

COMPARISON OF tDNA-INTERGENIC SPACER PCR AND RPOB-GENE SEQUENCING FOR SPECIES LEVEL IDENTIFICATION OF BOVINE COAGULASE-NEGATIVE STAPHYLOCOCCI

K. Supré^{1*}, S. De Vliegher¹, O. Sampimon³, R. Zadoks⁴, M. Vaneechoutte⁵, M. Baele², A. de Kruif⁴, F. Haesebrouck²

¹Dept. Reproduction, Obstetrics and Herd Health, Faculty of Veterinary Medicine, Ghent University, Belgium;

²Dept. of Pathology, Bacteriology and Poultry Diseases, Faculty of Veterinary Medicine, Ghent University, Belgium; ³Animal Health Service, Deventer, The Netherlands;

⁴Quality Milk Production Services, Cornell University, Ithaca, New York, USA;

⁵Dept. Clinical Chemistry, Microbiology and Immunology, Faculty of Medicine, Ghent University, Belgium;

*Corresponding author: karlien.supre@UGent.be

1. INTRODUCTION

In many European dairy farms that have adopted the 5- and 10-point mastitis prevention programs, the relative importance of coagulase-negative staphylococci (CNS) has increased. They are the predominant pathogens found in milk samples and are causing the majority of intramammary infections (imi) in fresh dairy heifers. The increase in CNS prevalence and incidence relative to traditional major pathogens, combined with changes in limits for bulk milk somatic cell count penalties and the fact that CNS are causing clinical mastitis also, warrant reconsideration of their historical designation "minor pathogen". On the other hand, protective characteristics of CNS have been reported. The confusion can partly be explained by the lack of (accurate) species identification. Accurate and low-cost identification is a prerequisite for epidemiological studies aiming at elucidating the relevance of the different CNS species in bovine mastitis. Current identification methods are largely phenotypic and based on reference strains of human origin. These methods may not be suitable for isolates of bovine origin. In this study, we have updated tDNA-intergenic spacer PCR (tDNA-PCR) for identification of bovine CNS species by extending the current library of the technique, followed by comparing the results with sequencing of the *rpoB*-housekeeping gene.

2. MATERIALS AND METHODS

2.1. Isolates

2.1.1. Updating tDNA-PCR library using bovine field isolates.

Ninety-four CNS-isolates originating from milk and 52 from teat apices were available. Both tDNA-PCR and gene sequencing were performed on all isolates. Peak patterns obtained with tDNA-PCR were added to the existing database, which consisted of reference CNS-strains. Gene sequencing was used as gold standard, implying that when tDNA-PCR identification was uncertain or did not correspond with sequencing identification, the latter was considered correct.

2.1.2. Comparison of tDNA-PCR and gene sequencing for the identification of bovine CNS.

One hundred CNS-isolates originating from milk and 48 from teat apices were available. All isolates were subjected to tDNA-PCR and gene sequencing. tDNA-PCR was performed using the updated library as described before.

2.2. Techniques

DNA-lysates were prepared by alkaline extraction (Baele *et al.*, 2000). tDNA-intergenic spacer PCR was performed as described (Vaneechoutte *et al.*, 1998; Baele *et al.*, 2000, 2001). The length of the PCR-products was analysed with capillary electrophoresis using an ABI-PrismTM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) (Vaneechoutte *et al.*, 1998; Baele *et al.*, 2000, 2001) and a software program was used for interpretation (Baele *et al.*, 2000). Sequencing of the *rpoB*-gene was performed as described (Drancourt and Raoult, 2002) with small modifications. Results were compared to online reference data using nucleotide-nucleotide BLAST. When there was no amplification with the *rpoB*-primers or less than 97% homology with reference strains was seen, additional *cpn60*-sequencing and if not sufficient *16S*-sequencing, was performed.

3. RESULTS

3.1. Updating tDNA-PCR library using bovine field isolates.

Nine isolates (6.2%) were unidentifiable with gene sequencing and were therefore temporarily ignored for further study. Of 21 (15.3%) of the isolates identified with gene sequencing, there was doubt about the tDNA-PCR identification. The peak patterns of these isolates were added to the library based on the sequencing identification. The updated library was used for the identification of the CNS-isolates in step 2.

3.2. Comparison of tDNA-PCR and gene sequencing for the identification of bovine CNS.

The results are presented in table 1 (milk samples) and 2 (teat apices). Overall, 12 isolates (8.1%) were unidentifiable with gene sequencing. The overall agreement (isolates from milk and teat apices) between tDNA-PCR and gene sequencing was 97.5%. When focussing on milk samples and teat apices separately, 96.6 and 100% of the identifications agreed in both tests, respectively.

Table 1: Comparison of tDNA-PCR and gene sequencing for identification of CNS-isolates originating from bovine milk samples.

tDNA-PCR identification	gene sequencing identification															Total		
	no identification	<i>S. capitis</i>	<i>S. caseolyticus</i>	<i>S. chromogenes</i>	<i>S. cohnii</i>	<i>S. epidermidis</i>	<i>S. equorum</i>	<i>S. fleuretti</i>	<i>S. haemolyticus</i>	<i>S. hyicus</i>	<i>S. nepalensis</i>	<i>S. saprophyticus</i>	<i>S. sciuri</i>	<i>S. simulans</i>	<i>S. succinus</i>		<i>S. warneri</i>	<i>S. xylosus</i>
no identification	2			3		1			1	1	1					1	1	11
<i>S. capitis</i>		1																1
<i>S. caseolyticus</i>			0	1														1
<i>S. chromogenes</i>				42					1									43
<i>S. cohnii</i>					1													1
<i>S. epidermidis</i>						11												11
<i>S. equorum</i>							4											4
<i>S. fleuretti</i>								2										2
<i>S. haemolyticus</i>									2									2
<i>S. hyicus</i>				1						5								6
<i>S. nepalensis</i>											0							0
<i>S. saprophyticus</i>												1						1
<i>S. sciuri</i>													1					1
<i>S. simulans</i>														3				3
<i>S. succinus</i>															2			2
<i>S. warneri</i>																3		3
<i>S. xylosus</i>																	8	8
Total MILK	2	1	0	47	1	12	4	2	3	7	1	1	1	3	2	4	9	100

Table 2: Comparison of tDNA-PCR and gene sequencing for identification of CNS-isolates originating from bovine teat apices.

tDNA-PCR identification	gene sequencing identification															Total		
	no identification	<i>S. capitis</i>	<i>S. caseolyticus</i>	<i>S. chromogenes</i>	<i>S. cohnii</i>	<i>S. epidermidis</i>	<i>S. equorum</i>	<i>S. fleuretti</i>	<i>S. haemolyticus</i>	<i>S. hyicus</i>	<i>S. nepalensis</i>	<i>S. saprophyticus</i>	<i>S. sciuri</i>	<i>S. simulans</i>	<i>S. succinus</i>		<i>S. warneri</i>	<i>S. xylosus</i>
no identification	7		1										1					9
<i>S. capitis</i>		2																2
<i>S. caseolyticus</i>			3															3
<i>S. chromogenes</i>	1			4														5
<i>S. cohnii</i>					2													2
<i>S. epidermidis</i>						0												0
<i>S. equorum</i>							5											5
<i>S. fleuretti</i>								2										2
<i>S. haemolyticus</i>	2								5									7
<i>S. hyicus</i>										2								2
<i>S. nepalensis</i>											0							0
<i>S. saprophyticus</i>												0						0
<i>S. sciuri</i>													7					7
<i>S. simulans</i>														1				1
<i>S. succinus</i>															1			1
<i>S. warneri</i>																0		0
<i>S. xylosus</i>																	2	2
Total MILK	10	2	4	4	2	0	5	2	5	2	0	0	8	1	1	0	2	48

4. DISCUSSION

When studying the impact of different CNS species on performances (udder health, milk production...) in dairy cattle, an accurate identification technique is required. Although no single test can offer fully reliable identification of bacterial species, gene sequencing is often seen as the gold standard. Unfortunately, the high cost and its labour intensiveness limit its use in large field studies for most routine laboratories. Phenotypic methods on the other hand are usually cheaper but lack accuracy. The results of this study show that tDNA-intergenic spacer PCR could be a good alternative for gene sequencing. It's a rapid, low-cost and easy to perform technique that has a high reproducibility if capillary electrophoresis is available (Baele *et al.*, 2001). The overall agreement between tDNA-PCR and gene sequencing was high.

Overall, a high number of CNS-isolates could not be identified with the gold standard (gene sequencing: *rpoB*, *cpn60*, *16S*), especially isolates originating from teat apices (20.3%). Possible explanations could be the presence of undefined species on teat apices, or strain differences between isolates from different origins. Still, availability of a complete reference database is a prerequisite and could be the bottleneck. Additional sequencing of the *tuf*-gene might give a definite answer (in progress).

To conclude, tDNA-PCR will be a useful tool for our field study aiming at elucidating the relevance of CNS imi in dairy cattle.

5. ACKNOWLEDGEMENTS

This research is funded by the Institute for the Promotion of Innovation through Science and Technology in Flanders (IWT-Vlaanderen, grant n° 61459). The authors acknowledge the Dutch Udder Health Centre, Deventer, The Netherlands, for financially supporting part of the analyses.

6. REFERENCES

- Baele, M., Baele, P., Vaneechoutte, M., Storms, V., Butaye, P., Devriese, L.A., Verschraegen, G., Gillis, M., Haesebrouck, F. Application of tRNA intergenic spacer PCR for identification of *Enterococcus* species. *J. Clin. Microbiol.* 2000; 38: 4201-4207.
- Baele, M., Storms, V., Haesebrouck, F., Devriese, L.A., Gillis, M., Verschraegen, G., De Baere, T., Vaneechoutte, M., Application and evaluation of the interlaboratory reproducibility of tDNA-intergenic length polymorphism analysis (tDNA-PCR) for identification of *Streptococcus* species. *J. Clin. Microbiol.* 2001; 39: 1436-1442.
- Drancourt, M., and Raoult, D. *RpoB*-gene sequence-based identification of *Staphylococcus* species. *J. Clin. Microbiol.* 2002; 40: 1333-1338.
- Vaneechoutte, M., Boerlin, P., Tichy, H.V., Bannerman, E., Jäger, B., Bille, J. Comparison of PCR-based DNA fingerprinting techniques for the identification of *Listeria* species and their use for atypical *Listeria* isolates. *Int. J. Syst. Bacteriol.* 1998; 48: 127-139.