Development of PCR assays for detection of *Streptococcus canis*

A.A. Hassan a, I.U. Khan b, A. Abdulmawjood a, C. Lämmler c,*

a Institut für Tierärztliche Nahrungsmittelkunde, Professur für Milchwissenschaften, Justus-Liebig-Universität Gießen, Ludwig Str. 21, 35390 Gießen, Germany  
b Department of Environmental Health, Toxicology Division, 3223 Eden Ave, Medical Center, University of Cincinnati, Cincinnati, OH 45267-0056, USA  
c Institut für Pharmakologie und Toxikologie, Justus-Liebig-Universität Gießen, Frankfurter Str. 107, 35392 Gießen, Germany

Received 18 September 2002; accepted 19 December 2002  
First published online 31 January 2003

Abstract

*Streptococcus canis* isolates, also including *S. canis* of artificially contaminated milk, could be identified by polymerase chain reaction (PCR) amplification using oligonucleotide primers designed according to species-specific parts of the 16S rRNA gene and, after sequencing, according to *S. canis*-specific parts of the 16S–23S rDNA intergenic spacer region and with oligonucleotide primers detecting an internal fragment of the group G streptococcal CAMP factor gene *cfg*. The 16S rRNA gene- and CAMP factor gene *cfg*-specific oligonucleotide primers could be used together in a multiplex PCR. No cross-reactivities could be observed with other group G streptococcal isolates or with any of the other control strains of various streptococcal species and serogroups. The PCR methods presented in this study allowed a rapid and reliable identification of *S. canis* and might help to improve the diagnosis of this bacterial species in animal and human infections.

© 2003 Published by Elsevier Science B.V. on behalf of the Federation of European Microbiological Societies.

Keywords: *Streptococcus canis*; 16S rRNA gene; 16S–23S rDNA intergenic spacer region; CAMP factor gene *cfg*; Multiplex polymerase chain reaction

1. Introduction

Streptococci of serological group G occur as either minute or large colony strains. The large colony form can be isolated from infections of animals and humans. The large colony group G streptococci isolated from animals were characterized and differentiated from those isolated from humans. They comprise a single species which was named *Streptococcus canis* [1]. The molecular and chemotaxonomic studies of Farrow and Collins [2], summarized by Schleifer and Kilpper-Bälz [3], revealed biochemical and genetic similarities between group G streptococci isolated from humans, the group C streptococcal species *S. dysgalactiae* and *S. equisimilis* and group L streptococci. According to Vandamme et al. [4] for group G streptococci isolated from humans the name *S. dysgalactiae* subsp. *equisimilis* was proposed. However, the characteristics of *S. canis* isolated from dogs and bovines, such as a positive α- or β-galactosidase reaction or a negative trehalose reaction, differed clearly from those of human group G streptococci [5,6]. *S. canis* is well known as a causative agent of bovine mastitis [7–9], as the cause of various infections of dogs and cats [10–12], from cases of septicaemia of harbor porpoises [13], rarely from human infections [14,15]. In dogs *S. canis* has been isolated from skin, urogenital and respiratory tract infections [10], otitis externa [16], from streptococcal toxic shock syndrome and necrotizing fasciitis [17–19] and also as part of the normal microflora of the uterus of bitches and female cats [20,21].

The identification of *S. canis* traditionally relies on the determination of biochemical properties and on serological grouping by use of Lancefield antisera. However, developments in nucleic acid technology such as polymerase chain reaction (PCR) have resulted in new methods that can be used for identification of bacteria.

The aim of the present study was the development of PCR and multiplex PCR assays targeted to *S. canis*-specific parts of the gene encoding the 16S rRNA, the 16S–23S rDNA intergenic spacer region and the gene *cfg* encoding group G streptococcal CAMP factor and the use of these PCR assays for the rapid and reliable identification of this species.
2. Materials and methods

2.1. Bacterial strains

A total number of 102 streptococci obtained from the institute’s strain collection were used in this study. The cultures included isolates of the species *S. canis* (16 isolates from canines, 12 isolates from bovines), *S. dysgalactiae* subsp. *equisimilis* (serogroup G) (*n* = 11), *S. dysgalactiae* subsp. *dysgalactiae* (serogroup C) (*n* = 6), *S. dysgalactiae* subsp. *dysgalactiae* (serogroup L) (*n* = 6), *S. pyogenes* (*n* = 4), *S. agalactiae* (*n* = 12), *S. equi* subsp. *equi* (*n* = 2), *S. equi* subsp. *zooepidemicus* (*n* = 3), *S. iberis* (*n* = 12), *S. parauberis* (*n* = 2), *S. porcinus* (*n* = 12) and *S. suis* (*n* = 4). The 16 canine isolates of *S. canis* were isolated from nose, uterus, spleen, stool, urine and vagina (*n* = 1, 1, 1, 4 and 8, respectively), the 12 bovine isolates were isolated from stool, urine and milk (*n* = 1, 1, 3 and 7, respectively).

2.2. PCR amplification of species-specific parts of the 16S rRNA gene, the 16S–23S rDNA intergenic spacer region and the CAMP factor gene cgf

Based on the sequence analysis of the V2 region of the 16S rRNA gene given by Bentley and Leigh [22] species-specific primers were designed using the computer program Oligo 4.0. Sequencing of the 16S–23S rDNA intergenic spacer region and the CAMP factor gene cgf was performed using facilities of the university in Gießen (Institut für Medizinische Mikrobiologie) with the MegaBACE 1000 DNA Sequencing System (Amersham Pharmacia Biotech Europe, Freiburg, Germany). The sequencing data were further studied and analyzed with computer program Chromas 1.42, 1996–1997 (Brisbane, Queensland, Australia) and program MegAlign, 1993–1997 (DNASTAR, Konstanz, Germany). Based on the sequencing results species-specific primers were designed using the primer design program. In addition, the sequencing results of the CAMP factor gene cgf described previously [23] were used to develop cgf-specific oligonucleotide primers. The target genes, the primer sequences and the thermal cycler programs are summarized in Table 1. The oligonucleotide primers were synthesized by MWG-Biotech (Ebersberg, Germany).

For DNA preparation five to 10 colonies of *S. canis* were suspended in 100 μl TE buffer (10 mmol l⁻¹ Tris–HCl, 1 mmol l⁻¹ EDTA, pH 8.0) containing 5 μl mutanolysin (10 U μl⁻¹) (Sigma, Deisenhofen, Germany) for 60 min at 37°C in a water bath and 10 μl proteinase K (14.8 mg ml⁻¹, Boehringer, Mannheim, Germany) for 120 min at 56°C. In parallel experiments mixed streptococcal cultures were used including: (1) *S. canis* (canine isolate), *S. dysgalactiae* subsp. *equisimilis* (serogroup G) and *S. agalactiae*; (2) *S. dysgalactiae* subsp. *equisimilis* (serogroup G), *S. iberis* and *S. parauberis*; (3) *S. canis* (bovine isolate), *S. dysgalactiae* subsp. *dysgalactiae* (serogroup C), *S. dysgalactiae* subsp. *equisimilis* (serogroup G) and *S. dysgalactiae* subsp. *dysgalactiae* (serogroup L); and (4) *S. canis* (bovine isolate), *S. porcinus* and *S. agalactiae*. For DNA preparation these bacteria were suspended in 100 μl TE buffer containing 7 μl mutanolysin (10 U μl⁻¹) for 60 min at 37°C in a water bath and 15 μl proteinase K (14.8 mg ml⁻¹) for 120 min at 56°C. After boiling for 10 min at 100°C the suspension was centrifuged (10,000×g, 15 s) and cooled before use.

The PCR reaction mixture (30 μl) contained 1 μl primer 1 (10 pmol μl⁻¹), 1 μl primer 2 (10 pmol μl⁻¹), 0.6 μl dNTP (10 mmol, MBI Fermentas, St. Leon-Rot, Germany), 3 μl 10× thermophilic buffer (Promega, Mannheim, Germany), 1.8 μl MgCl₂ (25 mmol) (Promega), 0.2 μl *Taq* DNA polymerase (5 U μl⁻¹, Promega) and 19.9 μl distilled water. Finally 2.5 μl DNA preparation was added to each reaction tube. The tubes were then subjected to 30 cycles on a thermal cycler (Techne-Progene, Thermodux, Wertheim, Germany) with the programs described in Table 1. For multiplex PCR the reaction mixture (30 μl) contained as first primer pair (16S rRNA gene) 0.6 μl primer can I (10 pmol μl⁻¹), 0.6 μl

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Oligonucleotide primer</th>
<th>Sequence (5′–3′)</th>
<th>Size of the PCR product (bp)</th>
<th>PCR programs</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S rRNA</td>
<td>can I</td>
<td>AGTGGTTAAACACATGTTAAGAA</td>
<td>1320</td>
<td>1*</td>
</tr>
<tr>
<td></td>
<td>can II</td>
<td>GATAGATGATTGGGTTGAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16S–23S rDNA intergenic spacer</td>
<td>c-I</td>
<td>TAAACCCGAAACGCTGTAAGATTA</td>
<td>215</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>c-II</td>
<td>ACCATATGTAGGGTTCCGTTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAMP factor cgf</td>
<td>camp-canis-I</td>
<td>CAAATTACTAAATAGTAGAACAG</td>
<td>238</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>camp-canis-II</td>
<td>CTCTCCTCAAACGGGTTG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*1 = 30× (94°C 60 s, 58°C 90 s, 72°C 90 s); 2 = 30× (94°C 10 s, 64°C 30 s, 72°C 10 s).
Fig. 1. Sequence alignment of a partial segment of the 16S rRNA gene, the complete 16S–23S rDNA intergenic spacer region and a partial segment of the 23S rRNA gene of *S. canis* isolated from dogs (G2567, G2643, G3232, G5093) and cows (H113/108, M113/38). The marked areas indicate the differences in nucleotide sequences. The region arrangement was performed according to Chanter et al. [24]; stuffer regions to achieve alignment are indicated by dashes.
primer can II (10 pmol µl⁻¹), and as second primer pair (CAMP factor gene *cfg*) 0.4 µl primer camp-canis-I (10 pmol µl⁻¹), 0.4 µl primer camp-canis-II (10 pmol µl⁻¹). In addition 0.6 µl dNTP (10 mmol), 3 µl 10× thermostable buffer, 1.8 µl MgCl₂ (25 mmol), 0.2 µl *Taq* DNA polymerase (5 U µl⁻¹) and 19.9 µl distilled water and 2.5 µl bacterial DNA was added. The multiplex PCR was performed using program 1 mentioned in Table 1. The presence of PCR products was determined by electrophoresis of 12 µl of the reaction product in a 2% agarose gel (Sigma) with Tris acetate electrophoresis buffer (TAE, 4.0 mmol l⁻¹ Tris, 1 mmol l⁻¹ EDTA, pH 7.8), a 100-bp DNA ladder (Gibco BRL, Eggenstein, Germany) or peQ-GOLD 100-bp DNA ladder (PEQLAB, Erlangen, Germany) as molecular marker.

For direct detection of *S. canis* DNA bovine sterile milk was artificially contaminated with the *S. canis* strain H113/108 at a concentration of 3 × 10⁵ CFU ml⁻¹. To determine the limit of detection of the PCR assays serial dilutions were prepared in TE buffer. The number of bacteria in the various dilutions was calculated after plating 100 µl of each sample on blood agar. Another 100 µl from each dilution was mixed with 500 µl sterile bovine milk. In all experiments one sample of sterile milk was included as negative control. The DNA extraction method was originally described for the detection of *Brucella* spp. in milk [26]. The contaminated milk samples (600 µl) were centrifuged (13 000 g, 5 min) the pellet washed two times with 400 µl NET buffer (50 mM NaCl, 125 mM EDTA, 50 mM Tris–HCl, pH 7.6) and centrifuged at 13 000 × g for 5 min. The pellet was dissolved with 100 µl NET buffer, containing 5 µl mutanolysin (10 U µl⁻¹) and incubated for 60 min at 37°C. Subsequently the following denaturing agents were added: 9 µl of 2.6 M 1 M NaOH solution and 18 µl of 24% sodium dodecyl sulfate (SDS) (Roth, Karlsruhe, Germany). After incubation for 10 min at 80°C 10 µl proteinase K (14.8 mg ml⁻¹) was added, incubated for 120 min at 56°C, and subsequently boiled for 10 min. The DNA was extracted with phenol–chloroform–isoamyl alcohol [27]. For this 200 µl phenol–chloroform–isoamyl alcohol 25:24:1 (Roti® Phenol/Chloroform, Roth) was added, the content mixed until an emulsion formed and centrifuged at 13 000 × g for 5 min. From the aqueous phase (upper phase) 170 µl was transferred to a fresh tube and the DNA recovered by adding 20 µl of 5 M 1 M NaClO₄ (Sigma) and 100 µl isopropanol (2-propanol, Roth) [28]. The suspension was mixed, incubated for 30 min at room temperature and centrifuged at 13 000 × g for 30 min. The supernatant was carefully discarded. The DNA was precipitated by addition of 500 µl 70% alcohol. The tubes were centrifuged at 13 000 × g for 5 min, the supernatant discarded and the DNA dried at room temperature. The DNA pellet was dissolved in 20 µl of sterile distilled water. For the PCR assays 2.5 µl of this DNA was used. The dilution process and the DNA extraction from artificially contaminated milk was repeated five times on different occasions.

3. Results

The oligonucleotide primers designed according to species-specific parts of the 16S rRNA gene yielded an amplicon with a size of 1320 bp for all *S. canis* investigated. *S. dysgalactiae* subsp. *equisimilis*, also belonging to Lancefield’s serogroup G, and all the other control strains of various species and serogroups were negative throughout. Sequencing the intergenic spacer region and partial segments of the 16S rRNA and the 23S rRNA gene revealed almost identical sequences for all six *S. canis* investigated (Fig. 1). This allowed the design of *S. canis*-specific oligonucleotide primers. With this primer pair all 28 *S. canis* could be detected yielding an amplicon with a size of 215 bp (Fig. 2). In addition, all *S. canis* could be identified by amplification of species-specific parts of group G streptococcal CAMP factor gene *cfg* with an amplicon size of 238 bp. Both PCR systems revealed no cross-reactivities with the control strains investigated. The three PCR assays also identified *S. canis* specifically with DNA artificially contaminated with DNA from other streptococcal species. A multiplex PCR using 16S rRNA gene- and *cfg*-specific oligonucleotide primers yielded specific amplicons with the expected sizes and allowed the detection of *S. canis*.
in one PCR assay (Fig. 3). All three PCR assays could additionally be used for detection of this species in artificially contaminated milk. The limit of detection of S. canis in milk by PCR was \(403 \pm 633\) CFU ml\(^{-1}\) for the 16S rRNA assay, \(300 \pm 107.5\) CFU ml\(^{-1}\) for the 16S–23S rDNA intergenic spacer region assay and \(143 \pm 92.2\) CFU ml\(^{-1}\) for the CAMP factor gene cfg assay.

## 4. Discussion

The amplification of species-specific gene sequences by PCR offers a rapid and sensitive diagnostic tool applicable in veterinary and human microbiology. Molecules most suited for these purposes appeared to be the gene encoding the 16S rRNA and the 16S–23S rDNA intergenic spacer region. Segments of both rRNA sequences are highly conserved while others vary. The variations are species-specific and sufficiently stable to allow investigations of phylogenetic relationships [22,29].

According to Jayarao et al. [30,31] the amplification of the 16S rRNA gene from genomic DNA and the subsequent digestion with endonucleases proved to be a simple and reliable technique for differentiation of various bacterial species of bovine origin. This technique had been previously used for differentiation of S. uberis and S. para-uberis [32], for identification of S. agalactiae [28] and the serologically heterogeneous species S. porcinus [33] and for demonstration of intraspecies variations of S. equi subsp. zooepidemicus [34]. The major disadvantage of this procedure is the additional time-consuming manipulation of the samples subsequent to the PCR.

A second strategy relies on the detection of species-conserved gene sequences that are present in all strains of the various species. Comparable to previous studies [25,28,29] the species-specific variations of the V2 region of the 16S rRNA gene and the 16S–23S rDNA intergenic spacer region allowed the design of species-specific primers for PCR-mediated identification of S. canis. Forsman et al. [29] used the sequence variations of the 16S–23S rDNA intergenic spacer region for identification of streptococci and staphylococci causing bovine mastitis. However, S. canis was not included in these studies. Sequencing the 16S–23S rDNA intergenic spacer region of six S. canis performed in the present study revealed no significant differences among the S. canis investigated and allowed the development of a S. canis-specific PCR. A third target gene selected in this study was the previously characterized CAMP factor gene cfg of S. canis [25]. Comparable to the CAMP factor gene cfb of S. agalactiae [25,35,36] sequence variations of the CAMP factor gene cfg of S. canis allowed the design of species-specific oligonucleotide primers. The occurrence of a CAMP-like synergistic hemolytic reaction of S. canis in close proximity to a β-toxin-producing Staphylococcus aureus has been described as a common property of this species [6,17,37]. The PCR amplification of species-specific gene sequences of S. canis demonstrated in the present study offers a rapid and sensitive possibility to identify this species even in cases of mixed infections. This might help to determine the prevalence of S. canis in animal and human infections. The 16S rRNA gene- and CAMP factor gene cfg-specific oligonucleotide primers could be used in a multiplex PCR assay increasing the specificity of the identification process. As a first clinical approach the PCR assays were used to identify S. canis from artificially contaminated milk. All three assays revealed comparable results. Besides a confirmation of the species identity of biochemically or serologically identified S. canis all three PCR assays could additionally be used for identification of this species directly from clinical material. A comparable molecular identification of S. canis was performed by Poyart et al. [38] and Whatmore et al. [15] by sequencing the genes encoding a manganese-dependent superoxide dismutase and the 16S rRNA gene, respectively. Sequencing the target genes useful for taxonomic applications gives the most accurate information about a species to be identified. However, sequencing bacterial isolates can only be carried out in more specialized laboratories. This is in contrast to a bacterial identification mediated by PCR. The latter is a common tool also in routine diagnostic laboratories.
References


