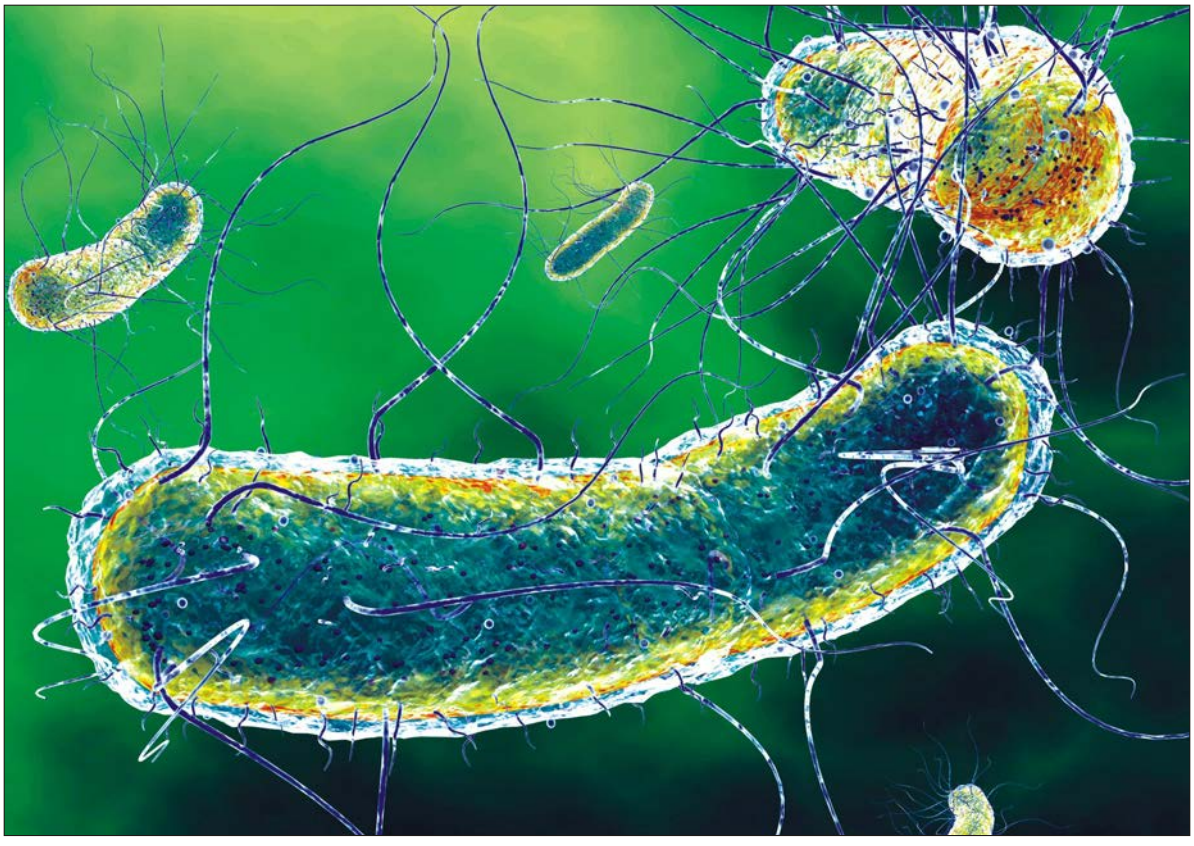

Current Research in Bacteriology

ISSN 1994-5426

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OPEN ACCESS Current Research in Bacteriology

ISSN 1994-5426
DOI: 10.3923/crb.2017.1.8

Research Article
Species Identification of Clinical Coagulase-negative Staphylococci Isolated in Al-Shifa Hospital Gaza using Matrix-assisted Laser Desorption/Ionization-time of Flight Mass Spectrometry

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Abstract

Background: Staphylococcal species, notably, coagulase negative staphylococci (CoNS) are considered important causative agents of hospital-acquired infections (HAI) associated with catheters and implanted medical devices. The increasing incidence of CoNS in HAI confirms the need for an accurate and simple identification method at the species level. Here, the capacity of MALDI-TOF MS was compare in comparison of VITEK-2 and ID32STAPH to accurately identify clinical CoNS species and to determine its ability to correctly distinguish between methicillin resistant CoNS (MR-CoNS) and methicillin sensitive CoNS (MS-CoNS). **Materials and Methods:** In this study, MALDI-TOF MS, VITEK-2 and ID32STAPH was compared for phenotypic identification of CoNS at species level. Eighty one clinical isolates of CoNS representing six species that collected from different clinical samples were analyzed. All CoNS isolates were tested by catalase, Pastorex™, coagulase, *nuc* and *mecA* genes. Statistics were performed with Statistical Package for Social Sciences (SPSS®) program version 20 (Chicago, IL, USA). **Results:** Our results showed correct species identification by MALDI-TOF MS was obtained in 98.8% in comparison to 96.3 and 75.3% correct identification for the VITEK-2 and ID32STAPH, respectively. Only one strain was identified by MALDI-TOF MS at the genus level. In addition, MALDI-TOF MS identified staphylococcal to subspecies level including *Staphylococcus hominis* subsp., *novobiosepticus* and subsp., *hominis*, *S. saprophyticus* subsp., *saprophyticus*. Also the single strain of *S. auricularis* was only identified by MALDI-TOF MS. There were 60 (74.1%) MR-CoNS that showed *mecA* positive and 21 (25.9%) were MS-CoNS. The MALDI-TOF MS technique was unable to discriminate between MR-CoNS and MS-CoNS, because the topology of dendrogram generated from the spectra of MR-CoNS and MS-CoNS strains were almost the same. **Conclusion:** These results confirm the value of MALDI-TOF MS as simple, accurate and rapid method for phenotypic identification of clinical CoNS isolates. However, this technique was unable to discriminate between MR-CoNS and MS-CoNS strains.

Key words: Coagulase negative staphylococci, species identification, MALDI-TOF MS, VITEK-2, ID32STAPH

Received: September 03, 2016 Accepted: October 14, 2016 Published: December 15, 2016

Citation: Nahed Ali Al Laham, 2017. Species identification of clinical coagulase-negative staphylococci isolated in Al-Shifa Hospital Gaza using matrix-assisted laser desorption/ionization-time of flight mass spectrometry. Curr. Res. Bacteriol., 10: 1-8.

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Competing Interest: The author has declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Hospital-acquired infections associated with catheters and implanted medical devices are most commonly caused by staphylococci, specially coagulase-negative staphylococci (CoNS). They are responsible for bacteremia, endocarditis, mediastinitis, meningitis and progressive joint destruction. Current methods for species identification are slow, costly and sometimes unreliable. To date there are 45 different *Staphylococcus* species with 24 subspecies. The clinical most relevant CoNS are *Staphylococcus epidermidis*, *S. lugdunensis*, *S. saprophyticus* and *S. capitis*[1,2]. The *S. epidermidis* is probably the major CoNS species causing nosocomial infections, although, other CoNS, such as *S. lugdunensis*, *S. schleiferi* and *S. saprophyticus* have been reported as even more pathogenic in a range of both nosocomial and community acquired infections[3,4].

Identification of CoNS in the clinical laboratory can be problematic as many of the conventional methods and automated systems currently used do not always distinguish the different CoNS species reliably due to variable expression of biochemical characters. Moreover, molecular methods targeting the 16S rRNA, *hsp*60, *femA*, *rpoB*, *gap*, *tuf* and *sodA* are favored for diagnostic purposes but these methods often are expensive and time-consuming techniques[2-6]. Recently, matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has shown to be a useful and simple method for the rapid identification of CoNS isolated in a routine clinical microbiology laboratory[3-10]. Starting from whole cells, cell lysates or bacterial extracts, it is possible to identify CoNS to species level within a few minutes based on mass analysis of the protein composition of the bacterial cell between 2,000 and 20,000 Da. A high quality reference database of spectra generated from well characterized strains and advanced analysis software for spectral pattern matching are essential to obtain accurate species identification. The output form the MALDI Biotyper, a frequently used MALDI-TOF MS instrument from Bruker® is a log score in the range of 0-3.0, computed by comparison of the peak list for an unknown isolate with the reference main spectra (MSP), containing information on average peak masses, average peak intensities and peak frequencies in the database. Bruker database version 3.1.1.0 includes 120 spectra of 37 different CoNS species[11-15].

The aim of this study was to confirm the identification capacities of MALDI-TOF MS to accurately identify clinical CoNS species and to compare its capacity to the phenotypic VITEK-2 and ID32STAPH diagnostic methods. In addition, the study aimed to determine the ability of MALDI-TOF MS to distinguish between methicillin resistant CoNS (MR-CoNS) and methicillin sensitive CoNS (MS-CoNS).

MATERIALS AND METHODS

Bacterial isolates: For the present study, 81 unique non-duplicate CoNS isolates representing six species associated with diverse clinical infections that collected from different clinical samples including pus, urine, blood, wound, vaginal and ear swabs were collected from the largest public tertiary referral hospital (Al-Shifa Hospital) in Gaza. These isolates were collected in 2012, out of a total unique 1121 bacterial isolates (March 1-July 31). Isolates were obtained directly from the clinical laboratory of Al-Shifa Hospital and represent complete capture of all CoNS isolates during the stated collection period. Although, a defined sampling strategy was not employed, the collected strains likely reflect the clinical epidemiology of CoNS in Gaza, since Al-Shifa Hospital is the primary referral hospital for patients from all areas of the Gaza Strip. Well typed strains including nineteen reference strains were included (Table 1). Ethical approval from the Helsinki committee at the ministry of health in Gaza Strip (Approval No. PHRC/HC/36/15) was obtained for performing the current study.

Identification of CoNS and MR-CoNS: The CoNS isolates were identified phenotypically based on colonial morphology, Gram stain and by using the following methods: Catalase test, tube coagulase test, Pastorex™ Staph Plus latex agglutination (Bio-Rad, Hercules, California) and the Staph ID 32 API system (bioMérieux, France) according to the manufacturer's instructions.

Table 1: Included reference strains

Species or subspecies	Strain ID
S. auricularis	ATCC 33753
S. capitis subsp., *capitis*	ATCC 27840
S. capitis subsp., *urealyticus*	ATCC 49326
S. cohnii subsp., *cohnii*	ATCC 29974
S. cohnii subsp., *urealyticus*	ATCC 49330
S. epidermidis	ATCC 14990, ATCC 12228, ATCC 14990
S. haemolyticus	ATCC 29970
S. hominis subsp., *hominis*	ATCC 27844
S. hominis subsp., *novobiosepticus*	ATCC 700236
S. lugdunensis	ATCC 43809
S. pettenkoferi	CCUG 51270
S. saccharolyticus	ATCC 14953
S. saprophyticus subsp., *saprophyticus*	ATCC 15305
S. scheiferi subsp., *coagulans*	ATCC 49545
S. scheiferi subsp., *scheiferi*	ATCC 43808
S. simulans	ATCC 27848
S. warneri	ATCC 27836

For VITEK-2 system, bacterial suspensions were prepared by suspending bacterial isolates in 0.45% saline to the equivalent of a 0.5-0.63 McFarland turbidity standard. The ID-GP identification card is a 64-well plastic card that includes 43 tests. Data were analyzed using VITEK-2 database, version 4.03 (bioMérieux, Marcy l'Etoile, France), according to the manufacturer's instructions.

The identification of four CoNS isolates that gave discrepant results at the species level was obtained by sequencing an internal fragment of the *sodA* gene as previously described[16,17]. The partial *sodA* gene was amplified and the PCR product sequenced as previously described[17]. The nucleotide sequences were analyzed using the GenBank database of $sodA_{int}$ sequences of Staphylococcus type strains. A multiplex PCR assay was used for detection of the *nuc* gene to exclude *S. aureus* and *mecA* gene for detection of methicillin-resistance among CoNS isolates[18].

MALDI-TOF MS: All isolates were streaked from stocks on tryptic soy agar (Lab M, Lancashire, UK) with 5% horse blood (E and O, Bonnybridge, Scotland) (TSA+B) and incubated overnight at 37°C. The MALDI-TOF MS analysis was performed on a pure subculture on TSA+B incubated overnight at 37°C in a CO_2 incubator.

Only the direct transfer method was used. Briefly, one colony of each bacterial strain was spotted on a ground steel MALDI target plate. Each strain was spotted two times. Spots were allowed to dry at room temperature. Subsequently, 1 µL of matrix (Bruker Daltonik GmbH, Bremen, Germany), a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile (Sigma-Aldrich) and 2.5% trifluoro-acetic acid (Sigma-Aldrich) was added to the spot, which was dried again. Afterwards, the target plate was introduced into the MALDI-TOF MS. Samples were analyzed with a Microflex LT MALDI-TOF MS instrument (Bruker Daltonik GmbH). Parameter settings were Ion Source (IS) 1 20 kV, IS2 18.5 kV, lens 8.5 kV, pulsed ion extraction 250 nucleotides and no gating. For spectral calibration, the Bruker® bacterial test standard (*E. coli* lysate) was used weekly. The measurements were performed in the automated mode using the software wizard (flex control version 3.0, MALDI Biotyper version 2.0 and MALDI Biotyper Automation Control version 2.0). Species identification was carried out with library version 3.1.1.0 containing MSP of 3290 reference strains (1805 different species). Ranking tables containing the 10 best matching MSP, sorted according to decreasing log score values, were obtained with the wizard. The acceptance criteria for identification results generally cited in the literature were used: A log score $\geq$1.7 and $\geq$2.0, respectively are the thresholds for a match at the genus and the species level. Scores of <1.7 were considered an unacceptable identification, according to the manufacturer's recommendations. These thresholds were empirically determined[4].

Statistics: Statistics were performed with Statistical Package for Social Sciences (SPSS®) program version 20 (Chicago, IL, USA). A p-value of less than 0.05 was considered significant.

RESULTS

Using the three phenotypic methods, all isolates were identified at the species level except four isolates. Discrepant results (n = 4) were reanalysed with MALDI-TOF MS. For confirmed discrepancies of these four CoNS, the sequencing of the *sodA* gene was performed and allowed the species identification of these four CoNS clinical isolates.

Species identification of reference strains: Reference strains (n = 19) were used to confirm the capacities of the MALD-TOF MS technology for species identification (Table 1). For all tested species at least one reference spectra was present in the Bruker database. MALDI-TOF MS was 100% correct in assigning the species. However, out of the 19 reference strains, six (*S. auricularis*, *S. cohnii* subsp., *cohnii*, *S. pettenkoferi*, *S. saccharolyticus*, *S. schleiferi* subsp., *coagulans* and *S. schleiferi* subsp., *schleiferi*) were identified only up to genus level and no reliable result was obtained for *S. cohnii* subsp., *urealyticus*.

Species identification of clinical CoNS strains: Eighty one clinical CoNS isolates were identified using MALDI-TOF MS, VITEK-2 system and ID32STAPH as described previously. Correct identification (ID) results were obtained for 98.8% (80/81), 96.3% (78/81) and 75.3% (61/81) with MALDI-TOF MS, VITEK-2 system and ID32STAPH, respectively. The correct ID for both MALDI-TOF MS and VITEK-2 system was significantly higher than the correct ID of ID32STAPH. Misidentification (Mis-ID) rates were 1.2% (1/81), 2.5% (2/81) and 18.5% (15/81), respectively. The percentage of strains with no-identification (no-ID) was 0.0% (0/81), 1.2% (1/81) and 6.2% (5/81), respectively. A correct ID (100%) of all strains in all three systems was not achieved. However with MALDI-TOF MS and VITEK-2 systems, it was obtained for three species, *S. hemolyticus*, *S. epidermidis* and *S. warneri* (Table 2).

Only MALDI-TOF MS identified staphylococcal to subspecies level including *S. hominis* subsp., *novobiosepticus*

and subsp., *hominis* and *S. saprophyticus* subsp., *saprophyticus*. Also the single strain of *S. auricularis* was only correctly identified by MALDI-TOF MS and was confirmed by sequencing *sodA* gene PCR product. However, one of the *S. hominis* isolates could not be identified using MALDI-TOF MS.

For the CoNS species that most frequently cause diseases in humans or are isolated from human samples, *S. hemolyticus* (n = 33), *S. epidermidis* (n = 26), *S. saprophyticus* (n = 12) and *S. hominis* (n = 07), MALDI-TOF MS gave a correct identification in 98.7% (77/78), VITEK-2 system in 97.4% (76/78) and ID32STAPH in 78.2% (61/78) of these isolates (Table 2).

Differentiation of MR-CoNS from MS-CoNS strains using MALDI-TOF MS: According to the *mecA* PCR results there were 60 (74.1%) MR-CoNS and the rest (21 and 25.9%) were MS-CoNS. Figure 1 and 2 showed that, the topology of the dendrogram generated by the MALDI Biotyper 2.0 software from the spectra of MR-CoNS and MS-CoNS strains (with or

Table 2: Identification obtained with the matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS), VITEK-2 system and ID32STAPH for the 81 clinical coagulase-negative staphylococci (CoNS) isolates

Identification system										
		MALDI-TOF MS			VITEK-2 system			ID32STAPH		
*Species	No.	ID (%)	Mis-ID (%)	No-ID (%)	ID (%)	Mis-ID (%)	No-ID (%)	ID (%)	Mis-ID (%)	No-ID (%)
S. hemolyticus	33	33 (100)	-	-	33 (100)	-	-	29 (87.9)	3 (9.1)	1 (3.0)
S. epidermidis	26	26 (100)	-	-	26 (100)	-	-	23 (88.5)	3 (11.5)	-
S. saprophyticus	12	12 (100)	-	-	11 (91.7)	1 (8.3)	-	6 (50)	6 (50.0)	-
S. hominis	7	6 (85.7)	1 (14.3)	-	6 (85.7)	1 (14.3)	-	3 (42.9)	3 (42.9)	1 (14.3)
S. warneri	2	2 (100)	-	-	2 (100)	-	-	0 (0)	-	2 (100)
S. auricularis	1	1 (100)	-	-	0 (0)	-	1 (100)	0 (0)	-	1 (100)
Total strains	81	80 (98.8) [§]	1 (1.2)	-	78 (96.3) [§]	2 (2.5)	1 (1.2)	61 (75.3)	15 (18.5)	5 (6.2)

No.: Number of isolates, ID: Correct identification, Mis-ID: Misidentification, No-ID: No identification, *Four isolates of CoNS were identified at the species level by sequencing the *sodA* gene (reference standard), [§]Statistically significant difference in percentage of correct identification between the identification systems (p-value <0.05)

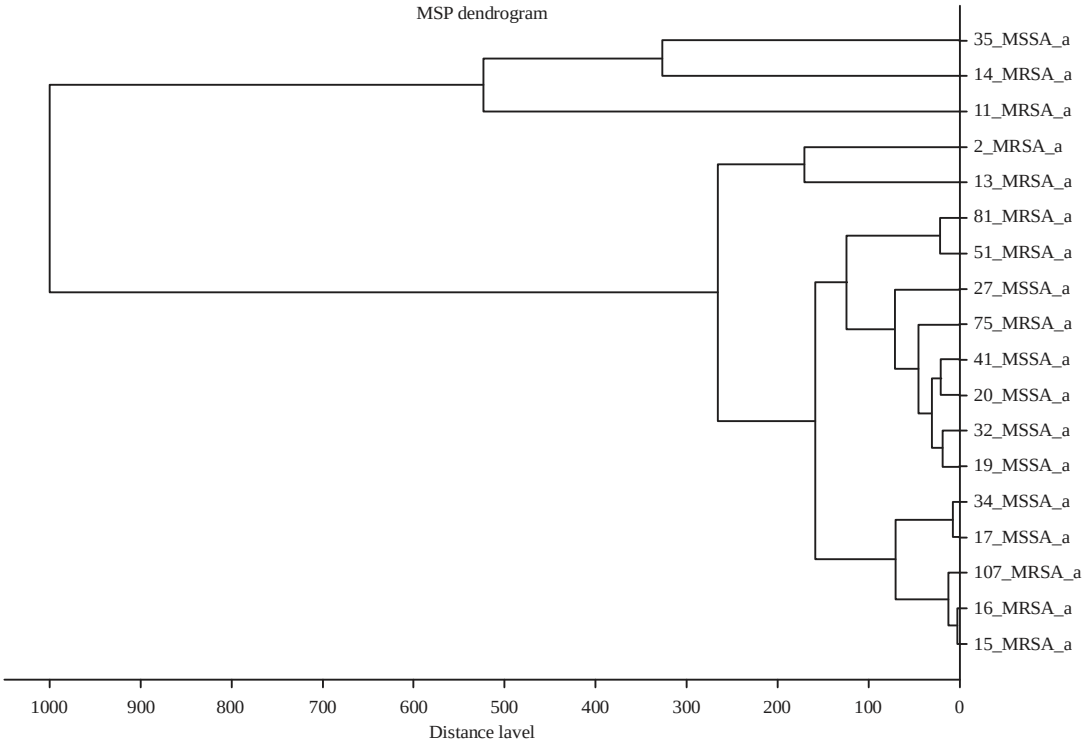

Fig. 1: Dendrogram representation of hierarchical cluster analysis that generated by the MALDI Biotyper 2.0 software from the spectra of selected MR-CoNS and MS-CoNS strains (without using formic acid as a matrix)

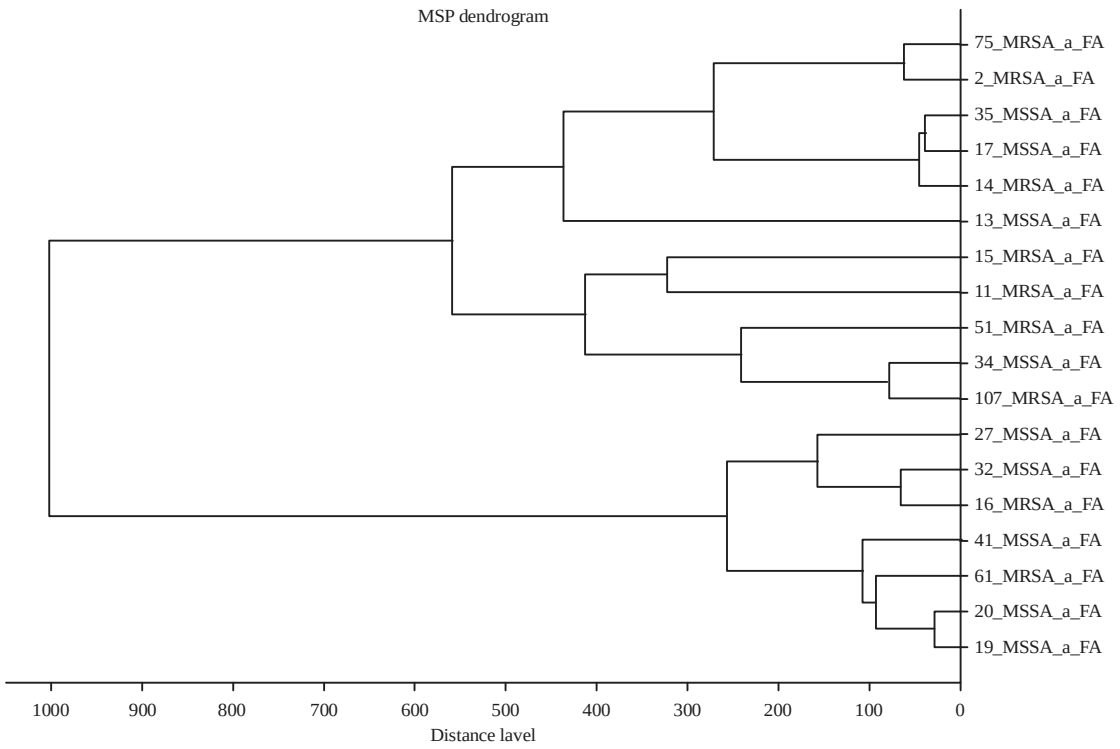

Fig. 2: Dendrogram representation of hierarchical cluster analysis that generated by the MALDI Biotyper 2.0 software from the spectra of selected MR-CoNS and MS-CoNS strains (with using formic acid as a matrix)

without using formic acid as a matrix) were almost the same which make us unable to discriminate between them.

DISCUSSION

The CoNS are normal inhabitants of the human skin that lately have revealed themselves as significant etiological agents causing nosocomial infections, particularly in medical devices. The use of MALDI-TOF MS for staphylococcal identification is now considered routine in laboratories compared with the conventional phenotypical methods previously used. This technique allowed the identification of approximately 85% of the CoNS strains, whereas only 14% of the CoNS strains were identified to the species level with phenotypic methods[4]. Also, MALDI-TOF MS had previously demonstrated its powerful tool for the identification of clinically relevant species of CoNS[3]. Bacterial identification is routinely achieved using phenotypically based techniques. However, those techniques remain time-consuming and sometimes of limited value[2-6], as for example for CoNS, where commercial identification kits identified only 37% of 177 CoNS isolates with the API 20 Staph system[3].

In this study and as first aim, we compared between MALDI-TOF MS, VITEK-2 and ID32STAPH for the identification of CoNS to the species level. Our results showed correct species identification by MALDI-TOF MS was obtained in 98.8% compared to 96.3 and 75.3% correct identification for the VITEK-2 and ID32STAPH respectively. Dupont *et al.*[2] achieved correct identification in 97.4, 79 and 78.6% using MALDI-TOF MS, phoenix and VITEK-2, respectively. In this study, Mis-ID rates were 1.2, 2.5 and 18.5%, respectively. The percentage of strains no-ID was 0.0, 1.2 and 6.2%, respectively. Comparable findings were achieved in Dupont *et al.*[2] study where they found that Mis-ID occurred in 1.3, 21 and 10.3% with MALDI-TOF MS, Phoenix and VITEK-2, respectively and the rates of no-ID results were low with all systems: 1.3, 0 and 0.9% with MALDI-TOF-MS, phoenix and VITEK-2, respectively. However, Delmas *et al.*[19] obtained a correct identification of only 71.2% for clinical and environmental CoNS with the VITEK-2 ID system and 82.5% when isolates belonging to species absent from the automate database were excluded. Nevertheless, overall identification accuracy for CoNS with VITEK-2 was in the range of 91-96[20-22]. In another study using MALDI-TOF MS, a total of 151 strains out of 152 (99.3%) CoNS were correctly identified at the species level and only one strain was identified at the genus level[6]. Spanu *et al.*[23] evaluated the use of MALDI-TOF MS for the identification of 450 blood isolates of the most relevant staphylococcal species,

using sequence analysis of the rpoB gene as the reference method. A correct species identification by MALDI-TOF MS was obtained in 99.3%, with only three isolates (0.7%) being misidentified. These findings are comparable to our results (correct species identification: 98.8%, misidentification: 1.2%).

In this study, MALDI-TOF MS correctly identified all six CoNS species to the species level, except for *S. hominis* (85.7%). Many studies showed comparable results, however, Dupont *et al.*[2] showed same results except for *S. hemolyticus* (85.8%). Only MALDI-TOF MS identified staphylococcal to subspecies level including *S. hominis* subsp., *novobiosepticus* and subsp., *hominis* and *S. saprophyticus* subsp., *saprophyticus*. Same results were obtained by Spanu *et al.*[23] where MALDI-TOF MS correctly identified all the staphylococcal subspecies studied, including *S. capitis* subsp., *capitis* and subsp., *urealyticus*, *S. cohnii* subsp., *urealyticus*, *S. hominis* subsp., *novobiosepticus* and subsp., *hominis*, *S. saprophyticus* subsp., *saprophyticus*, *S. schleiferi* subsp., *schleiferi* and *S. sciuri* subsp., *sciuri*[23].

The second aim of this report was to determine the ability of MALDI-TOF MS to correctly distinguish between MR-CoNS and MS-CoNS. The topology of dendrogram generated from the spectra of MR-CoNS and MS-CoNS strains were almost the same which make us unable to discriminate between them. The molecular available methods used to type strains, including pulsed-field gel electrophoresis and multilocus sequence typing are laborious and expensive to perform. Several studies have addressed the use of MALDI-TOF MS as a method for typing methicillin-resistant *S. aureus*[24-26], but there is no any previous report studying CoNS. While some studies showed the ability of MALDI-TOF MS to distinguish between methicillin-susceptible and resistant *S. aureus*[27,28], contradictory reports have been published on this issue[24,29]. Yet, the MALDI-TOF MS method revealed different clonal lineages of *S. epidermidis* that were of either human or environmental origin, which suggests that the MALDI-TOF MS method could be useful in the profiling of staphylococcal strains[6]. On the other hand, Bittar *et al.*[30] showed that MALDI-TOF MS can distinguish between positive and negative PVL and TSST *S. aureus* isolates by producing specific peaks of 4448 and 5302 m/z. But Szabados *et al.*[31] indicate that the peaks of 4448 and 5302 m/z are independent of the presence of PVL and TSST as they were found in all 104 MRSA strains with different PFGE in contrast to the aforementioned report. Moreover, they did not find evidence for an association of single protein peaks in the m/z range of 3000-10,000 Da with the presence of the toxins sea, seb, sec, sed, see, seg, seh, sei and sej. So, they suggest that a MALDI-TOF MS-based typing approach in PVL-positive *S. aureus* is clearly inferior and inapplicable compared to well-established molecular typing methods[31].

Finally and because MALDI-TOF MS is easy to perform, fast and relatively cheap, it is the preferred method for the species identification of clinical CoNS in routine clinical microbiology[5,11,12].

CONCLUSION

Many previously reported studies had demonstrated that MALDI-TOF MS is a powerful tool for the identification of clinically relevant species of CoNS and showed that it is a simple, accurate and rapid method for phenotypic identification of clinical CoNS isolates. Our results obtained in this study demonstrate the higher performance of MALDI-TOF MS in the identification of CoNS to the species level and even more to the subspecies level compared to VITEK-2 and ID32STAPH. However, this study revealed for the first time the inability of MALDI-TOF MS to correctly distinguish between MR-CoNS and MS-CoNS.

ACKNOWLEDGMENTS

The researchers would like to thank the German Academic Exchange Service (DAAD), Germany and the Erasmus Mundus External Cooperation Window (EM ECW lot III), Belgium, EU for support of my work during my visits to the University Clinic of Muenster, Germany and the Free University of Brussels, Belgium respectively. Also my thanks to the laboratory technologists at Al Shifa hospital for their kind help.

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