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Identification by MALDI-TOF Mass Spectrometry of Bacteria in Air Samples in a Biosafety Level 2 Laboratory

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Abstract: The implementation of an environmental monitoring program in a biosafety level 2 laboratory was evaluated in a multinational manufacturer of personal care products in Querétaro State, México. A total of six sites were monitored in the facility and microbiological air samples were collected. Nineteen bacterial genera were identified by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) and the most prevalent genera identified were *Staphylococcus aureus*, *Micrococcus luteus*, *Microbacterium oleivorans*, *Kocuria rosea*, *Citricoccus sp*. and *Arthrobacter Phenanthren ivorans*. The results showed that Gram negative bacteria, such as *Pseudomonas sp*. and *P. perfectomarina* were highly resistant to all the antibiotics tested, as were the Gram positive species, *Staphylococcus cohnii* and *S. xylosus*. Our results strongly suggest that bacterial characterization is important for this environmental monitoring program to assure the maintenance of acceptable air quality conditions.

Key words: multi-drug resistance, MALDI-TOF mass spectrometry, bacteria, multinational manufacturer, environmental monitoring program

1. Introduction

The global beauty market is a multibillion dollar industry. Personal care products, such as skin moisturizers, perfumes, lipsticks, fingernail polishes, eye and facial makeup preparations, shampoos, hair colors, tooth pastes, deodorants, etc., are required to be safe when consumers use them according to specifications on the labels [1]. Such products must satisfy the regulatory requirements for cosmetics or drugs. Generally, drugs must either receive premarket approval by the U.S. Food and Drug administration, by COFEPRIS in Mexico, or by the appropriate regulatory agencies in other countries.

Product testing is one way in which a multinational manufacturer might endeavor to ensure the safety of a cosmetic or drug product. Diverse microorganisms have the ability to grow in cosmetic products and those organisms may cause spoilage or chemical changes and injury to the consumers [2]. Microorganisms may be opportunistic pathogens, so that in the cosmetic industry microbial monitoring should include *Staphylococcus aureus*, *Streptococcus pyogenes*, *Pseudomonas aeruginosa*, *Candida albicans* and other potentially pathogenic microorganisms [2]. Standard microbiological activities involving infectious microorganisms should be conducted in a biosafety laboratory to minimize the risk of contamination.

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Normally, the cosmetic industry uses a biosafety level 2 laboratory for microbiological work, to comply with governmental regulations. Control and management of the biosafety level 2 laboratory is necessary to minimize the possibility of microbial contamination of cosmetic or drug products [3]. Most of the research on the distribution and spread of Gram positive and Gram negative bacterial strains found in indoor air samples has focused on efficient methods for classification and identification of those strains [4, 5]. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) has become an important technology and offers an attractive alternative to traditional laboratory protocols in industry for classification and identification of microorganisms [6-9]. The aim of the present study was to evaluate an environmental monitoring program in a multinational manufacturer of personal care products in Querétaro State, México to gain insight about the bacterial diversity and multi-drug resistance in air samples collected in a biosafety level 2 laboratory. To do this, we isolated and identified bacterial strains through MALDI-TOF MS.

2. Methods

2.1 Isolation and Identification of Bacteria

We collected the air samples in a biosafety level 2 laboratory of a multinational manufacturer of personal care products in Querétaro State, México. Six areas were sampled and the samples were designated EC8 (general material storage area), EC28 (culture media storage area), A32 (culture media preparation room), A33 (personal care products storage area), A34 (incubation room) and A35 (general work area). Active air sampling was accomplished with a SAS Super 100 & 180 microbial air sampler, where airflow is directed onto a standard Petri dish containing soya agar media. Bacterial isolates were identified by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry and when an organism could not be identified by referencing the appropriate database it was identified by analyzing 16S ribosomal deoxyribonucleic acid (rDNA) sequences. MALDI-TOF mass spectrometry was performed with a MicroFlex LT mass spectrometer (Bruker Daltonics, Germany) according to the manufacturer's protocols. Spectra were analyzed by using Bruker Biotyper 2.0 software and library (version 2.0, 3,740 entries; Bruker Daltonics). The identification score criteria used were those recommended by the manufacturer: a score ≥ 2.000 indicated species-level identification, a score between 1.700 and 1.999 indicated identification at the genus level, and a score < 1.700 was interpreted as no identification.

For molecular characterization of 16S ribosomal deoxyribonucleic acid (rDNA) sequences, the bacterial strains were grown in liquid soya medium at 35°C overnight and harvested and processed for DNA extraction using standard procedures. Amplification of a 16S rDNA gene sequence was performed by polymerase chain reaction (PCR) with the conserved eubacterial primers forward (fD1) and reverse (rD1) [10]. The reactions were performed in 30 µl volumes with PCR Master Mix (2X) (Fermentas, Lithuania). The amplification conditions using a C1000 Thermal Cycler (Bio-Rad Laboratories, Inc., Berkeley, CA, USA) were as follows: 94°C for 2 min, 30 cycles of 94°C for 30 s, 45°C for 40 s, and 72°C for 2 min, with a final 5 min elongation step at 72°C. The amplification products were purified using the DNA clean concentrator-5 kit (Zymo Research, CA, USA) according to the specifications of the manufacturer. Sequencing reactions were performed by the Macrogen Korea Institute (Seoul, Republic of Korea). The 16S rDNA sequences obtained were aligned against nucleotide sequences obtained from GenBank [11] and the Ribosomal Database Project (RDP) [12] using the ClustalX2 method [13].

The antibiotic susceptibility of each strain was determined by growing each strain in liquid soya medium. Cultures were incubated with shaking at 37°C until the suspension reached an optical density (OD)

between 0.4 and 0.5 at 600 nm. A total volume of 100 µl of each strain was spread in agar soya medium. Sterile filter paper discs (6 mm in diameter) impregnated with 5 µl of each antibiotic were placed on the agar plate and incubated overnight at 37°C. After overnight incubation, the diameter in mm of the inhibitory or clear zones around the disc was recorded. All tests were performed in triplicate and the antibacterial activity was expressed as the mean of inhibition diameters (mm) produced. The results were normalized (n/antibiotic with maximum mean of inhibition diameter produced, where n is the mean of inhibition diameters produced for each antibiotic). Values between 0 to 1 represents some grade of inhibition diameter produced, where green color represents maximum resistance (value of 0) and red color represents maximum sensitivity (value of 1) and cluster analyses were done using the software R package, version 3.0.2. Where a value of 0 (green colour) indicates resistance and a value of 1(red color) indicates sensitive. The following antibiotics and concentrations were used: (Am) ampicillin (2, 10 and 15 µg/mL), (Cb)carbenicillin (50, 100 and 150 µg/mL), (Km) kanamycin (5.20 and 50 µg/mL), (Rf) rifampicin (20, 40 and 80 µg/mL), (Cm) chloramphenicol (5, 25 and 50 µg/mL), (Pb) polymyxin (5, 10 and 15 µg/mL), (Sp) spectinomycin (25, 50 and 75 µg/mL), (St) streptomycin (150, 300 and 450 µg/mL) and (Tc) tetracycline (20, 40 and 80 µg/mL) . The sensitivity and resistance of each isolate were determined by the criteria of the National Committee for Clinical Laboratory Standards (1999).

3. Results

To determine the level of bacterial diversity in the biosafety level 2 laboratory and to examine whether that diversity was associated with multidrug resistance, we collected air samples from six sites in the facility. The identification results obtained are shown in Fig. 1. We identified nineteen bacterial genera from all the sampled sites. Gram positive genera isolated were *Agrococcus, Arthrobacter, Bacillus, Chryseomicrobium, Citricoccus, Corynebacterium, Exiguobacterium, Kocuria, Kytococcus, Microbacterium, Micrococcus, Planococcus, Planomicrobium, Sanguibacter,* and *Staphylococcus*. Gram negative genera isolated were *Pointibacter, Pseudomonas, Psychrobacter* and *Skermanella*. In total, 85% of bacterial genera were identified by MALDI-TOF mass spectrometry and 15% of bacteria genera by 16S rDNA sequencing. Taxonomic analysis indicated that the genus *Bacillus*, represented by five species, was most common, followed by *Staphylococcus* with four species, and *Arthrobacter* and *Kocuria* with three species.

The relative prevalence of the bacterial species is presented in Fig. 1. The most prevalent genera identified across all the sampled sites were *Staphylococcus aureus* and *Micrococcus luteus*. Some

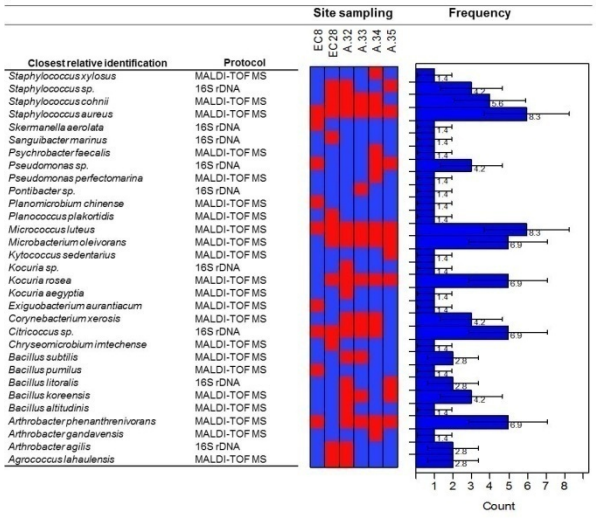

Fig. 1 The relative prevalence of bacterial diversity for each sampled site. Six areas were sampled and designated EC8 (general material storage area), EC28 (culture media storage area), A32 (culture media preparation room), A33 (personal care products storage area), A34 (incubation room) and A35 (general work area).Protocols of bacterial identification were MALDI-TOF MS and 16S rDNA. Red colour means presence of microorganism. The numbers at the right of the frequency diagram is percentage of total frequency relative. The lines at the right of the frequency diagram are error bars.

other bacterial genera, such as *Microbacterium oleivorans, Kocuria rosea, Citricoccus* sp. and *Arthrobacter phenanthren ivorans* were observed predominantly at five sampled sites. *Staphylococcus cohnii* was observed at four sampled sites. The prevalent genera identified in at least three site samples were *Staphylococcus* sp., *Pseudomonas* sp., *Corynebacterium xerosis* and *Bacillus korensis*. The prevalent genera identified in at least two site samples were *Bacillus subtilis*, *Bacillus litoralis*, *Arthrobacter agilis* and *Agrococcus lahaulensis*. A high percentage (51.6%) of the total number of genera was found in at least one site sample. Although the results show differences in bacterial diversity, the presence of multiple species for a given genus is the true determinant of the bacterial diversity. For example, site A32 shows the highest bacterial diversity with nine genera and seventeen species, followed by site A34 with nine genera and thirteen species. Site EC28 presented ten genera and twelve species while site A33 presented nine genera and eleven species and site A35 presented eight genera and ten species. Although the results show that the site EC8 with lower bacterial diversity, it presented nine genera similar to A32, but has only nine species.

Considering the bacterial identification results above, and the fact that microbes play an important role as opportunistic pathogens, we decided to investigate the potential impact of the isolated bacteria as multi-drug resistant organisms. Representative strains for each genus were analyzed. We first selected nine antibiotics with different intracellular targets, as explained in materials and methods. We obtained 837 antibiotic profiles and cluster analyses were done using the software R package, version 3.0.2. The results, presented as a heatmap (Fig. 2), show interesting associations between antibiotic resistance, genus and species. We observed five clusters of bacterial genera whose component species showed similar antibiotic responses. Group I species showed high resistance to polymyxin, spectinomycin, kanamycin,

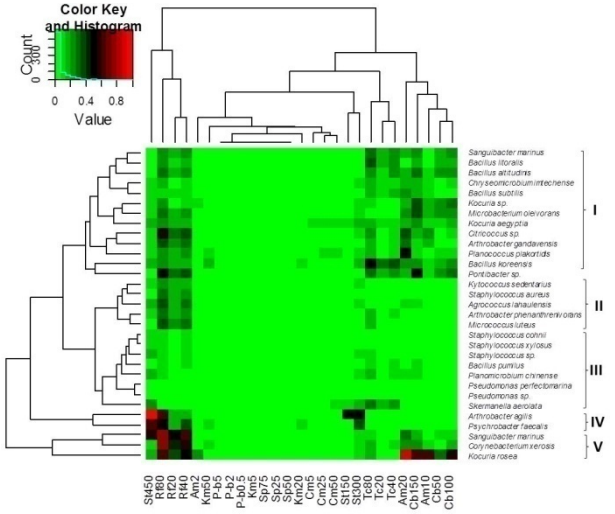

Fig. 2 Heatmap diagram of the antibiotic sensitivity results. The hierarchical clustering was based on 837 differential antibiotic profiles as described in Materials and Methods. The results were normalized (n/antibiotic with maximum mean of inhibition diameter produced, where n is the mean of inhibition diameters produced for each antibiotic).Values between 0 to 1 represents some grade of inhibition diameter produced, where green color represents maximum resistance (value of 0) and red color represents maximum sensitivity (value of 1). The bacterial strains tested are represented on the right of the vertical axis and antibiotic concentrations on the bottom of the horizontal axis.

chloramphenicol and streptomycin (green colour). On the other hand, Group I species were sensitive to rifampicin, tetracycline, ampicillin and carbenicillin. Group II and III species showed highest resistance to the antibiotics we tested. It was, however, somewhat surprising to observe that Gram negative bacteria, such as *Pseudomonas sp* and *P. perfectomarina* showed resistance to all antibiotics tested. Of the Gram positive species, *Staphylococcus cohnii* and *S. xylosus* showed highest resistance to the antibiotics. Group II species showed higher sensitivity to rifampicin than Group III.

In Groups IV and V; *Kocuria rosea, Corynebacterium xerosis, Sanguibacter marinus, Arthrobacter agilis* and *Psychrobacter faecalis* showed significant sensitivity to rifampicin. *Kocuria rosea* was the species that exhibited the greatest sensitivity to ampicillin and carbenicillin. The Gram positive species *Arthrobacter agilis* was the only one

to show sensitivity to all concentrations of streptomycin, while *Psychrobacter faecalis* showed slightly less streptomycin sensitivity.

4. Discussion

The results obtained from the active air sampling and identification by MALDI-TOF MS and 16S rDNA gene sequencing provided us with information about the bacterial diversity in a biosafety level 2 laboratory. The bacterial diversity encountered during our study appeared to be in agreement with previous reports on other indoor environments [18, 19], where the most frequently found microorganisms belonged to *Staphylococcus, Micrococcus, Microbacterium, Kocuria, Citricoccus, Arthrobacter* and *Bacillus* genera. However, we found that the most prevalent genera identified for all the sampled sites were *Staphylococcus aureus* and *Micrococcus luteus*, and the culture media preparation room (site A32) showed the highest frequency bacterial diversity (Fig. 1). It is important to note that our analysis of nineteen bacterial genera identified from all the sampled sites may have underestimated the extent of the bacterial diversity in the air samples we tested because of the strong likelihood that many of the species in those samples could not be grown in culture [14, 15]. A limitation of our study was that air samples were only tested for bacteria, but we did not calculate the number of isolates to know measures of species abundance for each sampled sites.

Recent developments in the field of environmental microbiology are the reasons for the establishment of environmental monitoring programs to assess and manage the risks from exposure to biological agents inside of biosafety level 2 laboratory, where is suitable for work involving agents that pose moderate hazards to personnel and the environment. Our study and others shows that accurate species identification is critical not only for the maintenance of environmental monitoring programs, but also to mitigate the risk presented by potential human pathogens with antibiotic multi-resistance in indoor environments [16-19].

Our results of bacterial identification have revealed, for example, that diverse physiologically versatile species that can be isolated from various environment [20-23]. *Agrococcus lahaulensis*, *Bacillus altitudinis*, *Citricoccus sp*, *Chryseomicrobium imtechense*, *Planomicrobium chinense*, *Planococcus plakortidis, Pointibacter sp.*, *Sanguibacter marinus* and *Skermanell aaerolata* have been isolated from diverse environments but have not yet been demonstrated to have beneficial industrial uses or to represent potential human pathogens [24-32].

However, our results of bacterial identification and antibiotic multi-resistance have revealed other species found in industrial environments or indoor environments as potential human pathogens, for example;*Bacillus pumilus, Kocuria*, *Micrococcus luteus*and *Staphylococcus* species [33-39]. Similarly, *Arthrobacter* species have been isolated from veterinary clinical sources [40, 41]. *Corynebacterium xerosis* is considered a commensal bacterium of human skin and mucous membranes and has also been isolated from veterinary sources, but its potential role as a pathogen has not been established [42]. *Kytococcus sedentarius* is usually part of the human skin flora and has on rare occasions been associated with serious infections [43, 44]. *Psychrobacter* and *Exiguobacterium* species have been isolated from the environment and are clinically not very relevant [45-47]. *Pseudomonas* species have also been isolated as opportunistic pathogens from humans [48, 49]. A striking feature of these species is their significant resistance to multiple antimicrobial agents. This resistance has been shown to result from the presence of multiple genetic determinants, such as three-component efflux systems of broad substrate specificity [48, 50-53]. It is noteworthy that in our study *Pseudomonas sp.* and *Pseudomonas perfectomarina* were the strains with the highest levels of multiple drug resistance (Fig. 2).

In conclusion, MALDI-TOF MS was used as a method for providing accurate and fast environmental species identification that can be used for routine detection with a cost-effective result in the biosafety level 2 laboratory in a multinational manufacturer of personal care products in Querétaro State, México. Multi-drug resistance microorganisms may be useful as indicators of potential health risk during an environmental monitoring program and by providing a massive reservoir of genetic information with important implications for the transfer of multi-drug resistance determinants in opportunistic bacteria. Our results suggest that microbial characterization is important for this environmental monitoring program to assure the maintenance of acceptable air quality conditions and more efforts are necessary to understand processes and risk management measures.

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