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# Development and validation of a new protocol for a rapid identification of frozen or no more viable microorganisms by Bruker MALDI-TOF Mass Spectrometry system

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Matrix-Assisted Laser Desorption Ionisation-Time of Flight Mass Spectrometry (MALDI-TOF/MS) became a promising and reliable tool for a fast and accurate microbial identification (ID). It is applied to fresh bacterial colonies and the use is expanding to direct analysis of clinical specimens [1]. The purposes of this study were: to develop and validate a new MALDI identification protocol, named "Wash Balls" extraction (WB), directly on frozen microorganisms, avoiding the microbial culture; to evaluate WB performance and applicability on no more viable frozen bacteria. A collection of 150 microorganisms, isolated from animal samples and stored at -20°C during the last 15 years, was cultured and tested following the Extraction Procedure Rev.4 as Gold standard (GS) using Bruker Microflex Lt® MALDI-TOF/MS. Each Cryobank™ (Mast Diagnostics, UK) or 20% glycerol-microbial suspension system was submitted to WB for validation. Briefly, 100 µL of 0.9% saline sterile solution was added to each freezing system, vortexed and the same amount transferred to an Eppendorf with 200 µL of sterile deionized water and 900 µL of ethanol. After centrifugation (14000 *rpm*) for 2 minutes, the supernatant was discarded. After drying at room temperature, 70% formic acid and pure acetonitrile were added to the pellet. Finally, 1µL of supernatant was pipetted onto a MALDI target and 1 µL of HCCA matrix solution was added. *Burkholderia cepacia* ATCC 25416, *Stenotrophomonas maltophilia* ATCC 13637, *Staphylococcus pseudintermedius* ATCC 49444 and *Escherichia coli* ATCC 25922 were used as control. The applicability of WB was tested on 64 no more viable frozen bacteria. Each strain was analyzed in double and twice by flexControl 3.4 software, and all spectra obtained were compared with the referent spectra of BDAL library using Biotyper 3.1 (Bruker Daltonics, Germany). Performance of WB was evaluated using SISA software, while differences between qualitative variables were analyzed by Chi-square test (STATA v. 13). Moreover, bioscore mean values were compared by Pearson's correlation ($R^2$). Among the 150 microorganisms, 94 different species were identified: 90 (60%) Gram positive, 55 (36.7%) Gram negative strains, and 5 (3.3%) yeasts. WB performance recorded a sensitivity of 98.6% and a specificity of 60%, with an accuracy of 96% and a substantial agreement (k=0.646). An ID correspondence of 92% was found between GS and WB. Identification at species level (bioscore >2.000) was obtained for 62.1% and for 77.8% (x=8.27, *P*=0.004) of microorganisms identified by GS and WB, respectively. In particular, WB showed a significant improvement in Gram positive ID (50% *vs* 78.4%; x=15.15, *P*=0.0001). A positive and strong correlation was recorded (r=0.97, $R^2$=0.95). Frozen and no more viable bacteria were identified by WB at genus (100%) and species (55%) levels. WB resulted in a valid, fast, cheap alternative to the GS. Moreover, it is a useful method to analyze and identify stored or no more viable microorganisms. The system and the time of freezing did not show spectrum differences and did not affect the ID. However, further studies will be carried out to evaluate the possible variations that the freezing process could cause to the microorganisms and their mass spectra.

[1] Tsuchida et al. Current status of Matrix-Assisted Laser Desorption/Ionization-Time-of-Flight Mass Spectrometry (MALDI-TOF MS) in clinical diagnostic microbiology, Molecules, 25:4775, 2020.