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Development of Specific and Rapid Detection of Bacterial Pathogens in Dairy Products by PCR

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ABSTRACT. A simple and specific method for direct detection of bovine mastitis pathogens (*Streptococcus agalactiae* (GBS), *Staphylococcus aureus* and *Escherichia coli*) in milk products, bacterial samples from milk and isolated bacterial DNA was developed. The method is based on polymerase chain reaction (PCR) using sequence-specific primers only for GBS and species-specific primers derived from 16S and 23S rRNA for all chosen species. The presence of the gene of surface immunogenic protein (Sip) in bovine GBS isolates, described previously only in human GBS isolates was confirmed. The GBS detection was performed with the sequence coding for surface immunogenic protein from GBS human isolates designated as Sip specific sequence (SSS); this sequence was selected for specific primer design. The sequence is unique for GBS and was designed from a consensus of all known *sip* genes. The specific identification was shown on a collection of 75 GBS bovine isolates from different localities in Slovakia. All isolates were positive to SSS, 16S and 23S rRNA sequence. The 16S and 23S rRNA PCR detection was also performed with *S. aureus* and *E. coli* isolates and specific PCR products were also detected. The detection limit of this assay for milk products was 6 CFU/μL (*i.e*. 6000 CFU/mL) for GBS and *E. coli*, and 16 CFU/μL for *S. aureus*. This rapid, sensitive and specific diagnostic method can be performed within hours and represents an innovative diagnostic tool for the detection of milk pathogens in dairy products.

Bovine mastitis, which is an inflammation of mammary gland, can have a clinical form with local and in some cases general clinical signs and milk abnormalities, or subclinical with production losses and lowered milk quality. Both forms produce significant economic losses due to rejected milk (less production), degradation of milk quality and higher production costs (Gruet *et al.* 2001; Tkáčiková *et al.* 2004; Vorobieva *et al.* 2005). The vast majority of mastitis causes are of bacterial origin and just five species of bacteria *Escherichia coli*, *Streptococcus uberis*, *Staphylococcus aureus*, *Streptococcus dysgalactiae* and *Streptococcus agalactiae*, account for almost 80 % of all diagnoses (Bradley 2002). In dairy animals *S. agalactiae* (GBS) is one of the major causes of intramammary infections (mastitis); it is frequently isolated from milk and may have a substantial impact to the quantity and quality of produced milk (Merl *et al.* 2003).

The suitability of a detection method for routine diagnosis depends on several factors such as specificity, sensitivity, cost, amount of time required and applicability to large numbers of milk samples. The common diagnosis of invasive GBS infection is based on identification of mammary gland pathogens by *in vitro* culture; however, this technique is labor-intensive and time-consuming (Baker and Edwards 2001; Shet and Ferrieri 2004). Therefore, new and time nonconsuming methods are preferred. Rapid diagnostic tests are based on identification of the GBS group-specific polysaccharide antigen from swab specimens, latex agglutination or enzyme-linked immunosorbent (ELISA) technology (Walker *et al.* 1992; Shet and Ferrieri 2004). Although they have good specificity (95 %), they tend to have low sensitivity (33–65 %), which increases only with heavy colonization, hence a negative test cannot rule out GBS colonization (Das *et al.* 2003; Shet and Ferrieri 2004). Recently, PCR method is mostly used for the detection of microorganisms in milk or in other organic samples; the overall approach is time-consuming due to the preceding isolation of genomic DNA or bacteria multiplying step in culture media are needed before. The identification could be confirmed also by PCR amplification of species-specific parts of 23S rRNA (Kawata *et al.* 2004) or 16S rRNA genes (Yildirim *et al.* 2002). The use of multiplex PCR as a rapid method has been described for detection of pathogens (Markowska-Daniel and Kowalczyk 2005; Růžičková *et al.* 2006; Zeng *et al.* 2006). Another pos-

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sibility is the use of PCR for the detection of genes coding for GBS cell-surface-associated proteins, *e.g.*, genes encoding the C_α and Rib (Flores *et al.* 1989; Stalhåmmar-Carlemalm *et al.* 1993; Navarre and Schneewind 1999), immunoglobulin A-binding β-antigen (*bac*), the α-antigen (*bca*), C_β antigen (Brady and Boyle 1989; Maeland *et al.* 1997; Navarre and Schneewind 1999), the laminin-binding surface protein (*lmb*), and the C5a peptidase (*scpB*) (Duarte *et al.* 2004). The other identified outer-surface proteins include R proteins (Lancefield and Perlmann 1952), X proteins (Rainard *et al.* 1991), glutamine synthetase (Suvorov *et al.* 1997), α-enolase (Pancholi and Fischetti 1998), Hsp70 (Hamel *et al.* 1996) and Sip protein (Brodeur *et al.* 2000). Sip (localized on the cell surface) is an antigen capable of raising a protective immune response and it was detected in human GBS isolates (Brodeur *et al.* 2000; Hughes *et al.* 2002). However, among these proteins, only Sip and C5a-ase are conserved at the gene level in the majority of GBS isolates (Brodeur *et al.* 2000; Tettelin *et al.* 2000; Maione *et al.* 2005).

Here we report on the development of a simple, rapid, specific and cheap method for detection of pathogens involved in bovine mastitis (GBS, *S. aureus* and *E. coli*) by PCR. Species-specific primer pairs from s*ip* gene sequences denoted Sip specific sequence (SSS) for GBS and from 16S and 23S rRNA for all three pathogens were designed to reach this objective. The method was carried out in milk (or dairy products) and isolated bacterial DNA. Finally, the sensitivity limits of PCR assays in samples were analyzed.

MATERIALS AND METHODS

Bacterial strains and media. Seventy-five streptococcal isolates were collected from different farms in Slovakia and were identified as *Streptococcus agalactiae* belonging to Lancefield's serological group B. The identification was performed by determining of cultural biochemical analyses according to the CAMP test (Christie, Atkinson and Munch-Petersen) and serological properties based on surface polysaccharide antigens (Skinner and Quesnel 1978). *S. dysgalactiae*, *S. uberis*, *S. pyogenes*, and *S. faecalis* were collected from bovine mastitis of animals from different farms all around Slovakia. The strains *Streptococcus gordonii* CCM1045, *S. salivarius* CCM4046, *S. thermophilus* CCM4757, *E. coli*, *Staphylococcus aureus*, *S. dysgalactiae*, *S. uberis*, *S. pyogenes*, and *S. faecalis* were obtained from the Collection of Microorganisms of the *Institute of Molecular Biology, Slovak Academy of Sciences* and *University of Veterinary Medicine*, Košice, Slovakia. Three types of growth media were used: Todd–Hewitt broth (THB), Luria–Bertani (LB) broth (Sambrook *et al.* 1989) and blood agar; media were purchased from *Imuna*, Slovakia. GBS, *S. aureus* and *E. coli* strains were grown overnight on a rotary shaker at 37 °C. If agar plates were used all media were solidified with 1.5 % agar (*Serva*).

PCR conditions. Reactions were performed in a final volume of 20 μL. The reaction mixture contained 0.5 U DyNAzyme^{TM} DNA polymerase, 0.4 μL 10 mmol/L dNTP Mix, 2 μL Optimized DyNAzyme^{TM} 10× reaction buffer (all *Finnzymes*), and 1 μL of each primer (25 μmol/L). The amplification program consisted of denaturation (5 min, 96 °C), 30 cycles of denaturation (1 min, 96 °C), annealing (1 min, 55 °C) and extension (2 min, 72 °C), followed by a final extension (8 min, 72 °C). Ten μL of PCR-amplified product was analyzed by electrophoresis on 0.9 % agarose gel and stained with ethidium bromide; this was done in 1× BBE (in mmol/L: boric acid 650, disodium tetraborate 29, EDTA 250; pH 7.8). Under these conditions genomic DNA (0.3 mg/L) or bacterial suspension (2 μL) was used as a template.

Multiplex PCR was performed under the same conditions; here, bacterial suspensions of all three pathogens were used with SAGA1 and SAGA2, Ecoli1 and Ecoli2, SAU1 and SAU2, SIP3(F) and SIP4(R) primers. The molecular-size markers, a 100-bp DNA ladder (*BioLabs*), λ DNA–*Mlu*I digest, and mixture of λ DNA *Hin*dIII digest and φX174 DNA *Hae*III digest (*Finnzymes*) were run concurrently. Agarose gels were visualized under UV transilluminator (296 nm).

PCR primers were designed from species-specific regions of the DNA coding for 16S and 23S rRNA based on the sequence of the entries available in *GenBank* database (*S. agalactiae* accession no. NC 004368, NC004368; *E. coli* accession no. AB035925, AB035926, D12649; *S. aureus* accession no. AY688035, AY688034, AY688033, AY688032, AY688031) (Table I).

The primer pairs for detection of SSS were designed as a consensus sequence from the comparison of *sip* genes sequence from human GBS isolate entries available in *GenBank* database (*S. agalactiae* accession no. AF151357, AF151358, AF151359, AF151361, AF151362, NC 004368). The primer pairs were synthesized by *PROLIGO Primers & Probes* (France).

Preparation of genomic DNA for PCR. The genomic DNA was obtained from cells, formally pelleted from 3 mL of overnight culture by centrifugation (133 Hz, 15 min). The cells were resuspended in 250 μL of GET buffer (in mmol/L: glucose 50, Tris-HCl-Cl 25, EDTA 10; pH 8). Five μL of lysozyme solution (10 g/L) and 10 mg/L of lysostaphine were added and mixed. After a 1-h incubation at 37 °C,

sodium dodecylsulfate (SDS; 10 g/L final concentration) was added and incubated (to achieve complete lysis) for 5 min at 50 °C, 5 min in an ice bath, and 5 min at 37 °C. Then the solution was deproteinized two times with equal volumes of phenol–chloroform (1 : 1) and chloroform–3-methylbutan-1-ol (24 : 1), again two times. DNA was precipitated by pouring the aqueous phase into 1 mL ethanol with 1/10 of volume of 3 mol/L potassium acetate (pH 7) and incubated for 1 h at –20 °C. Samples were washed with 70 % ethanol and DNA was resuspended in 50 μL of TE buffer (10 mmol/L Tris, pH 7.5; 1 mmol/L EDTA); 0.3 mg/L of isolated DNA was used for PCR.

Table I. Primers used

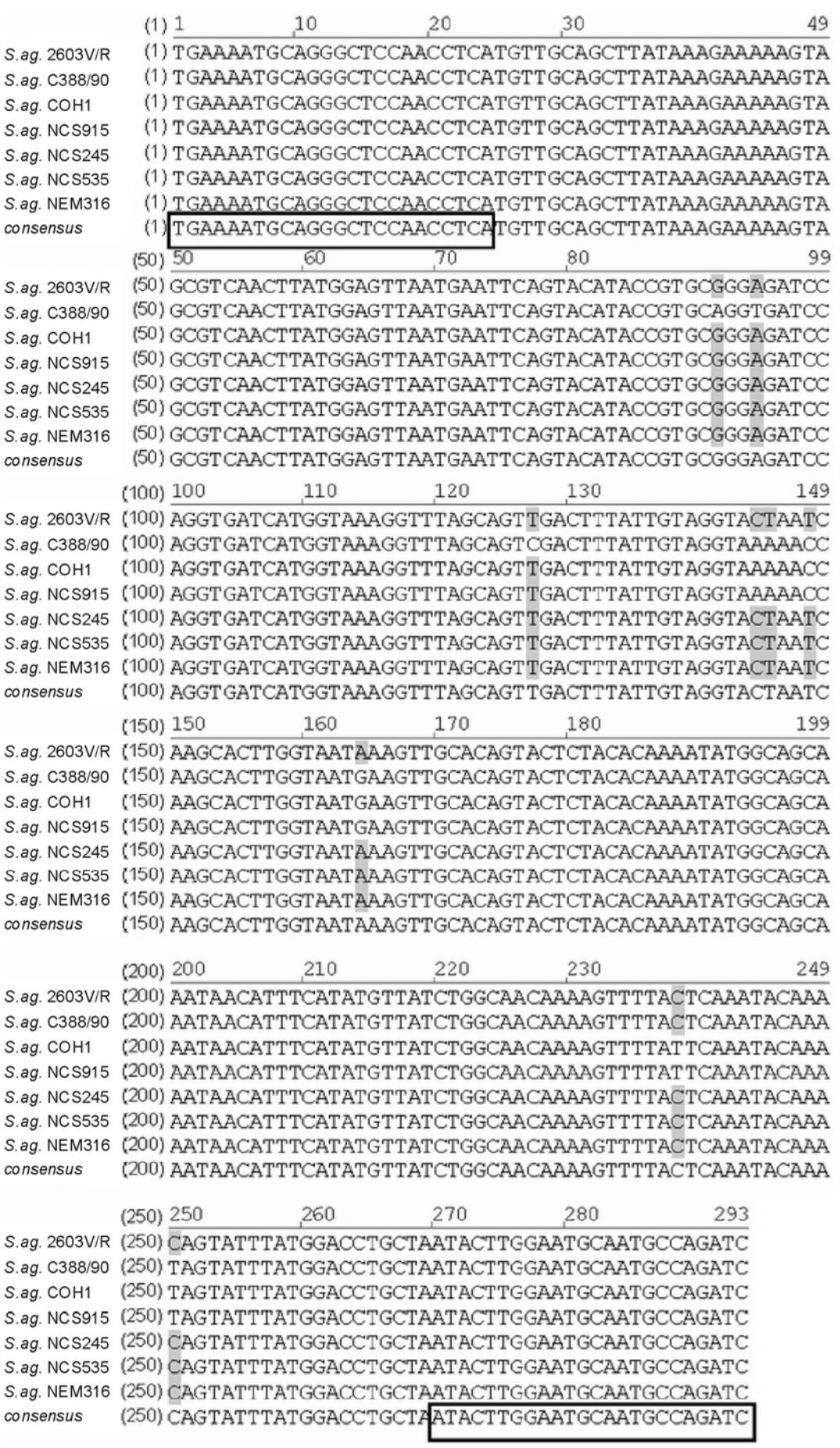
Strain(s)	Primer	Primer sequence	Product length, bp
GBS	SAGA1	5′-CGT TGG TAG GAG TGG AAA AT-3′	590
	SAGA2	5′-CTG CTC CGA AGA GAA AGC CT-3′	
	SIP3(F)	5′-TGA AAA TGC AGG GCT CCA ACC TCA-3′	293
	SIP4(R)	5′-GAT CTG GCA TTG CAT TCC AAG TAT-3′	
E. coli	Ecoli1	5′-GCT TGA CAC TGA ACA TTG AG-3′	660
	Ecoli2	5′-GCA CTT ATC TCT TCC GCA TT-3′	
S. aureus	SAU1	5′-GGA CGA CAT TAG ACG AAT CA-3′	1300
	SAU2	5′-CGG GCA CCT ATT TTC TAT CT-3′	

Preparation of bacterial suspension for PCR. One mL of overnight culture (GBS in THB, *S. aureus* and *E. coli* in LB) was transferred into a sterile Eppendorf tube and centrifuged (3 min, 167 Hz). The pellet was washed twice with sterile distilled water, once with 0.1 mol/L phosphate-buffered saline (pH 7.2) (PBS), and resuspended in 1 mL of deionized autoclaved water to allow the burst of bacterial cells. Two-μL samples of these preparations were used directly for PCR. With the aim to prepare sample with milk background, the pellet from an overnight culture was first resuspended in sterile bovine milk (UHT-treated) and then washed with water and PBS. The number of colony-forming units (CFU) needed for detection of PCR product was determined by cultivation.

DNA sequence analysis. DNA obtained from PCR amplifications were sequenced and compared with *GenBank* sequences using the BLAST method (Altschul *et al.* 1990).

RESULTS AND DISCUSSION

Detection of Sip specific sequence in GBS isolates. The most widely used methods of GBS detection depend on detectable amounts of streptococcal toxins (Ke and Bergeron 2001). Therefore, it was necessary to find a specific sequence of surface protein which is unique only for GBS, and use it for rapid and specific detection of GBS in biological material. Comparison of the GBS genomes with the genomes of two other pathogenic streptococci *S. pyogenes* and *S. pneumoniae* showed that most of the predicted GBS proteins had homologs in at least one of the two other species (Ferretti *et al.* 1998; Tettelin *et al.* 2001). Interestingly, these regions contain genes of many predicted surface proteins and putative virulence factors and include many of the genes that are unique to GBS (Glaser *et al.* 2002; Tettelin *et al.* 2002; Lindahl *et al.* 2005). One of the surface proteins specific for GBS is the surface immunogenic protein – Sip. We focused on the use of this protein as a specific marker for GBS strains. The nucleotide sequences of *sip* genes were found to be highly conserved and, at the nucleotide level, these genes showed differences which made them 98 % identical. BLAST comparison showed that the sequence of *sip* gene from human GBS isolates was not detected in other sequenced bacterial genomes (Brodeur *et al.* 2000). The comparison of *sip*-gene sequences from human GBS isolates from *GenBank* (accession no. AF151357, AF151358, AF151359, AF151361, AF151362, NC 004116, NC 004368) revealed a region with high homology (96.9 % identity). This specific sequence was not present in other sequenced bacterial genomes, which made it unique for GBS. The consensus sequence was named Sip-specific sequence – SSS (Fig. 1). The SSS is localized 948 bp downstream of the start codon in *sip* gene and is 293 bp large. BLAST comparison revealed that the SSS sequence is not present in other bacterial genomes; this was confirmed by PCR (*see below*). SIP3(F) and SIP4(R) primers were used for SSS detection. All of 75 GBS bovine isolates tested for the presence of SSS gave the same 293 bp fragment (Fig. 2). The presence of SSS was also tested in other streptococci: *S. pyogenes*, *S. faecalis*, *S. gor-*


(1) 1 10 20 30 49
S.ag. 2603V/R (1) TGAAAATGCAGGGCTCCAACCTCATGTTGCAGCTTATAAAGAAAAAGTA
S.ag. C388/90 (1) TGAAAATGCAGGGCTCCAACCTCATGTTGCAGCTTATAAAGAAAAAGTA
S.ag. COH1 (1) TGAAAATGCAGGGCTCCAACCTCATGTTGCAGCTTATAAAGAAAAAGTA
S.ag. NCS915 (1) TGAAAATGCAGGGCTCCAACCTCATGTTGCAGCTTATAAAGAAAAAGTA
S.ag. NCS245 (1) TGAAAATGCAGGGCTCCAACCTCATGTTGCAGCTTATAAAGAAAAAGTA
S.ag. NCS535 (1) TGAAAATGCAGGGCTCCAACCTCATGTTGCAGCTTATAAAGAAAAAGTA
S.ag. NEM316 (1) TGAAAATGCAGGGCTCCAACCTCATGTTGCAGCTTATAAAGAAAAAGTA
consensus (1) TGAAAATGCAGGGCTCCAACCTCATGTTGCAGCTTATAAAGAAAAAGTA
(50) 50 60 70 80 99
S.ag. 2603V/R (50) GCGTCAACTTATGGAGTTAATGAATTCAGTACATACCGTGCGGGAGATCC
S.ag. C388/90 (50) GCGTCAACTTATGGAGTTAATGAATTCAGTACATACCGTGCAGGTGATCC
S.ag. COH1 (50) GCGTCAACTTATGGAGTTAATGAATTCAGTACATACCGTGCGGGAGATCC
S.ag. NCS915 (50) GCGTCAACTTATGGAGTTAATGAATTCAGTACATACCGTGCGGGAGATCC
S.ag. NCS245 (50) GCGTCAACTTATGGAGTTAATGAATTCAGTACATACCGTGCGGGAGATCC
S.ag. NCS535 (50) GCGTCAACTTATGGAGTTAATGAATTCAGTACATACCGTGCGGGAGATCC
S.ag. NEM316 (50) GCGTCAACTTATGGAGTTAATGAATTCAGTACATACCGTGCGGGAGATCC
consensus (50) GCGTCAACTTATGGAGTTAATGAATTCAGTACATACCGTGCGGGAGATCC
(100) 100 110 120 130 149
S.ag. 2603V/R (100) AGGTGATCATGGTAAAGGTTTAGCAGTTGACTTTATTGTAGGTACTAATC
S.ag. C388/90 (100) AGGTGATCATGGTAAAGGTTTAGCAGTCGACTTTATTGTAGGTAAAAACC
S.ag. COH1 (100) AGGTGATCATGGTAAAGGTTTAGCAGTTGACTTTATTGTAGGTAAAAACC
S.ag. NCS915 (100) AGGTGATCATGGTAAAGGTTTAGCAGTTGACTTTATTGTAGGTAAAAACC
S.ag. NCS245 (100) AGGTGATCATGGTAAAGGTTTAGCAGTTGACTTTATTGTAGGTACTAATC
S.ag. NCS535 (100) AGGTGATCATGGTAAAGGTTTAGCAGTTGACTTTATTGTAGGTACTAATC
S.ag. NEM316 (100) AGGTGATCATGGTAAAGGTTTAGCAGTTGACTTTATTGTAGGTACTAATC
consensus (100) AGGTGATCATGGTAAAGGTTTAGCAGTTGACTTTATTGTAGGTACTAATC
(150) 150 160 170 180 199
S.ag. 2603V/R (150) AAGCACTTGGTAATAAAGTTGCACAGTACTCTACACAAAATATGGCAGCA
S.ag. C388/90 (150) AAGCACTTGGTAATGAAGTTGCACAGTACTCTACACAAAATATGGCAGCA
S.ag. COH1 (150) AAGCACTTGGTAATGAAGTTGCACAGTACTCTACACAAAATATGGCAGCA
S.ag. NCS915 (150) AAGCACTTGGTAATGAAGTTGCACAGTACTCTACACAAAATATGGCAGCA
S.ag. NCS245 (150) AAGCACTTGGTAATAAAGTTGCACAGTACTCTACACAAAATATGGCAGCA
S.ag. NCS535 (150) AAGCACTTGGTAATAAAGTTGCACAGTACTCTACACAAAATATGGCAGCA
S.ag. NEM316 (150) AAGCACTTGGTAATAAAGTTGCACAGTACTCTACACAAAATATGGCAGCA
consensus (150) AAGCACTTGGTAATAAAGTTGCACAGTACTCTACACAAAATATGGCAGCA
(200) 200 210 220 230 249
S.ag. 2603V/R (200) AATAACATTTCATATGTTATCTGGCAACAAAAGTTTTACTCAAATACAAA
S.ag. C388/90 (200) AATAACATTTCATATGTTATCTGGCAACAAAAGTTTTACTCAAATACAAA
S.ag. COH1 (200) AATAACATTTCATATGTTATCTGGCAACAAAAGTTTTATTCAAATACAAA
S.ag. NCS915 (200) AATAACATTTCATATGTTATCTGGCAACAAAAGTTTTATTCAAATACAAA
S.ag. NCS245 (200) AATAACATTTCATATGTTATCTGGCAACAAAAGTTTTACTCAAATACAAA
S.ag. NCS535 (200) AATAACATTTCATATGTTATCTGGCAACAAAAGTTTTACTCAAATACAAA
S.ag. NEM316 (200) AATAACATTTCATATGTTATCTGGCAACAAAAGTTTTACTCAAATACAAA
consensus (200) AATAACATTTCATATGTTATCTGGCAACAAAAGTTTTACTCAAATACAAA
(250) 250 260 270 280 293
S.ag. 2603V/R (250) CAGTATTTATGGACCTGCTAATACTTGGAATGCAATGCCAGATC
S.ag. C388/90 (250) TAGTATTTATGGACCTGCTAATACTTGGAATGCAATGCCAGATC
S.ag. COH1 (250) TAGTATTTATGGACCTGCTAATACTTGGAATGCAATGCCAGATC
S.ag. NCS915 (250) TAGTATTTATGGACCTGCTAATACTTGGAATGCAATGCCAGATC
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S.ag. NCS535 (250) CAGTATTTATGGACCTGCTAATACTTGGAATGCAATGCCAGATC
S.ag. NEM316 (250) CAGTATTTATGGACCTGCTAATACTTGGAATGCAATGCCAGATC
consensus (250) CAGTATTTATGGACCTGCTAATACTTGGAATGCAATGCCAGATC

donii CCM1045, *S. salivarius* CCM4046, *S. thermophilus* CCM4757, *S. dysgalactiae*, *S. uberis.* None of these strains showed the presence of this sequence, which makes it unique.

Detection of bovine mastitis pathogens in dairy samples. In parallel to GBS detection, a similar PCR method, based on 16S and 23S rRNA coding sequence, was established because segments of these rRNA sequences are highly conserved while the others vary (Forsman *et al.* 1997; Abdulmawjod and Lämmler 1999; Straub *et al.* 1999). Specific oligonucleotide primers based on 16S and 23S rRNA sequences of GBS, *S. aureus* and *E. coli* were used. The primer pair specificity was confirmed by the positive amplification of the DNA from GBS (SAGA1 and SAGA2), *S. aureus* (SAU1 and SAU2) and *E. coli* (Ecoli1 and Ecoli2); no amplification was observed when using bacteria with noncorresponding primer pairs. Detection limit of GBS DNA needed for PCR was 82 ng/L (*i.e.* 8.2×10^{-8} g/L), in case of *S. aureus* the detection limit was 1.5 ng/L DNA and with *E. coli* DNA it was 96 ng/L. The detection confirmed the specificity of PCR, and showed a potential use in diagnostics based on unique sequences. The only problem was the time-consuming preparation of DNA.

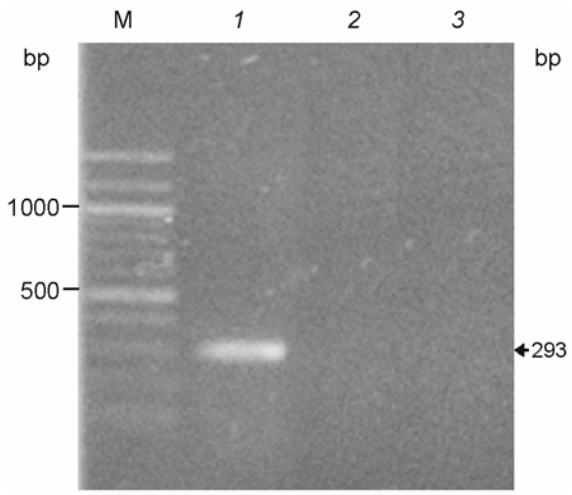

Fig. 2. Determination of specificity of primers SIP3(F) and SIP4(R) for SSS in PCR assay with bacterial cell suspension; *1* – *S. agalactiae*, *2* – *S. aureus*, *3* – *E. coli*; amplification products were analyzed on 0.9 % agarose gel, M – molecular size marker (100-bp DNA ladder (*BioLabs*).

To develop a rapid method for detection of these pathogens, the suspension of overnight-cultured bacterial cells after washing with water and PBS was used directly for PCR. The PBS was used to protect cell membranes from lysis during these steps, and to precipitate Ca^{2+} (which is known as PCR inhibitor; Bickley *et al.* 1996) contained in milk (when pellet from overnight culture was resuspended in it before washing steps). No differences were observed on PCR products amplification when the isolated DNA or bacterial cell suspensions were used in PCR reactions.

In addition, multiplex PCR mixture with primers SAGA1 and SAGA2, Ecoli1 and Ecoli2, SAU1 and SAU2, SIP3(F) and SIP4(R) was developed for rapid diagnostics. The multiplex PCR was performed with bacterial suspensions of all tested pathogens because PCR products of different sizes are very simple to distinguish among the microorganisms used. PCR products detected by this method were the same as products from single PCR (Fig. 3). The detection limits of the PCR performed on samples from overnight cultures were 3 CFU/μL (*i.e.* 3000 CFU/mL) for GBS, 8 CFU/μL for *S. aureus* and 3 CFU/μL for *E. coli*.

We also determined the influence of milk background on the limit of PCR detection. The level of sensitivity was diminished to 6 CFU/μL when the GBS cells were resuspended in milk before washing (Fig. 4A, B). Similarly, two-fold decrease of sensitivity was observed when the cells of *S. aureus* and *E. coli* were resuspended in milk before washing (Fig. 4C, D).

Fig. 1. Comparison of nucleotide sequences of SSS from *S. agalactiae* from strains 2603V/R (NC004116), C388/90 (AF151357), COH1 (AF151358), NCS915 (AF151362), NCS246 (AF151359), NCS535 (AF151361) and NEM316 (NC004368); differences are in *grey*; primers are boxed; forward SIP3(F) is in the beginning of the consensus sequence; reverse sequence of primer SIP4(R) is at the end of the consensus sequence.

Generally, the detection limit levels were very low and this assay can therefore be used as a diagnostic tool for bovine mastitis pathogens. Moreover, PCR is less labor-intensive and more rapid than bacteria culturing and conventional methods of bacterial identification.

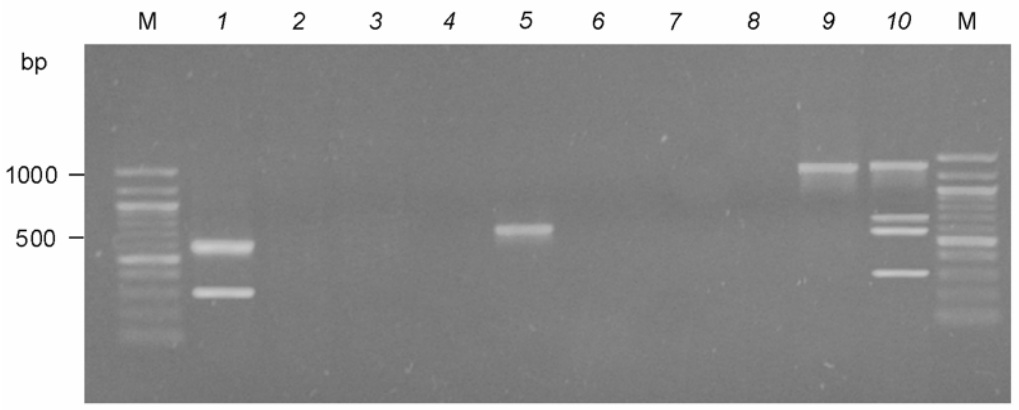

Fig. 3. Determination of specificity of primers in PCR assay with bacterial cell suspensions; amplification products of the different primer combinations were analyzed by electrophoresis in 0.9 % agarose gel. Lanes: *1–3* – *S. agalactiae* with primers SAGA1 and SAGA2 and SIP3(F) and SIP4(R) (*1*), Ecoli1 and Ecoli2 (*2*), SAU1 and SAU2 (*3*); *4–6* – *E. coli* with primers SAGA1 and SAGA2 (*4*), Ecoli1 and Ecoli2 (*5*), SAU1 and SAU2 (*6*); *7–9* – *S. aureus* with primers SAGA1 and SAGA2 (*7*), Ecoli1 and Ecoli2 (*8*), SAU1 and SAU2 (*9*); *10* – multiplex PCR with all 8 primers; M – 100-bp DNA ladder (*BioLabs*).

Recent development of PCR technology has provided a new detection platform for identification of bacteria. We present here a rapid diagnostic method for pathogen (*S. agalactiae*, *S. aureus* and *E. coli*) detection directly from milk (or dairy products) samples without cultivation and DNA isolation which provides cost and time reduction. Detection primers designed from SSS, and 16S and 23S rRNA sequences were successfully used (as shown with milk or diary products, and isolated DNA). The multiplex PCR provides a rapid, highly specific detection; it can be simply implemented into veterinary practice or food-microbiology laboratories.

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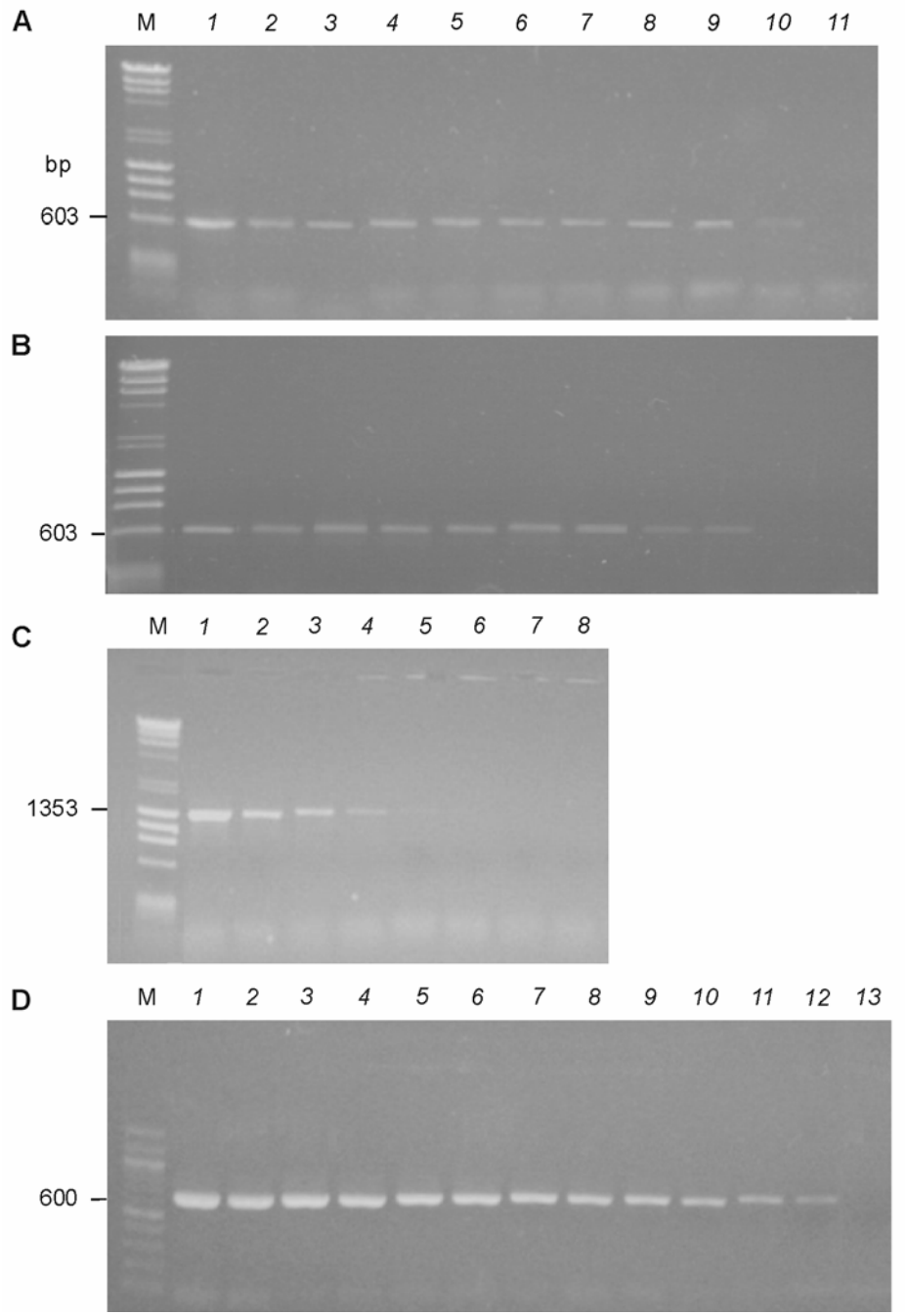

Fig. 4. Sensitivity of the PCR assay in detecting colony-forming units (in CFU/μL) *S. agalactiae* from two different media THB (**A**) and milk (**B**), *S. aureus* (**C**) and *E. coli* (**D**); concentrations (in CFU/μL) of bacteria used were: **A**, **B**: *1* – 1500, *2* – 770, *3* – 385, *4* – 192, *5* – 96, *6* – 48, *7* – 24, *8* – 12, *9* – 6, *10* – 33, *11* – 1.5; **C**: *1* – 82100, *2* – 537, *3* – 134, *4* – 33, *5* – 8, *6* – 2, *7* – 0.5, *8* – 0.125; **D**: *1* – 6210, *2* – 3080, *3* – 1540, *4* – 770, *5* – 385, *6* – 192, *7* – 96, *8* – 48, *9* – 24, *10* – 12, *11* – 6, *12* – 3, *13* – 1.5; amplification products were analyzed on 0.9 % agarose gel; M – molecular-size markers: A, B, C: mixture of λ DNA *Hin*dIII digest and φX174 DNA *Hae*III digest (*Finnzymes*), D: 100-bp DNA ladder (*BioLabs*).

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