cpn60 Gene Based Multiplex-PCR Assay for Simultaneous Identification of Streptococcal Species

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Abstract

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The *cpn60* genes of *Streptococcus agalactiae, Streptococcus dysgalactiae* and *Streptococcus uberis* were sequenced and a certain polymorphism of *cpn60* genes was revealed. Presumable species-specific pairs of primers were designed and their specificity was confirmed by PCR. Based on these data, the *cpn60* gene-based multiplex-PCR assay was developed. It was found to be effective for simultaneous identification of *S. agalactiae, S. dysgalactiae* and *S. uberis* strains.

Streptococci, cpn60 gene, multiplex-PCR

Bovine mastitis is a major cause of economic loss in dairy industry (Allmann et al. 1995; Keefe 1997). In the United States, for example, the economic loss is estimated to be approximately $1,8 billion annually. *Streptococcus agalactiae, Streptococcus dysgalactiae* and *Streptococcus uberis* are the major streptococcal species among causative agents of bovine mastitis (Keefe et al. 1997). Obviously, the rapid and specific identification of streptococci is desirable for prevention of infectious diseases and monitoring mastitis.

At present, ribosomal operon-based PCR is used for identification of many bacterial species, and numerous approaches have been applied for detection of mastitis-causative bacteria in milk products (Martinez et al. 2001; Phuektes et al. 2001; Riffon et al. 2001). However, due to high homology of the ribosomal operon genes among different species, the ribosomal operon based PCR can result in a false-positive diagnosis (Backman et al. 1999; Hassan et al. 2000; Meiri- Bendek et al. 2002). These observations reduce the value of ribosomal operon-based PCR approach for identification of causative bacterial species. Obviously, some other genes have to be tested as potential targets for species-specific diagnosis. Recently it was demonstrated that phylogenetic *cpn60* gene sequence analysis of streptococcal strains could be used for precise identification of several streptococcal species (Goh et al. 1998; Brousseau et al. 2001; Hung et al. 2005) that correlated with the ribosomal operon sequence analysis (Teng et al. 2002). However, *S. agalactiae, S. dysgalactiae* and *S. uberis* as important pathogens for animals and humans, were not tested using *cpn60* gene as a potential discriminative marker. The goal of the present study was to develop a *cpn60* gene based multiplex-PCR assay for simultaneous identification of these streptococcal species.

Materials and Methods

Bacterial strains

A total of 68 streptococcal strains were used in this study. Forty two *S. agalactiae* strains, nine *S. dysgalactiae* strains and seven *S. uberis* strains were isolated from dairy cows in different regions of the Czech Republic and Slovakia, and ten *S. agalactiae* strains were isolated from pregnant women in Russia. Bacteria were grown in Todd-Hewitt broth or on 1.5% horse blood agar at 37 °C overnight. Other animal pathogens, i.e., *Escherichia coli, Staphylococcus aureus* and *Salmonella typhimurium* were also used in this study.

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General DNA techniques

Most of the molecular genetic procedures were conducted according to previously published protocols (Maniatis et al. 1982). In order to prepare DNA samples for PCR, a single bacterial colony was resuspended in 100 µl of distilled water and incubated at 97 °C for 5 minutes. The samples were centrifuged, and 5 µl of supernatant was used in PCR reaction. PCR was carried out in the final volume of 25 µl using the primers listed in Table 1. A pre-PCR step at 94 °C for 2 min was applied. A total of 30 PCR cycles were used under the following conditions: denaturation at 94 °C for 30 s, annealing at 52 °C for 1 min and extension at 72 °C for 30 s. After the final cycle, the additional incubation at 72 °C for 10 min was done to complete the reaction. For a multiplex-PCR assay, all the primers were added in the same reaction mixture. Sequencing of PCR products was performed using ABI Prism™ 377 Perkin-Elmer Sequencer and Big Dye Terminator Kit (Applied Biosystems, USA).

Computer analysis of DNA

Nucleotide sequences of *cpn60* genes were accessed through the GenBank database (http://www.ncbi.nlm.nih.gov/entrez). Computer analysis of DNA was accomplished using BLAST (http://www.ncbi.nlm.nih.gov/BLAST) and MULTALIN. Sequences of the primers were designed using the computer program "OLIGO". The 100 bp ladder (New England Biolabs) was used as a DNA molecular weight standard. The sequences data reported in this study were deposited in the GenBank Nucleotide Sequence Database under accession numbers AY487423, AY487424, AY496926 and AY508648.

Results

Previously, the polymorphism of *cpn60* gene sequences was used as a marker for identification of *Streptococcus iniae* (Goh et al. 1998), for differentiation of mutans streptococci and *Streptococcus suis* strains (Brousseau et al. 2001; Hung et al. 2005), and for determination of evolutional relationship of 10 streptococcal species belonging to viridans group streptococci (Teng et al. 2002). The *cpn60* gene phylogenetic tree of streptococcal species was similar to the tree constructed based on 16S rRNA sequences (Brousseau et al. 2001; Teng et al. 2002) .

In this study we hypothesized that polymorphism of *cpn60* genes could be used for differentiation of *S. agalactiae, S. dysgalactiae* and *S. uberis* strains.

In order to analyze this hypothesis we compared the complete *S. agalactiae cpn60* gene with *cpn60* gene fragments of *S. dysgalactiae* and *S. uberis* available in GenBank database. After the comparison, a certain polymorphism of *cpn60* genes was revealed (Fig.1). Based on these data, the presumed species-specific primers were designed and used in PCR (Table 1).

All 52 *S. agalactiae* strains tested produced the expected 310 bp amplification products when analyzed by PCR with primers AGAFor and AGARev. It is important that both human and bovine *S. agalactiae* strains of different serotypes produced amplification products of the same size (Fig. 2). PCR analysis of *S. dysgalactiae* and *S. uberis* strains with pairs of the primers DYSFor, DYSRev and UBEFor, UBERev, respectively, revealed PCR products of the expected sizes 192 bp and 400 bp, respectively (Fig. 3). Specificity of each pair of primers was confirmed when other streptococcal species did not produce any PCR products (Fig. 3). Furthermore, specificity of primers was demonstrated by PCR analysis of other veterinary pathogens, i.e., *E. coli, S. aureus* and *S. typhimurium* (Fig. 2).

Three PCR products of different sizes (192 bp, 310 bp and 400 bp) were isolated from agarose gel and sequenced. As expected, their sequences corresponded to the previously published streptococcal *cpn60* gene sequences available in the cpnDB Database (http://cpndb.cbr.nrc.ca).

In order to evaluate the possibility of simultaneous identification of streptococcal species, the multiplex-PCR with three pairs of primers AGAFor, AGARev, DYSFor, DYSRev, UBEFor and UBERev was employed (Fig. 3). Results of this analysis demonstrated species-specificity of the selected primers and indicated the value of *cpn60* gene polymorphism for differentiation of *S. agalactiae, S. dysgalactiae* and *S. uberis* strains.

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                    342                                                 391
S. agalactiae       CATTCGTCGT GGTATTGAAA CAGCTGTTTC AGCAGCAGTT GAAGAGCTAA
S. dysgalactiae     TATTCGTCGT GGGATTGAAA CAGCAACAGC AACAGCCGTT GAAGCCTTGA
S. uberis           CATTCGTCGC GGTATTGAAA AAGCAACATC AGCCGCAGTT GAAGAACTAA

                    392                                                 441
S. agalactiae       AAGAGATTGC ACAACCAGTT TCAGGCAAAG AAGCTATTGC TCAAGTTGCA
S. dysgalactiae     AAGCTATTGC TCAGCCTGTT TCTGGTAAAG AAGCGATTGC TCAAGTTGCT
S. uberis           AAGCTATTGC TCAACCAGTT TCAGGAAAAG AGGCGATTGC CCAAGTAGCT

                    442                                                 491
S. agalactiae       GCTGTGTCTT CACGTTCTGA AAAAGTTGGG GAATATATTT CTGAAGCTAT
S. dysgalactiae     GCTGTGTCAT CTCGTTCTGA AAAAGTTGGA GAATACATCT CAGAAGCCAT
S. uberis           GCCGTGTCAT CACGTTCAGA AAAAGTTGGG GAGTATATCT CAGAAGCTAT

                    492                                                 541
S. agalactiae       GGAGCGCGTG GGTAATGATG GTGTTATCAC TATTGAAGAA TCGCGAGGTA
S. dysgalactiae     GGAACGTGTG GGCAATGACG GTGTCATTAC TATCGAAGAA TCACGTGGTA
S. uberis           GGAACGTGTA GGCAATGATG GTGTTATCAC AATTGAAGAA TCACGTGGTA

                    542                                                 591
S. agalactiae       TGGAAACAGA GCTTGAAGTT GTGGAAGGAA TGCAGTTTGA CCGTGGGTAC
S. dysgalactiae     TGGAGACAGA GCTTGAAGTG GTAGAAGGCA TGCAGTTTGA TCGTGGTTAC
S. uberis           TGGAAACAGA ACTTGAAGTG GTTGAAGGGA TGCAATTTGA CCGCGGATAC

                    592                                                 641
S. agalactiae       TTGTCACAGT ATATGGTAAC TGATAACGAG AAAATGGTCT CTGAACTTGA
S. dysgalactiae     CTGTCTCAAT ACATGGTCAC AGACAATGAA AAAATGGTTG CAGACCTTGA
S. uberis           TTATCACAAT ATATGGTAAC AGATAATGAA AAAATGGTTG CTGATCTTGA

                    642                                                 691
S. agalactiae       GAATCCGTAT ATCCTTATTA CAGATAAGAA AATTTCAAAT ATCCAAGAAA
S. dysgalactiae     GAACCCATTT ATCTTGATTA CTGACAAAAA AGTGTCAAAC ATCCAAGATA
S. uberis           AAACCCATTT ATCTTAATCA CAGATAAAAA AGTATCAAAT ATTCAAGAAA

                    692                                                 741
S. agalactiae       TTTTACCATT ATTAGAAGAG GTTCTTAAAA CAAATCGTCC GTTGCTAATC
S. dysgalactiae     TTCTCCCATT GCTTGAGGAA GTTCTTAAAA CCAACCGTCC ATTGTTGATT
S. uberis           TTTTACCGTT ATTGGAAGAA GTGCTTAAAA CCAGTCGTCC CCTTCTCATT

                    742
S. agalactiae       ATCGCTG
S. dysgalactiae     ATCGCCG
S. uberis           ATTGCAG
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Fig.1. Comparative analysis of cpn60 gene fragments of *S. agalactiae*, *S. dysgalactiae* and *S. uberis* strains. Species- specific primers used in this study correspond to underlined regions. Numbers correspond to nucleotide numbers in complete *S. agalactiae cpn60* gene.

Table 1. Primers used in the present study

Primer	Sequence (5'→ 3')
DYSFor	CGTGGGATTGAAACAGCAACAG
DYSRev	ACCACGTGATTCTTCGATAGTAATG
UBEFor	TCGCGGTATTGAAAAAGCAACAT
UBERev	TGCAATAATGAGAAGGGGACGAC
AGAFor	CGTCGTGGTATTGAAACAGCTGTT
AGARev	GGATATACGGATTCTCAAGTTCAGAG

Discussion

Bovine mastitis is a major cause of economic loss in dairy industry due to reduced milk yield and quality, discarded milk, expensive treatment etc. (Allmann et al. 1995; Keefe 1997). Streptococci can widely spread within the herd due to specific life style and the machine milking technology. Additionally, humans can be infected by streptococci due to the close contact of farm staff with the infected animals and raw milk tanks. Thus, early identification of streptococci in the milk product is necessary for effective animal treatment and for monitoring streptococcal diseases.

The ribosomal operon-based PCR is often used for identification of streptococcal species in milk products (Martinez et al. 2001; Phuektes et al. 2001; Riffon et al. 2001). However, due to the high homology of ribosomal operons, the false-positive ribosomal operon-based PCR results can occur that reduce the specificity of this approach to 0.87-0.96. For example, the false-positive results were observed when *S. agalactiae* and *S. pneumoniae* as well as *S. agalactiae* and *S. difficile* strains were analyzed (Backman et al. 1999; Hassan et al. 2000; Meiri-Bendek et al. 2002). When *S. agalactiae* and *S. dysgalactiae* were analyzed, no false-positive results were observed (Riffon et al. 2001), however, the 6 bp difference between species-specific PCR products resulted in difficulties during comparison (Phuektes et al. 2001).

Obviously, a larger number of species simultaneously analyzed by ribosomal operon-based PCR can lead to a higher possibility of an incorrect diagnosis. Therefore, the search of another potential discriminative marker is important for veterinary and dairy industry.

The Cpn60 protein, also known as GroEL and HSP60, is a 60 kDa heat-shock protein that assists in the correct folding of most bacterial proteins under both normal and stress conditions (Ranford and Henderson 2002). The Cpn60 proteins showed extensive sequence similarity in bacterial species; typically around 70% (Ranford and Henderson 2002). It is significantly smaller than the sequence similarity of 16S rRNA genes (91% - 93%) as it was determined for different streptococcal species (Bentley et al. 1991).

The cpn60 gene-based PCR assay developed in this study avoided the disadvantages of ribosomal operon-based PCR. It demonstrated high specificity and simplicity in interpreting the results. It is important to note that the previously described *scpB* gene-based PCR detection of *S. agalactiae* in human sera and blood (Dmitriev et al. 2004) cannot be used for the analysis of bovine *S. agalactiae* because the *scpB* gene was present only in 20% of bovine strains (Dmitriev et al. 1999; Franken et al. 2001). In contrast to *scpB* gene-based detection, the use of *cpn60* gene as a target in PCR can be effectively used for both human and bovine *S. agalactiae* strains (Fig. 2). The *cpn60* gene-based multiplex-PCR approach demonstrated the possibility of simultaneous identification of *S. agalactiae, S. dysgalactiae* and *S. uberis* in the same sample (Fig. 3). An additional advantage of this approach is significantly reduced cost of analysis in comparison with three independent PCR assays, which can be attractive for dairy industry.

Based on the results of this study, we also hypothesize that *cpn60* gene polymorphism can be used for identification of any other bacterial pathogens. Clearly, additional study is necessary to analyze this hypothesis.

In conclusion, the data of the present study demonstrate that *cpn60* gene-based multiplex-PCR assay can be effectively used for simultaneous identification of *S. agalactiae, S. dysgalactiae* and *S. uberis* strains. This novel approach can be employed for analysis of the milk products and for monitoring mastitis.

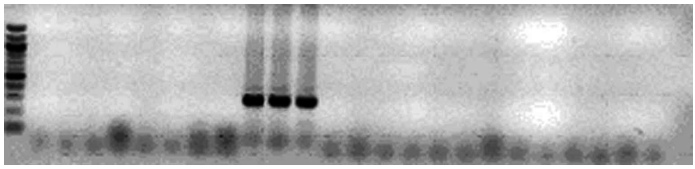

Fig. 2. PCR analysis of different bacterial species using *S. agalactiae* specific primers AGAFor and AGARev

Lane 1: 100 bp ladder;
Lanes 2-4: *S. uberis* strains;
Lanes 5-9: *S. dysgalactiae* strains;
Lane 10: human *S. agalactiae* strain;
Lanes 11-12: bovine *S. agalactiae* strains;
Lanes 13-14: *S. aureus* strains;
Lanes 15-21: *E. coli* strains;
Lanes 22-26: *S. typhimurium* strains.

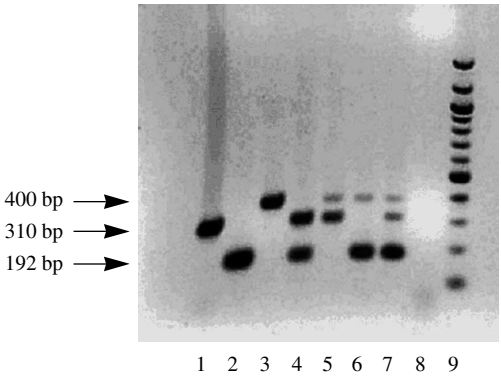

Fig. 3. Multiplex-PCR assay for simultaneous identification of *S. agalactiae*, *S. dysgalactiae* and *S. uberis* strains

Lane 1: sample containing *S. agalactiae*;
Lane 2: sample containing *S. dysgalactiae*;
Lane 3: sample containing *S. uberis*;
Lane 4: sample containing *S. agalactiae* and *S. dysgalactiae*;
Lane 5: sample containing *S. agalactiae* and *S. uberis*;
Lane 6: sample containing *S. uberis* and *S. dysgalactiae*;
Lane 7: sample containing *S. agalactiae*, *S. uberis* and *S. dysgalactiae*;
Lane 8: negative control;
Lane 9: 100 bp ladder.

Paralelná identifikácia génov cpn60 multiplex-PCR pre druhy streptokokov

Gény *cpn60 Streptococcus agalactiae*, *Streptococcus disgalactiae* a *Streptococcus uberis* boli sekvenované a poukázali na prítomnosť polymorfizmov. U navrhnutých špecifických primerov bola potvrdená ich špecifickosť pomocou PCR. Na základe týchto údajov bola vyvinutá cpn60 - multiplex - PCR. Pomocou navrhnutej PCR je možné paralelne identifikovať *Streptococcus agalactiae, Streptococcus disgalactiae* a *Streptococcus uberis*.

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