Construction of multiplex quantitative PCR for detection of streptococcal mastitis

JAKUB SURYNEK, ALES KNOLL, IRENA VRTKOVA
Department of Animal Morphology, Physiology and Genetics
Mendel University in Brno
Zemedelska 1, 613 00 Brno
CZECH REPUBLIC

xsurynek@mendelu.cz

Abstract: The objective of this study was to develop a multiplex quantitative PCR-based method for simultaneous detection of *Streptococcus agalactiae*, *Streptococcus dysgalactiae* and *Streptococcus uberis* in biological samples. The *cfb* gene for CAMP factor was used for detection of *S. agalactiae*. The *cpn60* gene was used for detection of *S. dysgalactiae* and the plasminogen activator gene for *S. uberis* detection. PCR for *S. dysgalactiae* performed optimally, but reactions of *S. agalactiae* and *S. uberis* showed poor and no amplification respectively.

Key words: mastitis, *Streptococcus agalactiae*, *Streptococcus dysgalactiae*, *Streptococcus uberis*, qPCR

Introduction

Intramammary infection, also known as mastitis, is the most frequently occurring and economically the most important infectious disease in dairy cattle. Besides health disorders of mammary gland, mastitis causes significant losses in milk yield, degradation of its nutritive and technological properties, fertility disorders and even systemic disease [1]. Numerous microorganisms have been described as causative agents of bovine mastitis. According to their epidemiology, mastitis pathogens can be divided into two groups, contagious and environmental. The primary reservoir of contagious pathogens is an infected udder whereas a contaminated environment is the primary reservoir of pathogens causing environmental mastitis. *Streptococcus agalactiae* is considered as typical contagious pathogen of the mammary gland, where it can survive for a long period of time. *Streptococcus uberis* is a typical environmental pathogen. *Streptococcus dysgalactiae* has been most commonly described as a contagious pathogen but it can also behave as an environmental pathogen [2, 3].

Traditionally, culture methods are considered gold standard in mastitis pathogens identification. However, in last twenty years introduction of molecular biology methods brought new possibilities – identification based on DNA using polymerase chain reaction. Historically, most PCR assays developed for identification of *Streptococcus* sp. targeted the 16S rRNA gene [4, 5, 6]. However, false positive results may occur due to high homology of ribosomal operons (91–93%) thus reducing specificity of this approach to 0.87–0.96 [7].

Polymorphism of *cpn60* gene was commonly used in phylogenetic studies of *Streptococcus* spp. and also differentiation of its species and strains. Product of this gene, the cpn60 protein, also known as GroEL or HSP60, is a 60 kDa heat-shock protein that assists in the correct folding of most bacterial proteins under both normal and stress conditions [8]. The cpn60 proteins showed extensive sequence similarity in bacterial species, typically around 70 %. Dmitriev et al. [7] successfully used polymorphism of *cpn60* for differentiation of *S. agalactiae, S. dysgalactiae,* and *S. uberis*.

For detection of *S. agalactiae* Gillespie and Oliver [9] *cfb* gene encoding the CAMP factor of *S. agalactiae* was used. In case of *S. uberis*, Sazonova et al. [10] described its plasminogen activator gene (*pauA*). Gillespie and Oliver [9] first used *pauA* gene for detection of *S. uberis*. Therefore aim of this study was to assess the performance of our own reaction for *S. dysgalactiae* together with reactions previously used by Gillespie and Oliver [9]. Shome et al. [11] carried out study of potencial molecular targets for detection mastitis pathogens and for detection of S. uberis they used also the *cpn60* gene.

Material and Methods

Bacterial strains

Gemonic DNA of three strains of streptococci was used for preparation of standards: *S. agalactiae* CAPM 5153, *S. dysgalactiae* CAPM 5548, and *S. uberis* CAPM 5675.

Table 1 Primers and probes for multiplex qPCR

Designation	Sequence (5´→3´)	Length, bp	Source
SagCAMP_F	AGCTCTATTAGAAGTACATGCT	22	
SagCAMP_R	CATTTGCTGGGCTTGATTATT	21	Gillespie and Oliver, 2005
SagCAMP_P	FAM-ATCAAGTGACAACTCCACAAGTGGTAA-BHQ1	27	
Sdycpn60_F	GCGATTGCTCAGCCTGTTTCT	21	
Sdycpn60_R	GGCTTCTGAAATGTATTCTCCAA	23	
Sdycpn60_P	Cy5-TTGCTGCTGTGTCATCTCGTTCTG-BHQ2	24	original design
SubpauA_F	AGAGGAATTCATCATGTTTTAACA	24	
SubpauA_R	AATTGTAGAAGAACCATTTGATGT	24	Gillespie and Oliver, 2005
SubpauA_P	HEX-AGCGTCTAACAACTCGGCCTTTG-BHQ1	23	

Primers and probes

Primers and probe for *S. dysgalactieae* were designed using OLIGO 4.0. Length of amplicon was designed to 95 bp. Previously published primers and probes were used in reactions of *S. agalactiae* and *S. uberis* (Tab. 1). Oligonucleotides were purchased from Generi Biotech (Hradec Kralove, Czech Republic)

Triplex qPCR for *S. agalactiae*, *S. dysgalactiae,* and *S. uberis*

Dilution series of *S. agalactiae, S. dysgalactie,* and *S. uberis* genomic DNA ranging from 10^7 to 10^1 genome copies were made. Amplification was carried out in 8-tube strips (Life Technologies, Foster City, CA, USA) using the ABI 7500 real-time PCR system and the PCR conditions were as follows: UDG pre-treatment at 50°C for 2 min, initial denaturation/activation at 95°C for 10 min, 45 cycles of denaturation at 95°C for 15 s and extension at 57°C for 1 min. Each 20-µl reaction contained 10 µl of TaqMan Gene Expression Master Mix (Life Technologies, Foster City, CA, USA), 600-nM primers, 250-nM probes and 2 µl of template. Reactions were run in triplicates (Fig. 2).

Results and Discussion

Optimal annealing temperature for multiplex qPCR

Compared with SagCAMP_F and SagCAMP_R, our original primers Sdycpn60_F and Sdycpn60_R gave strong PCR product of expected size 95 bp at all six annealing temperatures tested. Primers SagCAMP_F and SagCAMP_R gave much weaker PCR product whose intensity is comparable at temperatures 55, 56 and 57°C, and then it gradually decreases until no amplification at 60°C. For unknown reason, primers SubpauA_F and SubpauA_R gave no PCR product at any temperature. Annealing temperature of 57°C was selected for multiplex qPCR.

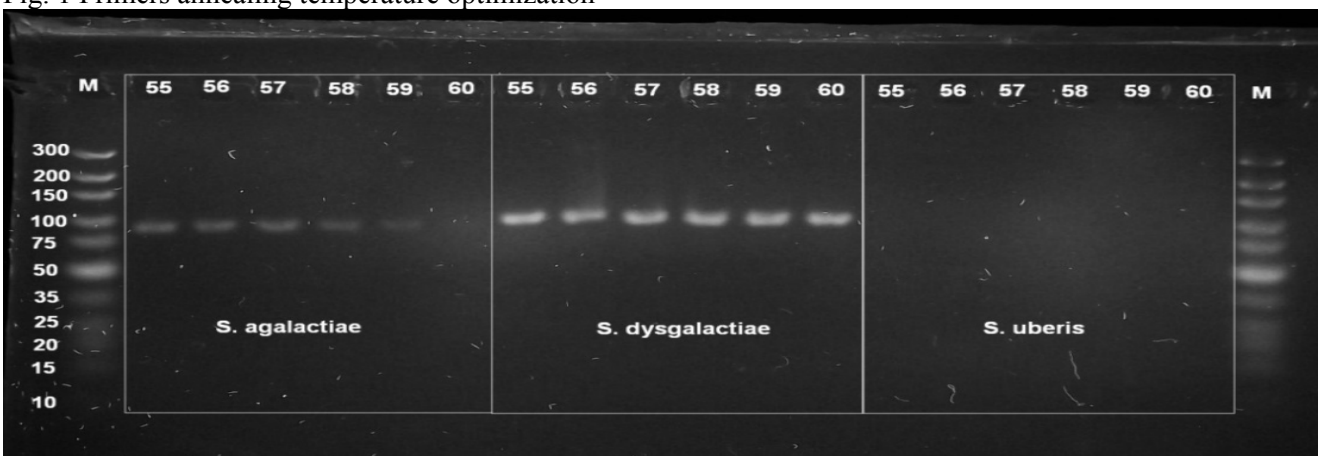
Performance of multiplex qPCR

Reaction of *S. dysgalactiae* performs well in multiplex. Amplification curves have correct shape; there is expected ΔCt of dilutions and good fit of replicates. However reaction of *S. agalactiae* does not meet these criteria and no reaction of *S. uberis* occured. These results are in congruence with results obtained in annealing temperature optimization by gel electrophoresis.

PCR detection of streptococcal mastitis

Muliplex PCR and multiplex qPCR assays that can simultaneously detect different mastitis-causing organisms in milk and other samples have been reported on. Phuektes et al. [4, 5] designed multiplex PCR assay for detection of *S. aureus*. *S agalactiae*, *S. dysgalactiae*, and *S. uberis*. Their assay was based on 16S rRNA genes. These assays might be not specific enough due to known problems of 16S rRNA-based bacterial identification. Different approach was chosen by Gillespie and Oliver [9] who in their multiplex qPCR used *cfb* gene coding CAMP factor of *S. agalactiae* and plasminogen-activator gene for *S. uberis* detection. However these reactions according to our results are not suitable for quantitative detection. Dmitriev et al. [7] were able to distinguish *S. agalactiae*, *S. dysgalactiae* and *S. uberis* in single tube based on sequences of their *cpn60* genes. Their results hold promise for design of multiplex qPCR for streptococcal mastitis detection.

Fig. 1 Primers annealing temperature optimization

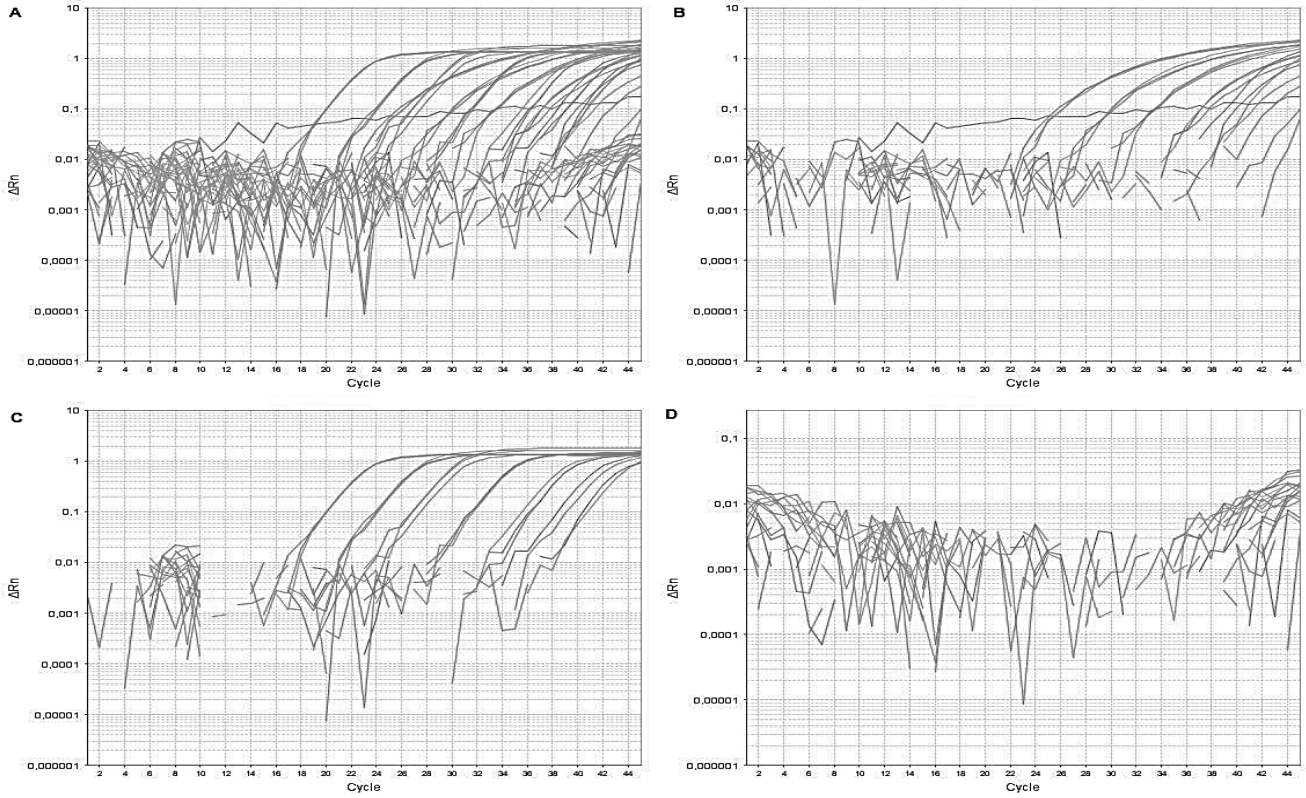


Legend: M – size marker (bp); figures indicate annealing temperature tested.

Conclusion

qPCR for *S. dysgalactie* targeting cpn60 gene seem to be performing well in multiplex conditions. Results of reactions of *S. agalactiae* and *S. uberis* are unsatisfactory. Primers and probes most likely need to be redesigned and tested for specificity. Another option could be to base the multiplex qPCR on *cpn60* gene and design primers and probes in sites with enough heterogeneity to distinguish species of interest from other *Streptococcus* species.

Fig. 2 Amplification plots of *S. agalactiae*, *S. dysgalactiae* and *S. uberis* reactions



Legend: A – combined plot of all three reactions, B – reaction of S. agalactiae, C – reaction of S. dysgalactiae, D – reaction of S. uberis.

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