Marine Pollution Bulletin xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Marine Pollution Bulletin

journal homepage: www.elsevier.com/locate/marpolbul

Characterization of *Enterococcus* species isolated from marine recreational waters by MALDI-TOF MS and Rapid ID API® 20 Strep system

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ARTICLE INFO

Article history:
Received 19 August 2016
Received in revised form 13 March 2017
Accepted 14 March 2017
Available online xxxx

Keywords:
Enterococci
Species identification
Gram-positive
Beaches
16S rRNA gene sequencing
MALDI-TOF MS

ABSTRACT

MALDI-TOF Mass Spectrometry Biotyping has proven to be a reliable method for identifying bacteria at the species level based on the analysis of the ribosomal proteins mass fingerprint. We evaluate the usefulness of this method to identify *Enterococcus* species isolated from marine recreational water at Brazilian beaches. A total of 127 *Enterococcus* spp. isolates were identified to species level by bioMérieux's API® 20 Strep and MALDI-TOF systems. The biochemical test identified 117/127 isolates (92%), whereas MALDI identified 100% of the isolates, with an agreement of 63% between the methods. The 16S rRNA gene sequencing of isolates with discrepant results showed that MALDI-TOF and API® correctly identified 74% and 11% of these isolates, respectively. This discrepancy probably relies on the bias of the API® has to identify clinical isolates. MALDI-TOF proved to be a feasible approach for identifying *Enterococcus* from environmental matrices increasing the rapidness and accuracy of results.

1. Introduction

Measurement of *Enterococcus* levels in waters is a traditional method used worldwide for quality assessment of recreational bathing water. However, the recommended *Enterococcus* measurement methods generally used such as EPA (Environmental Protection Agency) Method 1600 (USEPA, 2006) and Enterolert (IDEXX, Westbrook, ME, USA) are not designed to identify environmental *Enterococcus* species included in the Enterococci group that comprises approximately 23 species (Fergunson and Signoretto, 2011). *Enterococcus* spp. are Gram-positive, aerotolerant anaerobes and are present in high numbers (between 10^4 and 10^6/g wet weight) in the intestines of birds and mammals. Nevertheless, not all enterococci have a fecal link, and several species are associated with plants (Boehm, 2014).

It has been suggested that some *Enterococcus* species, such as *E. mundtii*, *E. casseliflavus* and *E. sulfurous,* are more abundant in environmental reservoirs, while *E. faecium* and *E. faecalis* may be more prevalent in human feces and urban sewage than other enterococcal species, appearing to be truly fecal indicators (Manero et al., 2002; Tyrell et al., 2002; Boehm, 2014). More recently, *E. casseliflavus* has been identified as the most common enterococcal species present in urban runoff (Fergunson et al., 2005; Moore et al., 2008), while *E. hirae* and *E. gallinarum* were linked to fecal pollution derived from pig farming activities as discussed elsewhere (Manero et al., 2002).

Currently, enterococci monitoring levels subsidize the assessment of the potential presence of pathogens in waters, being widely used in drinking water microbiology and in the sanitary analysis of recreational waters (CONAMA, 2012; USEPA, 2012). Usually, enterococci analysis made by standard water laboratories uses selective culture media, such as mEI (*Enterococcus* Indoxyl-β-D-Glucoside Agar) or Enterolert, which are able to quantify the presence of *Enterococcus* spp. isolates. It has been noticed that up to 10% growing colonies onto culture media plates do not belong to *Enterococcus* genus (Fergunson et al., 2010). Thus, identification of the growing colonies must be reassessed and standard procedures such as biochemical tests, like bioMérieux API® 20 Strep system or 16S rRNA gene sequencing has been used for identification of *Enterococcus* spp. at the species level.

Biochemical systems are limited in the sense that they are laborious and less reliable when testing environmental samples, once these tests are optimized to work with pure clinical specimens. For instance, API® 20 Strep system is able to identify only four species of *Enterococcus* genus (Applebaum et al., 1984), which is no suitable for environmental analysis where a broader enterococci diversity can be found. On the other hand, although 16S rRNA gene sequencing has been considered

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http://dx.doi.org/10.1016/j.marpolbul.2017.03.025
0025-326X/

the standard methodology for identifying most bacterial species, it is expensive, time-consuming and laborious in screening microbes in a large number of samples.

Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) has allowed the identification of microbial species in a few seconds. This methodology relies in the analysis of the mass spectra pattern of peptides and small proteins (20–20,000 Da), mainly ribosomal proteins, from microbial cells, which are specific for each microbial species (Holland et al., 1996; Krishnamurthy et al., 1996). The general principle of this technique is to break and ionize bacteria proteins that will be detected according to its mass-to-charge ratio (m/z). It will generate a spectrum that will be compared to a spectra database (Benagli et al., 2011). In the present study, Biotyper 3.0 software (Bruker Daltonics) was employed to identify the subjected bacteria. Currently, Bruker's database comprises >5000 bacteria species, including clinical and environmental isolates. Important to say, that the Bruker's database uses a proprietary algorithm to perform the comparison of the samples, and demands a prior calibration of the Mass Spectrometer with the BTS reagent (Bacteria Test Standard, Bruker Daltonics, Germany) which is a standard compound of *E. coli* lyophilized ribosomal proteins.

In recent years, MALDI-TOF MS has been increasingly used to identify clinically relevant bacteria (Krishnamurthy et al., 1996; Benagli et al., 2011); however, publications with environmental isolates are scarce. In this study, we evaluated the application of MALDI-TOF MS to identify enterococci species isolated from marine recreational waters. Besides the difficulty to suggest a direct link of a single host-specific indicator, the analysis of the distribution of enterococci species in the environment may assist the determination of fecal pollution sources, posing as a simplified, low-cost, microbial source tracking method.

2. Materials and methods

2.1. Recreational water samples collection and isolation of presumptive enterococci cells

Seawater samples were collected for regulatory monitoring purposes during the weekends of January 2008. Sampling was made at 1 m depth using a 500 mL sterile plastic bottles, as recommended by the Standard Methods for the Examination of Water and Wastewater in its Section 9060 (APHA, 2005). Samples were cooled down (ice packs) for transportation and processed within 24 h. Fig. 1 shows the location of the sampling sites throughout Brazilian coast. Membrane filtration technique was employed for presumptive identification of enterococci colonies from the seawater as described in the USEPA Method 1600 (USEPA, 2006). For EPA Method 1600, 1–100 mL volumes of sample were membrane-filtered onto mEI as previously suggested (Fergunson et al., 2005), up to five enterococci characteristic colonies (blue halos) per sample were randomly selected and subcultured onto brain heart infusion (BHI). After 18 to 24 h of incubation at 35 °C, cells were stored in 15% glycerol BHI at −80 °C for further analysis. *Enterococcus* genus screen tests were performed as follow: Gram stain; catalase test; growth and hydrolysis in bile esculin; growth at 45 °C; growth in BHI 6.5% NaCl.

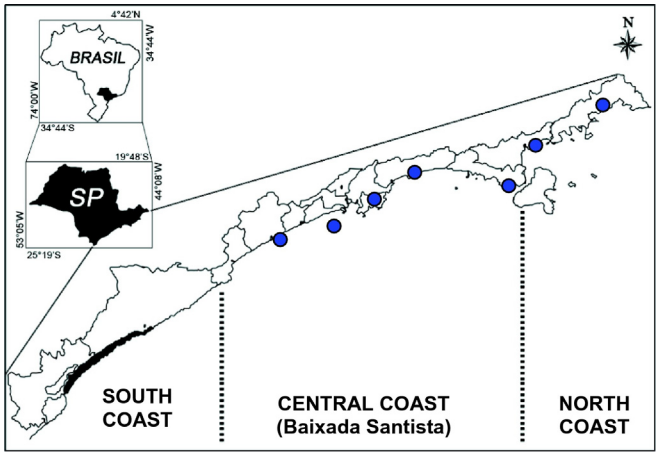

Fig. 1. Map of São Paulo State Coast. Black dots represent the beaches sampled for this study.

2.2. Identification of enterococci isolates by API® Strep20 system

The stored isolates were partially thaw and a loop of the culture was sub cultured onto BHI agar at 35 °C for 24 h and then identified by using API® Strep 20 System (2003), according to the manufacturer's instructions. Biomérieux's Analytical Profile Index WEB software (https://apiweb.biomerieux.com/) was used to generate the identification of the isolates. According to the manufacturer, strain identification (ID) to the species level was divided into four subgroups: (i) excellent species identification, percentage ID of ≥99.9%; (ii) very good species identification, ≥99.0%; (iii) good species identification, ≥90.0%, and (iv) acceptable species identification, ≥80.0%.

2.3. Enterococci identification by MALDI-TOF MS

MALDI-TOF MS analysis using the Bruker's MALDI Biotyper software was used to identify the enterococci presumptive isolates. Ethanol/formic acid protein extraction protocol was employed according to the manufacturer's instructions. Briefly, cells from single colonies were recovered by scraping the agar plates and transferred to a 1.5 mL microtube containing 300 μL of sterile water. Cells pellet were then washed with 900 μL of cold absolute ethanol and then centrifuged at 10,000*g* for 2 min at room temperature (25 °C). The supernatant was discarded and the pellet air-dried. Dried pellets were thoroughly mixed with 50 μL of 70% formic acid (Merck, USA) and 50 μL of acetonitrile (Sharlau, Spain). The suspension was centrifuged at 10,000*g* for 2 min and 1 μL of the supernatant containing the protein extracts was spotted into the target plate (96 Micro Scout Plate - 96 MSP, Bruker Daltonics, Germany) and let it dry at room temperature. After the drying step, each sample was overlaid with 1 μL of matrix solution, which consisted of a saturated solution of cyano-4-hydroxi cinnamic acid (Bruker Daltonics, Germany) in 50% acetonitrile and 2.5% trifluoracetic acid. Sample was allowed to air dry at room temperature to promote crystallization of the complex protein/organic matrix prior mass spectrometry readings. Spectra readings were performed with a Microflex LT series mass spectrometer (Bruker Daltonics, Germany) using FlexControl software (3.1 version, Bruker Daltonics, Germany). For further analysis, spectra were recorded in the positive linear mode (laser frequency: 20 Hz; ion source 1 voltage: 20 kV; ion source 2 voltage: 18.6 kV; lens voltage: 7.5 kV; mass range: 2000 to 20,000 Da). For each spectrum, 100 shots in 20-shots steps from different positions in the target spot were collected and analyzed. Spectra were internally calibrated by using BTS (Bacterial Test Standard, Bruker Daltonics) every time that the spectrometer was used. Raw spectra of each sample was exported to Biotyper software 3.0 version (Bruker Daltonic) and processed by standard pattern matching with default settings and with the microbial spectra library provided by Bruker Daltonics. Results were reported in a raking table provided by the Biotyper software and the outcomes of the pattern-matching analysis were expressed as an arbitrary ID (identification) scores ranging from zero to 3 given by the software algorithms. As suggested by the software manufacturer, interpretation of the scores was considered as following as: score <1.7 as "non-reliable Biotyper ID"; 1.7 < score < 1.9 as reliable Biotyper ID at genus level, and score > 1.9 as reliable Biotyper ID at species level.

Please cite this article as: Christ, A.P.G., et al., Characterization of *Enterococcus* species isolated from marine recreational waters by MALDI-TOF MS and Rapid ID API® 20 Strep system..., Marine Pollution Bulletin (2016), http://dx.doi.org/10.1016/j.marpolbul.2017.03.025