Tittle: Increase of daptomycin resistant cells in a subpopulation of MRSA is caused by *vraR* overexpression, thicker cell wall and lower membrane depolarization

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Abstract:

Daptomycin (DAP) resistance mechanism involving membrane and cell wall changes has been reported in methicillin-resistant *Staphylococcus aureus* (MRSA). SA90 and SA88 are isogenic clinical MRSA isolated from the same patient, with the same pulsotype and SCC*mec* element, which differed in DAP susceptibility after 48 hours incubation, suggesting DAP resistance in SA88. It was of interest to compare both isolates to characterize the mechanism involved in this phenotype. DAP minimal inhibitory concentration (MIC) was confirmed by broth microdilution method with incubation up to 48 hours. Time-kill assays with 0, 1, and 2 mg/L DAP and population analysis profile (PAP) for DAP were determined. Usually involved in DAP resistance, *graRS*, *vraRS*, *rpoB* and *mprF* genes were sequenced. Cell wall thickness by transmission electron microscopy (TEM) and expression of *graR*, *vraR*, *rpoB*, *mprF*, and *dltA* genes by qPCR was determined with and without 0.5 mg/L DAP. Membrane depolarization with 32 mg/L DAP was measured by fluorescence of $DiSC_3(5)$. DAP MIC for SA90 was 1 mg/L even after 48h incubation, but that of SA88 was 1 mg/L and 2 mg/L after 24h and 48h incubation, respectively. Both persisted in low DAP concentration in the time-kill assays. PAP of both isolates showed growth beyond DAP breakpoint suggesting heteroresistance. However, SA88 had a higher area under the curve (AUC) than that of SA90. No mutation was found in the described genes. Without treatment, *vraR* was more expressed in the SA88 strain, while *rpoB* and *mprF* were downregulated in this strain when compared with SA90. In the presence of 0.5 mg/L DAP, SA90 *graR* was overexpressed in almost 5 times (p=0.008), but this was not observed in SA88. *vraR* gene downregulated in both isolates, but it was still slightly increased in SA88 (p=0.015). DAP downregulated *rpoB*, *dltA* and *mprF* genes in both isolates, but this was more pronounced in SA90 resulting in the same expression levels when comparing both isolates. TEM revealed thicker cell wall in SA88, with or without DAP. Cell membrane of SA88 reached lower depolarization levels than that of SA90. In conclusion, both isolates were heteroresistant. However, *vraR* overexpression could lead to the thicker cell wall and this, together with the lower membrane depolarization in SA88, could explain its higher AUC. More DAP resistant cells in a subpopulation increase the selection chance of such cells among the 5×10^5 CFU/mL used for MIC determination by broth microdilution method.