Antibiotic resistance in *Staphylococcus equorum*: phenotypic and genomic analyses

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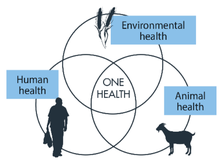
Introduction

Fermented dairy products may play a key role in the dissemination of antibiotic resistance genes (ARGs) within the food chain[1]. While the presence of ARGs in commensal bacteria do not involve a direct risk, these genes can be transferred to pathogens, which would be a cause of health concern.



One-health approach: all environments of the food chain are interconnected.

Cheeses contain a complex mixture of bacterial populations among which, *Staphylococcus equorum* has been detected as a majority species in traditional blue-veined cheeses made of raw milk[2].

Selected strains have already been proposed as starters or adjunct cultures although little is known about antibiotic resistance in this species.

Blue-veined cheese

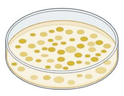
Objective

In the present work, the antibiotic resistance-susceptibility profile of 30 *S. equorum* strains to 16 antibiotics was tested by broth microdilution. To link phenotypic resistance with a genetic basis, 13 strains were subjected to genome sequencing and analysis.

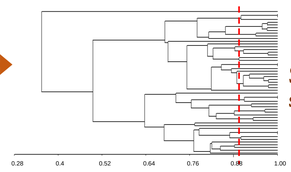
Materials and methods

- **Bacterial isolates, culture conditions and typing**

53 *S. equorum* isolates



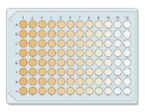
BHI with 2.5% NaCl, 32ºC 48 h



Strains selection

RAPD and rep-PCR analyses

- **Antibiotic testing and tentative cut-offs for *S. equorum***



Broth microdilution method

Strain classification into susceptible or resistant by comparing MIC values to breakpoints:

EUCAST European Committee on Antimicrobial Susceptibility Testing — European Society of Clinical Microbiology and Infectious Diseases

Clinical and Laboratory Standards Institute

Suggestion of *S. equorum* species-specific cut-offs based on the MIC distribution from this work and those from Marty et al.[3]

ECOFFinder

NRI

- **Genome sequencing and analysis for ARG**

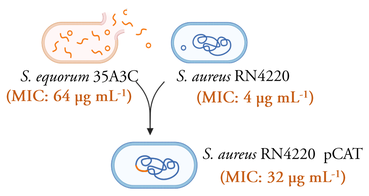
DNA isolation

DNA sequencing

ARGs were investigated against the databases:
- CARD
- ResFinder
- AMRFinderPlus

- **Transformation of plasmid DNA**

Plasmid DNA was purified and electrotransformed in *Staphylococcus aureus* RN4220. Transformants were selected with chloramphenicol.



Results and discussion

The genotyping studies revealed wide genetic diversity: 30 strains among the 53 *S. equorum* isolates at 90% of similarity. Strains were then tested for phenotypic antibiotic resistance by broth microdilution resulting in low MICs for the majority of the antibiotics. MIC values were compared with EUCAST and CLSI cut-offs to distinguish susceptible from resistant strains (Table 1). Four strains displayed MICs compatible with acquired (and possibly transferable) resistance to erythromycin (three strains) and chloramphenicol (one strain).

Antibiotics	Number of strains with a MIC value (µg mL^{-1})														*Staphylococcus* spp. cut-offs[a]				*S. equorum* cut-offs
	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	EUCAST		CLSI		This work[b]
															S (≤)	R (>)	S (<)	I (=)	R (>)	R (≥)
Erythromycin				3	14	6	3	1			2	1			1	1	0.5	1-4	8	**2**
Clindamycin			2	8	7	7	5	1							0.25	0.25	0.5	1-2	4	**4**
Chloramphenicol								3	23	3		1			(-)		8	16	32	**32**
Penicillin G	7	4	12			2	4	1							(-)		0.12	(-)	0.25	**0.5**
Quinupristin-dalfopristin				2	11	16	1								1	1	1	2	4	**4**
Rifampicin			27	3											0.06	0.06	1	2	4	**0.5**

Table1.- Distribution of MICs of 6 out 16 antibiotics to 30 *S.equorum* strains. Antibiotics gentamicin, kanamycin, neomycin, streptomycin, vancomycin, ciprofloxacin, tetracycline, linezolid, ampicillin, and trimethoprim were omitted from the table because MIC values to these antibiotics were all below the breakpoints and, therefore, strains were considered susceptible. Coloured, number of strains considered as resistant by the EUCAST (in yellow) and by CLSI (in orange). For clindamycin, quinuspristing–dalfopristin, and rifampicin, MIC values were above the EUCAST breakpoints, but below those established by CLSI; *S. equorum* strains were considered susceptible to these antibiotics. Regarding penicillin G, MICs were above the CLSI cut-offs in seven strains, but variability in the assay was noted and additional testing suggested the presence insensitive mutants.

The MIC distribution for most antibiotics showed a normal curve (Fig. 1). MIC distributions of this study and others from literature were analysed with ECOFFinder and NRI programs, which gave similar results. This analysis allowed us to suggest resistance-susceptibility cut-offs (Table 1).

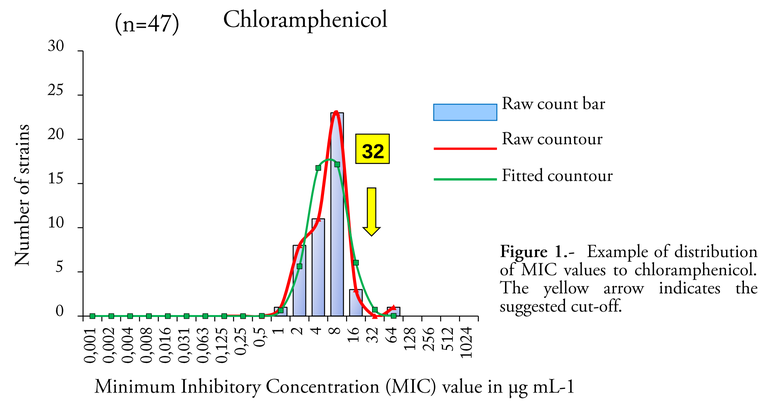


Figure 1.- Example of distribution of MIC values to chloramphenicol. The yellow arrow indicates the suggested cut-off.

Genome sequencing and analysis of 13 strains (either susceptible or resistant) revealed genes related to resistance to six classes of antibiotics but not always associated with a resistant phenotype. In that sense, a *blaR1-blaZI* operon (encoding a penicillinase), was identified in five ampicillin- and penicillin-susceptible strains, and a *bla* gene (coding for a beta-lactamase), was detected in all strains. The latter gene was polymorphic and protein variants may explain the differential phenotypic resistance to these antibiotics. An *lnu*(A) gene, related to lincomycin resistance, and a *norA* gene, involved in fluoroquinolone resistance, were also identified, although no correlation with MIC values to these antibiotics was observed. An *mph*(C) gene, encoding for a macrolide transferase, was detected in two strains that also carried an *mrs*(A) gene encoding an ABC-type ribosomal protection protein (the latter gene was also detected in other five strains). Variants of Mrs(A) provide high erythromycin resistance (MIC 24-64 µg mL $^{-1}$). Although disrupted in some, a *fosB/fosD* gene, encoding for a fosfomycin-inactivating enzyme, was identified in all strains. This gene was also polymorphic and protein variants might account for MIC differences to this antibiotic.

In contrast to those genes, a plasmid-located *cat* gene, encoding a chloramphenicol acetyltransferase, was detected in a strain resistant to chloramphenicol (MIC 64 µg mL $^{-1}$). The plasmid content of the strain was electroporated into *S. aureus* cells. Transformant strains harboured the pCAT plasmid (Fig. 2) and showed, as compared to the host strain, an MIC increased for chloramphenicol from 4 to 32 µg mL $^{-1}$.

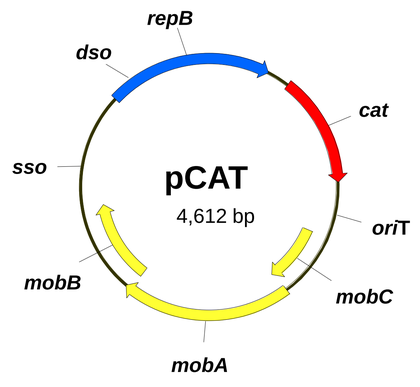


Figure 2.- Scheme of the genetic map of pCAT harbouring the *cat* gene that provides chloramphenicol resistance.

Conclusions

- *S. equorum* strains displayed little resistance to the tested antibiotics, but solely in a minority of the strains the resistance phenotype was supported by the presence of ARGs.
- The *S. equorum* resistome is composed of intrinsic and acquired genes.
- Starter and adjunct cultures should be free of transferable ARGs. Therefore, the presence of such genes in candidate strains should be thoroughly examined.

References

[1] Alexa et al. 2020. *mSystems*, *5*(1), 10-1128.
[2]Meugnier et al. 1996. *Int. J. Food Microbiol.*, *31*(1-3), 325-331.
[3] Marty et al. 2012. *Int. J. Food Microbiol.*, *159*(2), 74-83.

Acknowledgements

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