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BOOK of ABSTRACTS

Relevance of *tcrYAZB* operon acquisition for *Enterococcus* survival to high copper concentrations under anaerobic conditions

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Background: Among *Enterococcus*, the acquired *tcrYAZB* operon is the most frequently described Cu tolerance (CuT) mechanism, encoding an efflux pump of the P_{1B-3}-ATPases subgroup activated by Cu^{2+}/Cu^{+}. It is often adjacent to *cueO* (multicopper oxidase) and involved in the detoxification of Cu^{+} to Cu^{2+} under aerobiosis. The *tcrB+cueO* genes are frequently co-dispersed among unrelated MDR-*Enterococcus* from diverse sources and species (mainly *E. faecium*). The method proposed by Aarestrup et *al*[1] to determine the MIC^{CuSO4} among *Enterococcus* is the most used, but with variable results among studies. Currently, the need to develop standard methodologies important for application in biocide tolerance surveillance programs and for helping to establish regulatory policies is of utmost importance. Considering the Cu^{+} role in *tcrYAZB* function, we hypothesize that the use of Aarestrup *et al*[1] method under a reduced environment (modifying the classical atmosphere of aerobiosis to anaerobiosis) could identify more accurately the acquisition of *tcrYAZB*. A first proposal of $CuSO_4$ tolerance cut-off to differentiate *Enterococcus* with *tcrYAZB* operon is also given.

Methods: *Enterococcus* (n=225; 1997-2012, mostly MDR; diverse species and clonal lineages) comprised 79 isolates *tcrB±cueO*$^{+}$, 16 *cueO*$^{+}$ and 130 without CuT genes. They were collected from human, animal, food and environmental sources. MIC_{CuSO4} were done by agar dilution method using in all assays two sets of freshly prepared Mueller-Hinton-II agar plates supplemented with 0.25, 0.5, 1, 2, 4, 8, 12, 16, 20, 24, 28, 32, 36mM $CuSO_4$ concentrations (pH=7.2) and incubated (18-20h) under anaerobiosis and aerobiosis for comparison. The *tcrYAZB* operon characterization was performed by PCR+sequencing.

Results: Under aerobiosis it was more difficult to clearly differentiate *tcrB*$^{+}$/*tcrB*$^{-}$ isolates. *E. faecium* showed higher differences between isolates *tcrB+* and *tcrB-* (MIC_{50} MIC_{90}=28/36mM and 16/20mM, respectively) and *E. faecalis* the least (MIC_{50}/MIC_{90}>36mM for both in *tcrB*$^{+}$ and *tcrB*$^{-}$). Although small in number, *tcrB*$^{+}$ isolates from other species seem to have higher MIC than *tcrB*$^{-}$ ones in most cases. Of note is the growth of several *E. faecium, E. faecalis* or *E. hirae* in the same $CuSO_4$ concentrations (mostly in 20mM; 32->36mM in *E. faecalis*), independent of *tcrB* carriage. However, under anaerobiosis an association between *tcrB* occurrence and highest $CuSO_4$ tolerance levels was more evident, with most *tcrB*$^{+}$ isolates with MIC≥16mM and *tcrB*$^{-}$/*cueO*$^{+}$ or without genes with MICs≤12mM. The few MIC≤12mM*tcrB*$^{+}$ isolates did not amplified *tcrYAZB*, contrasting with MIC≥16mM-*tcrB*$^{+}$ isolates, suggesting a non-functional gene cluster.

Conclusions: Under anaerobiosis the acquisition of *tcrYAZB* is a clear advantage for *Enterococcus* survival to high Cu concentrations. The *tcrYAZB*$^{+}$ *Enterococcus* could be identified using Aarestrup *et al* agar dilution method under anaerobiosis and a $CuSO_4$ tolerance cut-off ≥16mM. A better awareness about the role of reducing environments (e.g., gut/piggery-waste lagoons) under multiple stressors (e.g., the more toxic Cu^{+}, antibiotics) for an enhanced survival and selection/persistence of particular CuT-MDR strains is needed.

[1] Aarestrup FM, Hasman H. *Vet Microbiol* 2004; 100:83-9