in recent years. Seng *et al*. [14] isolated *Staphylococcus* species with antimicrobial resistance from different surfaces at a university, where objects with high contact, such as books, showed the highest percentage of contamination. Previously, these species were considered to be innocuous microorganisms; however, the rates of infections of these opportunistic bacteria in the hospital environment have increased, with *Staphylococcus epidermidis* and *Staphylococcus haemolyticus* being the most significant species [15].

Therefore, due to the prevalence of resistant bacteria in both the hospital environment and the community, the objective of this research was to molecularly identify cultivatable palmar microbiota among patients' companions who passed through the waiting room of a hospital and to examine the antibiotic resistance of these microbiota.

Methodology

Collection of samples

A cross-sectional study was carried out in June 2016. Of the 30 individuals approached, 25 who claimed to be healthy companions of people with health problems and were present in the waiting room of a second-level hospital in Culiacan, Sinaloa, Mexico, volunteered; patients and hospital staff were excluded. Additionally, the participants were informed about the aim of the study. Samples were collected from the palmar surface (including the interdigital surface) using swabs previously moistened with injectable water under sterile conditions. The samples were stored in 15 mL threaded tubes and aseptically transported to the laboratory for further processing and microbial isolation.

Isolation and characterization of bacteria

Each of the 25 samples was cultivated in blood agar medium (MCD LAB, Mexico) and incubated at 37 °C for 24 hours. The colony-forming units (CFU) and macroscopic characteristics of each colony, such as its border, colour, and shape, were determined for each culture. Microscopic characterization of isolated colonies was performed using Gram staining. Colonies with representative or unique morphological characteristics were isolated. Each isolated colony was grown in 1 mL of Luria-Bertani broth with incubation at 37 °C for 24 hours.

Genomic DNA extraction

Genomic DNA was extracted using the Wizard SV Genomic DNA Purification System Kit (Promega, USA) according to the manufacturer's instructions for Gram negative bacteria. To rupture the cell wall of Gram-positive bacteria, enzymatic treatment with 60 µL of 10 mg/mL lysozyme (BioChemica, United Kingdom) and 2 µL of 5000 U/mL mutanolysin (Sigma–Aldrich, USA) was performed in a 480 µL solution of disodium EDTA (Promega, USA) with incubation at 37 °C for one hour.

Amplification of the V4 and V6 regions of the 16S rRNA gene of isolated bacteria

The V4 and V6 regions of the 16S rRNA gene were amplified using the primers 16S-V4-515F/806R [16] and 16S-V6-784F/1061R [17] with modifications of the annealing temperature (51 °C and 57 °C, respectively) as reported by Zhang *et al*. [18]. Each reaction consisted of 1.5 µL of each primer at 0.6 µM, 25 µL of GoTaq Green Master Mix (Promega, USA), 5 µL of DNA and 17 µL of nuclease-free water. Each PCR product was directly purified with the Wizard SV Gel and PCR Clean-up System Kit (Promega, USA), and 1 µL of the purified product was verified by electrophoresis in 1% agarose gel stained with 1 µL of 1X GelRed (Biotium Inc. USA). Seven microlitres (~50 ng/µL) of the purified product was sequenced in the 5'-3' direction by a commercial Sanger dideoxy sequencing service in an ABI 3730xl System (Macrogen, South Korea).

Sequence analysis

Chromas V 2.6.5 software was used to analyse the quality of the electropherogram and edit the sequences of the V4 and V6 fragments of the 16S rRNA gene. The V4 and V6 fragments were compared individually and concatenated with the 16S RefSeq of the National Center for Biotechnology Information (NCBI), for which the Megablast algorithm was used. The sequence with the highest percent identity (E value) was selected as the most similar. Total and maximum scores were used for molecular characterization of the isolated

bacteria. Furthermore, identity was also calculated manually based on the nucleotides analyzed by the alignment algorithm through the following formula:

$$I_{(V4-V6)} = \frac{(M_{V4} + M_{V6})}{(N_{V4} + N_{V6})} \times (100\%)$$

where $I_{(V4\text{-}V6)}$ represents the identity of the concatenated regions, M_{V4} indicates the identities in V4, M_{V6} the identities in V6, N_{V4} is the nucleotides evaluated from V4 and N_{V6} is the nucleotides evaluated from V6.

Bacterial phylogenetic analysis

A search for sequences corresponding to the taxonomic neighbours of the isolated bacteria was performed using MOLE-BLAST, and a multiple sequence alignment was retrieved from the search. The alignment was curated in Unipro UGENE software, version 1.29 [19], using the MUSCLE algorithm in standard mode [20]. Gap columns with a percentage > 20% were removed. A phylogenetic tree was constructed based on the Bayesian inference (BI) and maximum likelihood (ML) methods. The substitution model was determined through the MEGA6 program [21]. BI was performed in BEAUti/BEAST software, version 1.8.4 [22], with the Tamura–Nei model with a gamma distribution and 5 substitution rates across sites. A Markov chain Monte Carlo (MCMC) analysis of 2.5 × 10^7 generations was applied, sampling every 1000 states and discarding 10% of the generations as burn-in. The resulting phylogenetic tree was viewed and edited in FigTree, version 1.4.3 [23]. Analysis by the ML method was performed using the PhyML tool in NGPhylogeny (available at https://ngphylogeny.fr/tools; Lemoine *et al*. [24]). The Kimura 2-parameter model with 5 gamma rates and 1000 bootstrap replicates was used for tree inference. Tree topology was refined with STR and NNI searches. The final tree was built and edited in TreeGraph2, version 2.14.0 [25], using BI for the base topology and ML for additional support values. Conflicting topologies from both trees were discarded.

Determination of antibacterial resistance

Antibacterial susceptibility was evaluated by microdilution and disk diffusion methods. Each isolate was grown on nutrient agar plates for 24 hours at 37 °C. Subsequently, five colonies were taken from the plates, inoculated into 7 mL of tryptic soy broth (MCD LAB, Mexico) and incubated for 2 hours at 35-37 °C. One millilitre of the culture was taken and centrifuged at 16,000 × g for 30 seconds. The pellet was resuspended in 1 mL of saline, vortexed, and diluted (1:20) for quantification by absorbance measurement. Absorbance readings of 0.08-0.1 (λ = 625 nm) were considered equivalent to a bacterial concentration of 0.5 on the McFarland scale (10^8 CFU/mL).

For the microdilution sensitivity test in broth, 10 µL of quantified solution was diluted in 990 µL of Mueller–Hinton broth (1:100) (Conda, Spain). From this final solution, 100 µL (5 × 10^4 CFU) was deposited in 96-well microplates with the addition of six twofold serial dilutions of each antibiotic, namely, tetracycline (TET) (1 to 32 µg/mL), ciprofloxacin (CIP) (0.125 to 4 µg/mL), erythromycin (ERY) (0.5 to 16 µg/mL), chloramphenicol (CHL) (2 to 64 µg/mL) and gentamicin (GEN) (1 to 32 µg/mL), which were previously prepared according to the Clinical and Laboratory Standards Institute [26]. The procedure was performed in triplicate for these samples as well as for the growth controls and sterile controls for each bacterium and antibiotic used. The microplates were incubated at 37 °C for 18 hours, and the absorbance at 630 nm was measured with an EL × 800 microplate reader (BioTek, USA) at 0 and 18 hours of incubation. Absorbance results were collected by Gen5 software.

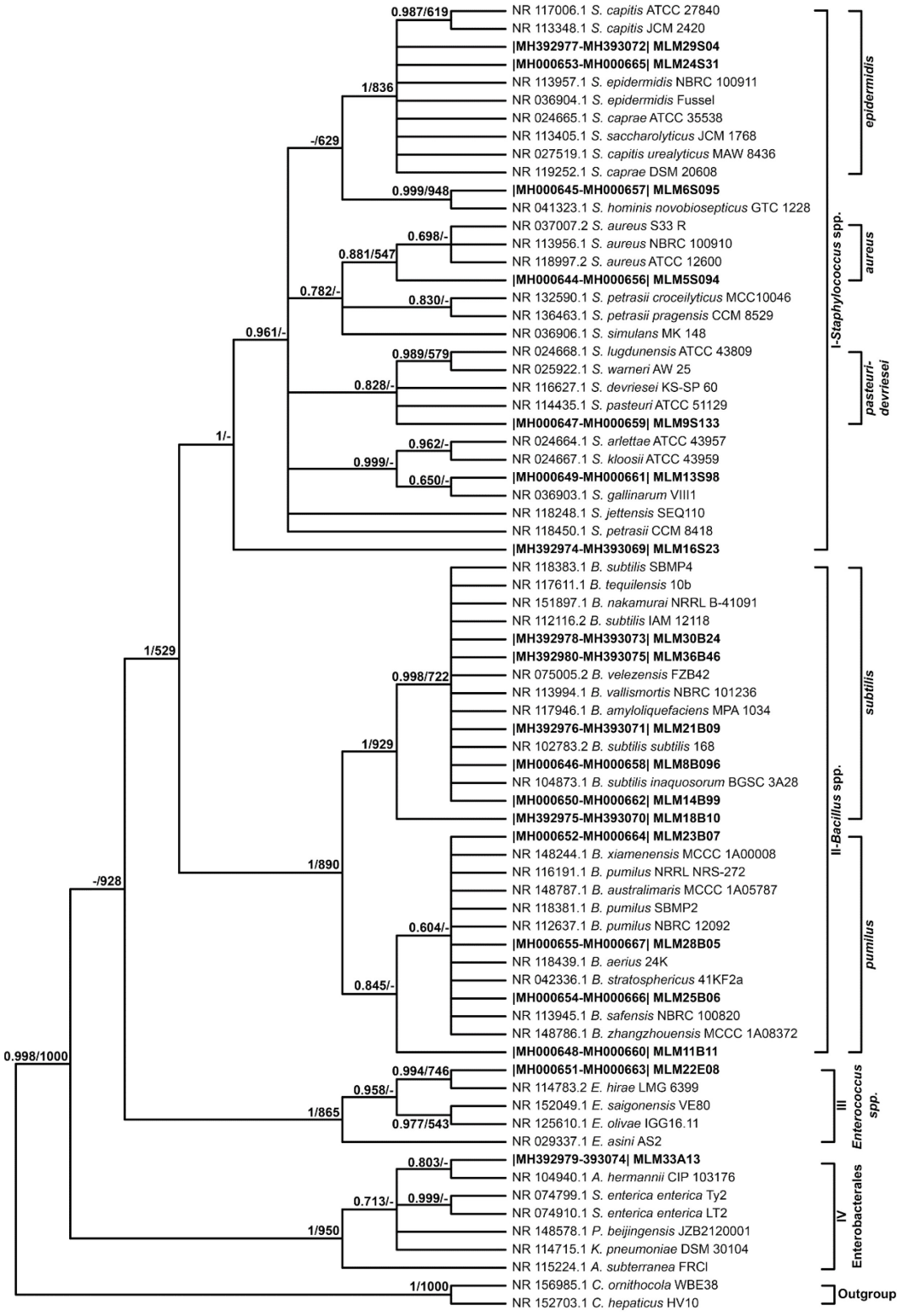
For the disk diffusion sensitivity test, Muller–Hinton agar plates (MCD LAB, Mexico) were inoculated with the previously quantified isolate using moistened swabs. A set of +/- IDLAB Sensidisks (Diagnostic Research-Gutiérrez Ramos Abel, Mexico) was placed on plates with the antibiotics amikacin (AMK) (30 µg), ampicillin (AMP) (30 µg), cephalothin (CEF) (30 µg), cefotaxime (CTX) (30 µg), dicloxacillin (DCX) (1 µg), ceftriaxone (CRO) (30 µg), netilmicin (NET) (30 µg), nitrofurantoin (NIT) (300 µg), penicillin (PEN) (10 U), and trimethoprim-sulfamethoxazole (SXT) (1.25/23.75 µg). The plates were incubated for 18 hours at 35 °C. Inhibition halos were interpreted according to the Clinical and Laboratory Standards Institute [26,27] and European Committee on Antimicrobial Susceptibility Testing [28]. *Staphylococcus aureus* subsp. *aureus* (ATCC 25923) and *E. coli* (ATCC 25922) were used as quality controls for both trials. Multiple antibiotic resistance (MAR) indexes for isolates and taxonomic groups were calculated using the following formulas:

$$MAR_{(Isolate)} = \frac{a}{b}$$

$$MAR_{(Taxa)} = \frac{a}{b(c)}$$

where *a* represents the number of antibiotics to which an isolate/taxon is resistant, *b* represents the number of antibiotics tested and c represents the number of organisms corresponding to said taxon [29].

Figure 1. Phylogenetic tree generated by Bayesian inference and maximum likelihood from the concatenated V4 and V6 hypervariable regions of the 16S rRNA gene.



The tree shows the support for posterior probabilities ≥ 0.6/bootstrap values ≥ 500, indicated on the respective branch. The strains isolated in this study are highlighted in bold, and the GenBank accession numbers are indicated for V4-V6. Four clades stand out, of which clades I and III are subdivided into 6 and 2 subgroups, respectively. The species *Campylobacter ornithocola* and *Campylobacter hepaticus* are shown as outgroups.

Statistical analysis

The minimum inhibitory concentration (MIC) of each antibiotic for each bacterium was considered to be the lowest concentration where the difference between the absorbance values obtained at 0 and 18 hours of incubation were not significantly different from those of the negative control of each sample. Significant differences were detected using one-way analysis of variance (ANOVA) ($\alpha = 0.05$) and Duncan's a posteriori test in STATISTICA software (version 7, 94-2006).

To determine the profiles and patterns of antibiotic resistance across the strains and taxonomic groups, hierarchical clustering analyses were performed with the unweighted pair group method with arithmetic mean (UPGMA) approach in Past3 [30]. The categorical values of antibiotic susceptibility were tested individually considering 1000 bootstrap replicates and a 50% bootstrap node cut-off.

Ethics approval

Due to the lack of an institutional ethics committee, such approval was not available. However, a letter of authorization was presented by the Faculty of Biology, Autonomous University of Sinaloa, indicating the assessment and correct application of the protocols and methodologies presented in the study and asserting that the study posed no risk to the health of participants. Likewise, no personal data (such as names, ages and other information of a private nature) of the participants were collected and/or disclosed. The authorization letter is attached as a separate file.

Results

Molecular identification

All (100%) of the samples showed bacterial growth; however, only 80% showed quantifiable growth, with values between 80 and 1600 CFU per plate. Among the quantifiable samples, 19 types of colonies were isolated based on their macroscopic and microscopic characteristics, 94.7% of which were colonies of Gram-positive bacteria and 5.3% of which were colonies of Gram-negative bacteria.

The bacterial isolates were subjected to amplification and sequencing of the V4 and V6 fragments of the 16S rRNA molecular marker, and each of the sequences was registered in the GenBank database (Table 1). BLAST comparison of the concatenated sequences of both regions showed that 36.8% of the isolates were related to species of the genus *Staphylococcus*, 52.6% were related to species of the genus *Bacillus* (predominantly *B. subtilis* (31.6%) and *B. pumilus* (21%)), one strain was associated with the genus *Enterococcus* (5.3%), and another strain was associated with species of the family

Table 1. GenBank accession numbers of the V4 and V6 sequences of the isolates obtained.

Strain	This study V4/V6	Species with the highest % identity[a]	GenBank accession no.	V4		V6		% Identity V4 and V6 concatenated[b]
				E Value	% Identity	E Value	% Identity	
MLM5S094	MH000644/MH000656	*Staphylococcus aureus*	NR_037007.2	4.00E-119	99	1.00E-118	99	98.7
MLM6S095	MH000645/MH000657	*Staphylococcus hominis*	NR_041323.1	4.00E-119	99	5.00E-98	96	97.8
MLM9S133	MH000647/MH000659	*Staphylococcus pasteuri*	NR_024669.1	1.00E-114	98	3.00E-130	99	98.6
MLM13S98	MH000649/MH000661	*Staphylococcus gallinarum*	NR_036903.1	2.00E-118	98	2.00E-127	99	99
MLM16S23	MH392974/MH393069	*Staphylococcus petrasii*	NR_132590.1	2.00E-106	96	5.00E-103	96	96.1
MLM24S31	MH000653/MH000665	*Staphylococcus epidermidis*	NR_113957.1	5.00E-114	99	6.00E-133	99	99.6
MLM29S04	MH392977/MH393072	*Staphylococcus epidermidis*	NR_113957.1	1.00E-118	99	2.00E-116	100	99.8
MLM8B096	MH000646/MH000658	*Bacillus subtilis*	NR_024931.1	1.00E-119	99	4.00E-134	100	99.4
MLM14B99	MH000650/MH000662	*Bacillus subtilis*	NR_024931.1	9.00E-116	99	2.00E-122	99	99.4
MLM18B10	MH392975/MH393070	*Bacillus subtilis*	NR_024931.1	2.00E-102	95	5.00E-123	100	97.7
MLM21B09	MH392976/MH393071	*Bacillus subtilis*	NR_024931.1	2.00E-118	99	9.00E-126	100	99.8
MLM30B24	MH392978/MH393073	*Bacillus subtilis*	NR_024931.1	9.00E-116	98	2.00E-122	100	98.8
MLM36B46	MH392980/MH393075	*Bacillus subtilis*	NR_024931.1	3.00E-115	99	5.00E-123	100	99.4
MLM11B11	MH000660/MH000648	*Bacillus pumilus*	NR_115334.1	2.00E-103	96	2.00E-127	100	98.1
MLM23B07	MH000652/MH000664	*Bacillus pumilus*	NR_115334.1	3.00E-115	99	2.00E-122	98	98.7
MLM25B06	MH000654.2/MH000666	*Bacillus pumilus*	NR_115334.1	2.00E-121	99	2.00E-122	99	99
MLM28B05	MH000655/MH000667	*Bacillus pumilus*	NR_115334.1	2.00E-118	99	3.00E-131	99	99.6
MLM22E08	MH000651/MH000663	*Enterococcus hirae*	NR_114452.1	2.00E-117	99	3.00E-131	100	99.4
MLM33A13	MH392979/MH393074	*Atlantibacter hermannii*	NR_104940.1	2.00E-117	99	2.00E-123	99	99.4

[a] The name and accession number of the species that showed the highest % identity are indicated; [b] Value of the % identity obtained from the individual and comparison of concatenated V4 and V6 regions from the GenBank database.

Enterobacteriaceae (5.3%). Most of the strains yielded percentages higher than 98%, except for the MLM6S095 and MLM18B10 strains, with 97.8 and 97.7%, respectively, and the MLM16S23 strain, with 96.1% (Table 1).

Regarding the samples belonging to the genus *Staphylococcus*, strains MLM5S094, MLM6S095, MLM9S133, MLM13S98, and MLM16S23 shared between 96 and 99% identity with *S. aureus*, *S. hominis* subsp. *novobiosepticus*, *S. pasteuri*, *S. gallinarum,* and *S. petrasii,* while both strains MLM24S31 and MLM29S04 were related to the species *S. epidermidis*, *S. saccharolyticus*, *S. caprae,* and *S. capitis*.

For samples of the genus *Bacillus*, strains MLM8B096, MLM14B99, MLM18B10, and MLM21B09 showed between 97.7 and 99.8% identity with *B. subtilis* but also with *B. vallismortis*, *B. amyloliquefaciens*, *B. tequilensis*, *B. velezensis,* and *B. nakamurai*. In addition to the aforementioned species, the MLM30B24 and MLM36B46 strains also showed the same percent identity (98.8 and 99.4%, respectively) with *B. mojavensis*, *B. malacitensis*, *B. axarquiensis,* and *B. halotolerans*. Strains MLM11B11, MLM23B07, MLM25B06, and MLM28B05 showed 98.1-99.6% identity with *B. pumilus*, *B. altitudinis*, *B. safensis*, *B. stratosphericus*, *B. aerius*, *B. xiamenensis*, *B. zhangzhouensis,* and *B. australimaris*.

On the other hand, strain MLM33A13 showed 99.4% identity with *Atlantibacter hermannii,* and strain MLM22E08 showed 99.4% identity with the species *Enterococcus hirae*, *E. mundtii*, *E. durans,* and *E. faecium*.

Phylogenetic analysis

A phylogenetic tree was obtained from 73 sequences (19 of the concatenated V4 and V6 sequences from this study and 54 from the NCBI 16S RefSeq database) with 466 nucleotides, the topology of which consisted of 4 clades and an external group composed of the species *Campylobacter ornithocola* and *Campylobacter hepaticus* (Figure 1).

Clade I belongs to the genus *Staphylococcus*. Strains MLM24S31 and MLM29S04 are related to the species corresponding to the *S. epidermidis* group, including strains of *S. caprae, S. capitis, S. saccharolyticus,* and *S. epidermidis* (NR_119252.1, NR_027519.1, NR_113405.1, NR_024665.1, NR_036904.1, NR_113957.1, NR_113348.1, and NR_117006.1). The homology of strain MLM6S095 and *S. hominis novobiosepticus* (NR_041323.1) is observed as a well-supported dichotomy (0.999 ppB/948 BS). MLM5S094 comprises the *S. aureus* group (NR_113956.1, NR_037007.2, and NR_118997.2). The MLM9S133 strain is observed within the *S. pasteuri-devriesei* group, which is related to the species *S. devriesei, S. pasteuri, S. lugdunensis,* and *S. warneri* (NR_116627.1, NR_114435.1, NR_024668.1, and NR_025922.1). The MLM13S98 strain is associated with *S. gallinarum* (NR_036903.1). The MLM16S23 strain is shown as a branch paraphyletic to the *Staphylococcus* group, indicating its relationship with the genus but lack of resolution at the species level (Figure 1).

Clade II corresponds to the genus *Bacillus*, in which a dichotomy is observed that divides the genus into two species subgroups: *B. subtilis* and *B. pumilus*. The MLM30B24, MLM36B46, MLM21B09, MLM8B096, and MLM14B99 strains are related to the *B. subtilis* subgroup, represented by the species *B. subtilis, B. tequilensis*, *B. nakamurai, B. velezensis, B. vallismortis,* and *B. amyloliquefaciens* (NR_075005.2, NR_112116.2, NR_102783.2, NR_113994.1, NR_117611.1, NR_151897.1, NR_117946.1, NR_104873.1, and NR_118383.1). The MLM18B10 strain is found on a branch paraphyletic to the *B. subtilis* subgroup. The MLM23B07, MLM28B05 and MLM25B06 strains are related to the *B. pumilus* subgroup, represented by the homologous species *B. xiamenensis*, *B. pumilus*, *B. australimaris, B. aerius*, *B. stratosphericus*, *B. safensis,* and *B. zhangzhouensis* (NR_148244.1, NR_116191.1, NR_148787.1, NR_118381. 1, NR_112637.1, NR_118439.1, NR_042336.1, NR_113945.1, and NR_148786.1). MLM11B11 also constitutes a divergent branch of this group (Figure 1).

Clade III belongs to the genus *Enterococcus*, in which the MLM22E08 strain shows homology with the species *E. hiare* (NR_114783.2). This strain is associated with the species *E. saigonensis* and *E. olivae* (NR_152049.1 and NR_125610.1) as a sibling group and with the paraphyletic strain *E. asini* (NR_029337.1) (Figure 1).

Clade IV corresponds to the Enterobacterales order. Strain MLM33A13 is homologous to *Atlantibacter hermannii* (NR_104940.1) and related to the species *S. enterica enterica, P. beijingensis,* and *K. pneumoniae* (NR_074799.1, NR_074910.1, NR_148578.1, and NR_114715.1) (Figure 1).

Antimicrobial susceptibility and antibiotic resistance patterns

The average MAR index was 0.31, ranging from 0.20 to 0.53. Furthermore, 100% of the strains in this study that shared identity with the genus

Staphylococcus showed resistance to PEN, AMP, and DCX, and 28.6% showed resistance to NET, SXT, ERY, and GEN. Of all the *Staphylococcus* strains, 42.9% presented intermediate resistance against CTX and 28.6% against CRO. The strains related to *S. epidermidis* MLM24S31 and MLM29S04 presented resistance to seven of 15 drugs, showing resistance against 46.7% of the drugs and intermediate resistance against 13.3% of the drugs. *Staphylococcus* presented an MAR index of 0.28. (Table 2).

All (100%) of the strains in this study that shared species-level identity with the *Bacillus* genus (MAR index = 0.32) presented resistance to AMP and PEN. In addition, 90% of the strains were resistant to DCX and 50% to NET, and one to three strains presented resistance against CEF, CTX, AMK, SXT, TET, ERY,

Table 2. Antimicrobial susceptibility testing of the strains isolated in this study against 15 antibiotics by broth microdilution and disk diffusion.

		Disk diffusion							Broth microdilution									Total
		AMP	**DCX**	**PEN**	**CEF**	**CTX**	**CRO**	**NET**	**AMK**	**NIT**	**SXT**	**TET**	**CIP**	**ERY**	**CHL**	**GEN**	**%R**	**%I***
S. aureus	MLM5S094	1	1	1	-	I*	-	0	0	0	0	0	0	0	0	0	20	6.7
S. hominis	MLM6S095	1	1	1	-	-	-	0	0	0	0	0	0	0	0	0	20	0
S. pasteuri	MLM9S133	1	1	1	-	-	-	0	0	0	0	0	0	0	0	0	20	0
S. gallinarum	MLM13S98	1	1	1	-	-	-	0	0	0	0	0	0	0	0	0	20	0
S. petrasii	MLM16S23	1	1	1	-	-	-	0	0	0	0	0	0	0	0	0	20	0
S. epidermidis	MLM24S31	1	1	1	-	I*	I*	1	0	0	1	0	0	1	0	1	46.7	13.3
S. epidermidis	MLM29S04	1	1	1	-	I*	I*	1	0	0	1	0	0	1	0	1	46.7	13.3
	%R	100	100	100	0	0	0	28.6	0	0	28.6	0	0	28.6	0	28.6		
	%I*	0	0	0	0	42.9	28.6	0	0	0	0	0	0	0	0	0		
B. subtilis	MLM8B096	1	-	1	1	I*	-	0	0	0	0	1	0	I*	I*	0	26.7	20
B. subtilis	MLM14B99	1	1	1	1	1	-	1	I*	0	0	0	I*	0	0	1	46.7	13.3
B. subtilis	MLM18B10	1	1	1	-	-	-	1	1	0	0	0	0	1	0	0	40	0
B. subtilis	MLM21B09	1	1	1	-	-	-	1	I*	0	0	0	0	0	0	0	26.7	6.7
B. subtilis	MLM30B24	1	1	1	-	I*	I*	0	I*	0	0	0	0	0	0	0	20	20
B. subtilis	MLM36B46	1	1	1	-	-	I*	0	0	0	0	0	0	0	0	0	20	6.7
B. pumilus	MLM11B11	1	1	1	-	-	-	0	0	0	0	0	0	0	0	0	20	0
B. pumilus	MLM23B07	1	1	1	-	I*	I*	1	0	0	1	1	0	1	0	1	53.3	13.3
B. pumilus	MLM25B06	1	1	1	-	I*	I*	1	0	0	1	0	0	1	0	1	46.7	13.3
B. pumilus	MLM28B05	1	1	1	-	-	-	0	0	0	0	0	0	0	0	0	20	0
	%R	100	90	100	20	10	0	50	10	0	20	20	0	30	0	30		
	%I*	0	0	0	0	40	40	0	30	0	0	0	10	10	10	0		
E. hirae	MLM22E08	1	1	1	1	1	-	1	-	0	-	0	1	0	0	-	46.7	0
A. hermannii	MLM33A13	1	1	1	1	I*	0	0	I*	0	0	0	0	-	0	0	26.6	13.3
Total	%R	100	94.7	100	21.1	10.5	0	42.1	5.3	0	21.1	10.5	5.3	26.3	0	26.3		
	%I*	0	0	0	0	42.1	31.6	0	21.1	0	0	0	5.3	5.3	5.3	0		
ATCC™ 25922		15	17a	12a	18	33	31	22	25	24	23	0.5	0.016	16a	8	1		
ATCC™ 25923		27	15a	26	35	30	28	23	23	22	24	0.5	0.12	0.25	16	0.5		

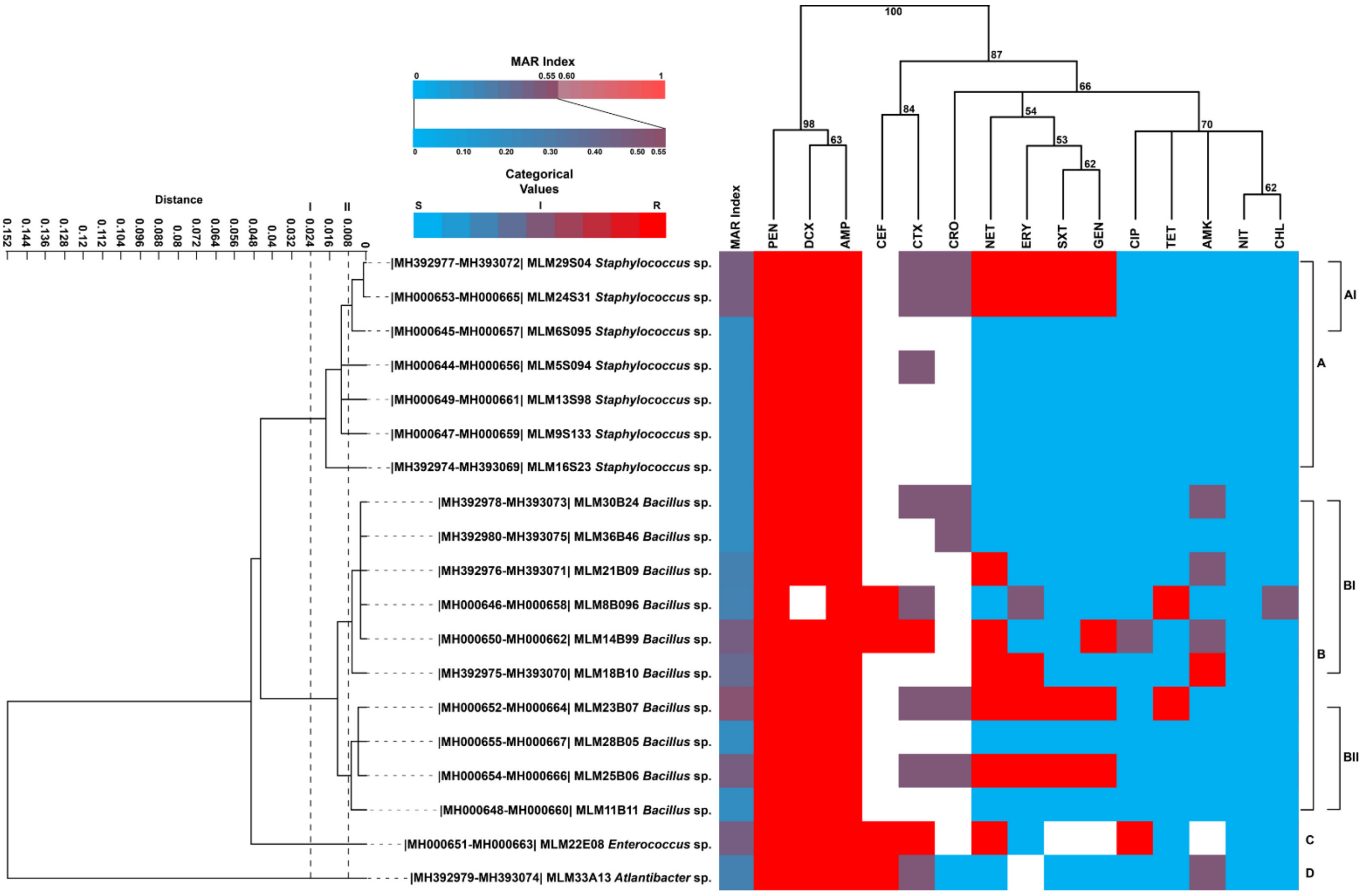
The results were interpreted according to CLSI 2019 [26], with some exceptions. For amikacin and netilmicin testing of *Staphylococcus*, the sensible interpretation criteria reported by EUCAST 2019 [28] were used. For cephalothin, cefotaxime and ceftriaxone, the sensible interpretation criteria from CLSI 2012 [27] were used since no such values were reported in CLSI or EUCAST 2019 [26,28]. For interpretation of the Sensidisks of *Bacillus* spp., *Staphylococcus* interpretation criteria were used. (-) Undefined susceptibility. For *Enterococcus*, the aminoglycosides (AMK, GEN and NET), the cephalosporins (CEF, CTX of CRO) and SXT are not clinically effective according to CLSI 2019 [26], so they are not reported as susceptible. (a) Indicates that there is no quality value for the strain with the said antibiotic according to CLSI 2019 [26]. 0 = sensitive, 1 = resistant, I* = intermediate, %R = percentage of resistance, %I* = percentage of intermediate resistance.

and GEN. The MLM23B07, MLM14B99, MLM25B06, and MLM18B10 strains were the most highly resistant to the drugs used, with 53.3%, 46.6%, 46.6%, and 40% resistance, respectively. The MLM8B096, MLM21B09, MLM30B24, MLM36B46, MLM11B11, and MLM28B05 strains showed resistance against three to four of the 15 antibiotics (Table 2). Forty percent of the *Bacillus* strains showed intermediate resistance against CTX and CRO, 30% showed resistance against AMK, and 10% showed resistance against CIP, ERY, and CHL. The MLM8B096 and MLM30B24 strains showed the highest intermediate resistance level (20%) against the drugs, followed by MLM14B99, MLM23B07 and MLM25B06 (13.3%) and MLM21B09 and MLM36B46 (6.7%).

The MLM22E08 strain (MAR index = 0.47), which shared identity with *Enterococcus* sp., presented resistance to seven of the 15 drugs tested. The strain that shared identity with *Atlantibacter* sp. (MLM33A13) showed resistance against 26.6% of the drugs and intermediate resistance against 13.3% of the drugs (Table 2).

The results of UPGMA-based hierarchical clustering analysis revealed a resistance pattern to PENs across all strains (Figures 2 and 3). Resistance patterns to NET, ERY, SXT, and GEN were observed in strains related to *S. epidermidis* and *B. pumilus*. Susceptibility patterns were found for AMK, CIP, TET, NIT, and CHL. Regarding hierarchical clustering of taxa, *Staphylococcus* spp., *Bacillus* spp., *B. pumilus*, *B. subtilis,* and *S. epidermidis-hominis* were the taxonomic

Figure 2. UPGMA-based hierarchical clustering and heatmap of antibiotic susceptibility values for isolated bacteria.



The left dendrogram shows taxonomic associations based on the phylogenetic tree inferred in this study. The tree distances indicate the taxonomic clustering of strains. The upper dendrogram indicates the hierarchical clustering of antibiotic susceptibility values with bootstrap node support (node cut-off < 50). The central heatmap indicates the different categorical values of susceptibility marked by coloured bars (white = undefined susceptibility, blue = sensitive, purple = intermediate, red = resistant). An MAR index heatmap is also presented using a two-bar scale. The main bar indicates the observed MAR index values, and the secondary bar details the MAR index values. TET = tetracycline, CIP = ciprofloxacin, ERY = erythromycin, CHL = chloramphenicol, GEN = gentamicin, AMK = amikacin, AMP = ampicillin, CEF = cephalothin, CTX = cefotaxime, DCX = dicloxacillin, CRO = ceftriaxone, NET = netilmicin, NIT = nitrofurantoin, PEN = penicillin, SXT = trimethoprim-sulfamethoxazole, R = resistant, I = intermediate, S = sensitive.

groups with the most extensive antibiotic resistance profiles (Figure 3).

Discussion

Dynamic movement in the hospital environment is a factor determining the transfer of pathogens and resistance genes, with hand contact being the principal means of transmission [5,8]. In the present study, the molecular identification and evaluation of the resistance to different antibiotics of bacteria isolated from the palmar area of patients' companions who were present in the waiting area of a hospital were performed.

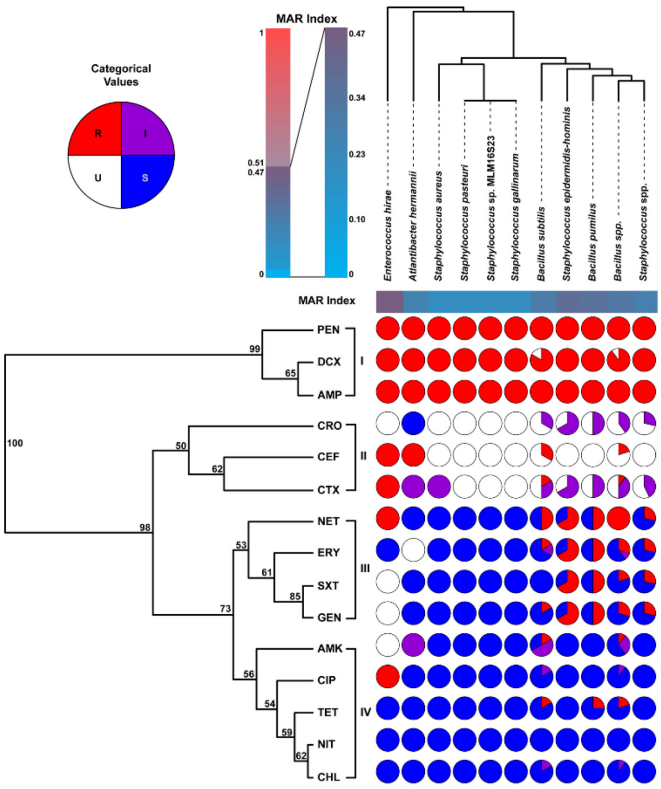
Phylogenetic analysis by Bayesian inference and ML generated a robust tree with high node support values, grouping similar sequences and indicating close relationships. The *Staphylococcus* group (clade I) showed species variability, highlighting *S. aureus* and the great diversity of coagulase-negative staphylococci. The topology was similar to that in previous reports using the complete 16S rRNA gene and other molecular markers (dnaJ, rpoB, and tuf gene fragments) [31]. The *Bacillus* group (clade II) displayed a dichotomy with the representative subclades *B. subtilis* and *B. pumilus*. This classification is consistent with that in previous reports [32,33]. In the *Enterococcus* group (clade III), the MLM22E08 strain shared high identity with the species *E. hirae, E. mundtii, E. durans,* and *E. faecium*. According to the complete analysis of the 16S rRNA gene of *Enterococcus*, this strain belongs to the same taxon as *E. faecium* [34]. In *Enterobacterales* (clade IV), the MLM33A13 strain had particularly high homology with the *A. hermannii* CIP strain. Recently, this strain was identified as a new genus within the family through concatenation of multilocus 16S rRNA and atpD-gyrB-infB-rpoB [35]. Meanwhile, two strains showed low percent identity and were paraphyletic within the *Staphylococcus* clade (MLM16S23) and *B. subtilis* subclade (MLM18B10). Based on these criteria, these strains could be new bacterial taxa within their genera.

The bacterial diversity found herein is consistent with that described in previous scientific literature, both on the hands of health personnel [4,6,10] and in the community [9,36]. Similar to findings of Ragusa *et al*. [13] and Cohen *et al*. [11], who evaluated surfaces that visitors regularly come in contact with, much of the bacterial load detected in this study corresponded to the normal hand microbiota. Nevertheless, in the literature, commensal species also correspond to principal opportunistic and nosocomial pathogens, such as *S. aureus* and *S. epidermidis*, and transitory bacteria, such as *Enterobacteriaceae* [1,2].

In this study, antibiotic susceptibility testing showed that 100% of the strains were resistant to AMP, while 94.7% were resistant to DCX and PEN. These drugs represent the first line of treatment for infections in primary care, so this result has implications for the treatment of infections caused by these resistant bacteria [2,37,38].

The resistance shown by species of clinical importance, such as *S. aureus*, *S. epidermidis*, *Enterococcus* species, and the transitory *Atlantibacter* species, varied among groups of antibiotics. The strain

Figure 3. UPGMA-based hierarchical clustering and frequency heatmap of antibiotic susceptibility values for isolated bacteria.



The left dendrogram indicates the hierarchical clustering of antibiotic susceptibility values with bootstrap node support (node cut-off < 50) and antibiotic resistance patterns (I-IV). The upper dendrogram show the clustering of taxa by susceptibility profiles. The circles on the heatmap indicate the relative frequencies of each susceptibility value shown by the upper-left circle (white = undefined susceptibility, blue = sensitive, purple = intermediate, red = resistant). An MAR index heatmap is also presented with a two-bar scale. The main bar indicates the MAR index values observed, and the secondary bar shows details of these values. I = resistance pattern to penicillins; II= resistance pattern to cephalosporins; III = resistance pattern to NET, ERY, SXT, and GEN; IV = resistance pattern to AMK, CIP, TET, NIT, and CHL. TET = tetracycline, CIP = ciprofloxacin, ERY = erythromycin, CHL = chloramphenicol, GEN = gentamicin, AMK = amikacin, AMP = ampicillin, CEF = cephalothin, CTX = cefotaxime, DCX = dicloxacillin, CRO = ceftriaxone, NET = netilmicin, NIT = nitrofurantoin, PEN = penicillin, SXT = trimethoprim-sulfamethoxazole, R = resistant, I = intermediate, S = sensitive.

belonging to the *S. aureus* taxon (MLM5S094) showed high sensitivity, except against the PEN group, a result that is consistent with the findings of Sánchez *et al*. [39], who conducted a community susceptibility study where the *S. aureus* strain was found to be sensitive to most drugs, except for TET. In contrast, susceptibility studies of *S. aureus* strains isolated from clinical samples showed multiresistance patterns [9,40].

The strains associated with the *S. epidermidis* group represent an important reservoir of mobile genetic elements that can cause resistance to not only β-lactam antibiotics but also other antibiotic families and can be transferred to related species, such as *S. aureus*, leading to the emergence and spread of resistant strains [41]. A multiresistance profile has been observed in isolates from both clinical samples [42-44] and environmental samples [14]. The ubiquity of *S. epidermidis* and other coagulase-negative staphylococci is attributed to the fact that these bacteria are part of the skin microbiota and are easily disseminated; this also positions these bacteria among the most prevalent nosocomial pathogens [15].

Similarly, six strains of the *Bacillus* group (MLM8B096, MLM14B99, MLM18B10, MLM30B24, MLM23B07, and MLM25B06) showed a multiresistance profile, exhibiting varying resistance patterns, a phenomenon that has also been reported by other authors [45,46]. Although the species found in this study (*B. subtilis* and *B. pumilus*) are infrequent pathogens, there is evidence of infections caused by these bacteria in immunocompromised individuals [47], in addition to their being considered a possible source of antibiotic resistance gene transfer [46].

On the other hand, *Enterococcus* species and members of the *Enterobacteriaceae* family are bacteria that belong to the normal microbiota of the human and non-human animal intestine, and other media behave as potential reservoirs for colonization by these bacteria via faecal contamination [48]. These bacteria have shown an increase in antibiotic resistance secondary to various intrinsic and acquired mechanisms that predispose them to antibacterial evasion. The most common mechanisms regarding intrinsic resistance include PEN-binding proteins for *Enterococcus* and β-lactamases, such as those encoded by *ampC* genetic determinant in *Enterobacteriaceae* [49,50]. In this study, the multi-drug-resistant strains of *Enterococcus* (MLM22E08) and *Atlantibacter* (MLM33A13) showed no susceptibility to PEN, cephalosporin, and aminoglycoside antibiotics, and MLM22E08 additionally showed resistance to a fluoroquinolone (CIP). Members of both groups, mainly isolates from patients with urinary tract infections, have been reported to have high resistance to PENs and aminoglycosides [51-53]. Likewise, resistant *Enterococcus* strains have also been found to be potential contaminants from the hands of health personnel, fomites, and hospital surfaces [10].

In general, 52.6% of the strains in this study showed multidrug resistance. MAR indexes were found to be ≥ 0.20, not exceeding 0.53 for strains and 0.47 for taxonomic groups. A value of 0.20 is associated with high-risk sources for which antibiotic use is predominant, and ≥ 0.40 is associated with human faecal contamination [29]. Notoriously, hospital settings are places of constant antibiotic use against a wide range of bacterial organisms, which generates selective pressure over multiple antibiotic-resistant organisms, enhancing the emergence of difficult-to-treat nosocomial infections and the potential dissemination of these bacteria from the hospital to the community [54]. Although we observed antibiotic-resistant bacteria on the palmar skin surfaces of patients' companions in a hospital, we did not necessarily determine the real origins of these bacteria, which gives rise to the following question: Did these bacteria emerge from the hospital environment, or were they introduced by the companions from the community? The importance of antibiotic resistance and its spread is increasingly being recognized, both in clinical settings and in the community [55]. The evolution of antibacterial-resistant bacteria is attributed to a multitude of factors, highlighting the widespread use of antimicrobials and their inappropriate applications, such as self-medication [8,38,56]. Control of the transmission of pathogens is based on compliance with preventive measures such as hand hygiene, use of protective equipment, cleaning and decontamination [57]. Programmes have been established in hospitals for the implementation of various prevention practices; however, these programmes are aimed primarily at healthcare workers [58], while the policies aimed at visitors are not as rigorous. Furthermore, individuals sometimes show hostility and refuse to comply with recommendations for prevention [59], or there is a lack of awareness regarding the importance of compliance with control and hygiene practices [60].

Importantly, this research highlighted bacteria resistant to antibacterial drugs, mainly PENs, that are commonly part of the normal microbiota of people who accompany patients in medical consultations. Thus, these people could be carriers and transmitters of opportunistic pathogens.

Conclusions

The dissemination of bacteria resistant to antibiotics by patients' companions in hospitals is a dynamic process that could contribute to the increased burden of nosocomial infections as well as to their failed treatment. Specifically, the presence of the hospital-associated pathogens *Enterococcus*, coagulase-negative *Staphylococcus,* and *Enterobacteriaceae* with well-known patterns of antibiotic resistance, mainly to beta-lactams and, to a lesser extent, aminoglycosides and folate pathway antagonists, residing on the palmar surfaces of visitors creates an unnoticed vehicle for the spread of these germs and their subsequent antibiotic resistance. This increases antibiotic resistance in clinical and societal settings, as patients' companions return to the community from hospitals upon concluding their visit. The dissemination and acquisition of these bacterial organisms show the need for visitors to practice adequate control and hygiene measures, including hand, common-use objects, and surface decontamination, to reduce the spread of these bacteria and their antibiotic resistance mechanisms inside and outside the hospital environment.

Furthermore, this research provides evidence of potential new bacterial taxa that reside on the palmar skin surface and could be found in hospital environments, functioning as potential novel agents of nosocomial infections. Future research on the dynamics of bacterial shedding in hospitals should address this previously overlooked issue.

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Authors' Contributions

SCL contributed to the study design, data analysis, interpretation and writing of the first and subsequent drafts of the manuscript. MJCP and LJDD contributed equally to experimental activities, data analysis, interpretation and writing of the first and subsequent drafts of the manuscript. JTGG contributed to the design and performance of phylogenetic analyses, data analysis, interpretation and writing of the first and subsequent drafts of the manuscript. LIPG contributed to data analysis, interpretation and writing of the first and subsequent drafts of the manuscript.

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