Molecular identification and further characterization of *Arcanobacterium pyogenes* isolated from bovine mastitis and from various other origins

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ABSTRACT

The present study was designed to identify phenotypically and genotypically 61 *Arcanobacterium pyogenes* isolated from bovine mastitis and from various other origins. The *A. pyogenes* isolates showed the typical cultural and biochemical properties of this species and displayed CAMP-like synergistic hemolytic activities with various indicator strains. The species identity could be confirmed genotypically by amplification and sequencing of the superoxide dismutase A encoding gene *sodA* of reference strains representing 8 species of genus *Arcanobacterium* and subsequent design of *A. pyogenes* *sodA* gene-specific oligonucleotide primer. The *A. pyogenes* *sodA* gene-specific oligonucleotide primer allowed, together with previously described *A. pyogenes* 16S-23S rDNA intergenic spacer region-specific oligonucleotide primer, a reliable molecular identification of all 61 *A. pyogenes* of various origins. The additionally performed PCR-mediated amplification of 5 known and putative virulence factor encoding genes revealed that 100, 20, 87, 75, and 98% of the *A. pyogenes* carried the genes *plo*, *cbpA*, *nanH*, *nanP*, and *fimA*, which allowed an individual strain characterization. This might help to elucidate the role the putative virulence factors play in bovine mastitis and in various other infections caused by this bacterial pathogen.

Key words: *Arcanobacterium pyogenes*, mastitis, molecular identification, virulence gene

INTRODUCTION

Arcanobacterium pyogenes is a worldwide known pathogen of domestic ruminants and pigs causing mastitis, abortion, and a variety of pyogenic infections

(Lämmle and Hartwigk, 1995; Moore et al., 2010). As summarized by Jost and Billington (2005), this bacterial pathogen is also able to cause disease in a large number of animal species, including antelopes, bison, camels, chicken, deer, elephants, gazelles, horses, macaws, reindeer, turkeys, and wildebeest, and also in companion animals such as dogs and cats. The *A. pyogenes* from companion animals were isolated from feline otitis externa and canine cystitis (Billington et al., 2002). A septicemia case of a farmer in Brazil caused by *A. pyogenes* had been described previously (Levy et al. 2009). A first case of endocarditis in a man with no identified animal contact was described by Plamondon et al. (2007). These authors also summarized 13 human cases of *A. pyogenes* infections.

According to a proposal of Yassin et al., (2010) *A. pyogenes*, *Arcanobacterium abortus suis*, *Arcanobacterium bernardiae*, *Arcanobacterium bialowiezense*, and *Arcanobacterium bonasi* should be reclassified in the newly described genus *Trueperella*. The genus *Arcanobacterium* should be restricted to *Arcanobacterium hemolyticum*, *Arcanobacterium hippocoleae*, *Arcanobacterium phocae*, and *Arcanobacterium pluranimalium*.

Arcanobacterium pyogenes possesses several known and putative virulence factors that may contribute to its pathogenic potential. These virulence factors include a hemolysin which is known as pyolysin and promotes hemolytic activities for red blood cells and cytolytic activities for immune cells (Jost and Billington, 2005); 2 neuraminidases (NanH and NanP), necessary for adhesion to epithelial cells (Jost et al., 2001, 2002); a collagen-binding protein (CbpA), necessary for adhesion to collagen rich tissues (Esmay et al. 2003); and fimbriae, which also might be involved in the adhesion process (Jost and Billington, 2005).

The identification of *A. pyogenes* is usually performed by cultural and biochemical properties. As shown previously, a PCR-mediated identification of 2 isolates of this bacterial species isolated from a gecko and a bearded dragon could be conducted by using 16S rDNA, the

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Table 1. Origin of the 61 *Arcanobacterium pyogenes* isolates investigated in the present study

Animal species	n	Origin/clinical diagnosis and additional information ¹
Bovine	15	Milk/mastitis
Bovine	14	Uterus/endometritis and fertility problems (3); placenta/septicemia (1); cervix/vaginal discharge and fertility problems (1); lung/bronchopneumonia and pleuropneumonia (2); lung/septicemia (1); kidney/septicemia (1); urine/pyelonephritis (1); feces/septicemia (1); dermoid cyst/abscessation (1); nostril abscess/bronchopneumonia and respiratory disorders (1); inguinal region/n.d. (1)
Pig	13	Lung/bronchopneumonia and pleuropneumonia (4), lung/septicemia (1), lung/n.d. (1); liver/septicemia (2); cervix/abortion, vaginal discharge, and fertility problems (2); ventricle/opisthotonus (1); ear abscess/perichondritis of ear cartilage (1); hind leg/abscessation (1)
Small ruminants		
Sheep	4	Kidney/septicemia (1); skin/n.d. (2); n.d. (1)
Goat	1	Lung/septicemia
Wild ruminants		
Elk	1	Rumen/septicemia
Yak	1	Lung/thorax edema
Deer	2	Lung/pneumonia (1); brain/septicemia (1)
Mountain Reedbuck	1	Tonsils/n.d.
Horse	1	Wound/n.d.
Companion animals		
Dog	2	Vagina/vaginitis and vaginal discharge (1); ear/n.d. (1)
Cat	2	Ear/otitis and abscessation (1); anal gland/perineal hernia (1)
Rabbit	1	Lung/septicemia
Rat	1	Liver/septicemia
Reptiles		
Bearded dragon	1	Lung/septicemia
Gecko	1	Intestine/enteritis, septicemia

¹Numbers of isolates are in parentheses. n.d. = no data available.

16S-23S rDNA intergenic spacer region (**ISR**), the *plo* encoding gene *plo*, and the β subunit of RNA polymerase encoding gene *rpoB* as molecular targets (Ülbeği-Mohyla et al., 2010b).

The objective of the present study was to investigate the usefulness of the previously investigated molecular targets ISR and *plo* and, comparatively to the molecular identification of bacteria of genus *Mycobacterium* (Zolg and Philippi-Schulz, 1994), the superoxide dismutase A encoding gene *sodA* for molecular identification of *A. pyogenes* isolated from bovine mastitis and from several other origins. The *A. pyogenes* isolates were also investigated for the potential virulence factor encoding genes *cbpA*, *nanH*, *nanP*, and *fimA*.

MATERIALS AND METHODS

A total of 71 bacterial cultures were investigated in the present study. The cultures included 10 reference strains of 9 species of genus *Arcanobacterium* (Hassan et al., 2009; Ülbeği-Mohyla et al., 2010a,b). The other 61 *A. pyogenes* isolates were obtained from bovine mastitis (n = 15), nonmastitic origin of bovines (n = 14), from pigs (n = 13), small ruminants (n = 5), wild ruminants (n = 5), from a horse (n = 1), from companion animals (n = 4), from a rabbit (n = 1), a rat (n = 1), and from reptiles (n = 2). The *A. pyogenes* of reptile origin has been described previously (Ülbeği-Mohyla et

al., 2010b). The origin of the isolates is summarized in Table 1.

The *A. pyogenes* isolates were investigated for cultural and biochemical properties and for CAMP-like synergistic hemolytic activities as described by Hassan et al. (2009) and Ülbeği-Mohyla et al. (2009). For molecular identification, the superoxide dismutase A encoding gene *sodA* of *A. bernardiae* DSM 9152^T, *A. hemolyticum* DSM 20595^T, *A. phocae* DSM 10002^T, *A. pluranimallium* DSM 13483^T, and *A. pyogenes* DSM 20630^T was amplified with the universal oligonucleotide primer pair described by Zolg and Philippi-Schulz (1994), and of *A. abortus* DSM 19515^T, *A. bonasi* DSM 17163^T, and *A. hippocoleae* DSM 15539^T by designing a novel oligonucleotide primer pair using the *sodA* sequences obtained in the present study. The DNA extraction, sequencing, and alignment studies were performed as described previously (Hassan et al., 2008, 2009). The amplified *sodA* genes were compared by dendrogram analysis. The *sodA* gene sequence of *A. pyogenes* additionally allowed the design of a species-specific oligonucleotide primer pair with the help of OLIGO 4 primer analysis software (version 4.0). The *A. pyogenes* isolates were also investigated using the *A. pyogenes* ISR specific oligonucleotide primers described previously (Ülbeği-Mohyla et al., 2010b). The oligonucleotide primers used in this study were synthesized by MWG Biotech/Operon (Ebersberg, Germany).

In addition, all *A. pyogenes* were investigated by amplification of pyolysin-encoding gene *plo*, collagen-binding protein-encoding gene *cbpA*, neuraminidase (NanH and NanP)-encoding genes *nanH* and *nanP*, and the fimbriae-encoding gene *fimA*. The oligonucleotide primer sequences, the thermal cycler PCR programs, and the expected amplicon sizes are summarized in Table 2.

The statistical analysis of the distribution of the putative virulence genes was performed with the statistical program package BMDP (Dixon, 1993); the comparison of the gene distribution between *A. pyogenes* of different animal origins was done by 4-fold table followed by the 2-sided Fisher exact test.

RESULTS AND DISCUSSION

All 61 bacterial isolates investigated in the present study could be identified phenotypically and genotypically as *A. pyogenes*. Comparable to the *A. pyogenes* reference strains and to previous findings (Ülbeği-Mohyla et al., 2009), all *A. pyogenes* isolates displayed a synergistic CAMP-like hemolysis with staphylococcal β -hemolysin and *Rhodococcus equi*, *A. hemolyticum*, and *A. phocae* indicator strains, a liquefaction of Loeffler agar, and a cross-reaction with streptococcal serogroup G-specific antisera. The latter are well-known properties of *A. pyogenes* (Bisping and Amsberg, 1988; Lämmle and Hartwig, 1995). In addition, all *A. pyogenes* showed the typical biochemical properties of this species (data not shown), which generally corresponded to the findings of Ding and Lämmle (1992) and Ramos et al. (1997).

With the available universal *sodA* gene-specific oligonucleotide primers, the *sodA* gene of *A. pyogenes* and of 7 additional species of genus *Arcanobacterium* (excluding *A. bialowiezense*) could be amplified and sequenced. Comparable to the previously described ISR, 23S rRNA and *rpoB* sequence data (Hassan et al., 2008; Ülbeği-Mohyla et al., 2010a,b), gene *sodA* appeared to be a novel target for molecular identification of bacteria of genus *Arcanobacterium*. The *sodA* gene sequence data of 8 species of genus *Arcanobacterium* were given to GenBank (FN562416, AM989465, FM955147, AM983534, FM242696, AM946391, AM989466, and AM949566). A dendrogram analysis of gene *sodA* of 8 species of genus *Arcanobacterium* is shown in Figure 1. Sequencing *sodA* additionally allowed the design of *A. pyogenes*-specific oligonucleotide primers. The *A. pyogenes* *sodA* gene-specific oligonucleotide primer and the previously described *A. pyogenes* ISR-specific oligonucleotide primer (Ülbeği-Mohyla et al., 2010b) could be used for PCR-mediated identification of all 61 *A. pyogenes* investigated in the present study and both *A.*

pyogenes reference strains. A typical amplicon of the *A. pyogenes* *sodA* gene-specific PCR is shown in Figure 2. The *A. pyogenes* *sodA* gene-specific oligonucleotide primer showed no cross-reactions with reference strains representing the remaining 8 species of genus *Arcanobacterium*, indicating that both *A. pyogenes*-specific oligonucleotide primer pairs could successfully be used for molecular identification of this species.

Amplification of the known and putative virulence factor-encoding genes revealed that 100, 20, 87, 75, and 98% of the *A. pyogenes* isolates carried the genes *plo*, *cbpA*, *nanH*, *nanP*, and *fimA*, respectively. Gene *plo* expresses the cholesterol-dependent pyolysin, which is well known as a major virulence factor of this species (Ding and Lämmle, 1996; Billington et al., 1997; Jost and Billington, 2005) and, because it is generally present in all *A. pyogenes* isolates, could be used for molecular identification of this species (Billington et al., 2002; Ertaş et al., 2005; Ülbeği-Mohyla et al., 2010b).

Jost and Billington (2005) additionally described a collagen-binding protein CbpA, which seems to promote the adhesion of these bacteria to collagen-rich tissues. According to the findings of Pietrocchi et al. (2007), CbpA also binds to fibronectin. However, collagen and fibronectin bind on different subsites of CbpA. As shown in the present study, the putative virulence factor CbpA-encoding gene *cbpA* seems to be present in *A. pyogenes* isolated from pigs (54%), companion animals (25%), and reptiles (100%), but rarely in *A. pyogenes* from bovine origin (7%). *Arcanobacterium pyogenes* from small and wild ruminants and from horse, rabbit, and rat did not carry gene *cbpA*. As shown in Table 3, the differences in the presence of *cbpA* between isolates from ruminants and pigs appeared to be significant ($P = 0.0004$). According to the DNA dot blotting results of Esmay et al. (2003), *cbpA* was found in *A. pyogenes* of bovine (49%), porcine (40%), and avian (100%) origin. Isolates from canines and from feline origin did not carry *cbpA*. Silva et al. (2008) could detect *cbpA* in all 57 investigated *A. pyogenes* isolated from cows with uterine infections. The results of the present study corresponded to the findings of Santos et al. (2010) who found *cbpA* only in 1.4% of the *A. pyogenes* isolated from bovine origin. However, in contrast to Silva et al. (2008) and Santos et al. (2010), in the present study, *cbpA* could also be found for reference strain *A. pyogenes* DSM 20630^T (ATCC 19411), originally isolated from a pig. These differences could possibly be explained by the differing oligonucleotide primer pairs used for amplification of this potential virulence gene. Using the *cbpA*-specific oligonucleotide primer proposed by Silva et al. (2008), *A. pyogenes* DSM 20630^T was negative (data not shown). However, using the *cbpA*-specific oligonucleotide primer of the present study, *A. pyogenes*

Table 2. Oligonucleotide primer sequences and PCR conditions used in the present study

Oligonucleotide primer	Primer sequence	Program ¹	Size of PCR product (bp)	Reference
<i>sodA</i> Z205 ²	5'-AGCTTCACCACAGCAAGCACCA-3'	1	489	Zolg and Philippi-Schulz (1994)
<i>sodA</i> Z212	5'-TCGG(T)CCCAGTTCACGACG(A)TTCCA-3'	2	285	Present study
<i>sodA1-F</i> ³	5'-GGCGA(C)T(C)TTTTC(G)CAAGATCAACCAG-3'			
<i>sodA1-R</i>	5' GTTGGCCTGCTGGTCGAAAAGCTGGAA-3'	3	199	Present study
<i>sodA F</i> (<i>Arcanobacterium pyogenes</i>)	5'-CGAGCTCGCCGACGCTATTGCT-3'			
<i>sodA R</i> (<i>A. pyogenes</i>)	5'-GAGCATGAGAATCGGGTAAGTGCCA-3'	4	122	Ülbegi-Mohyla et al. (2010b)
ISR F (<i>A. pyogenes</i>)	5'-GTTTTGCTTGTGATCGTGGTGGTTATGA-3'			
ISR R (<i>A. pyogenes</i>)	5'-AAGCAGGCCACGCGCAGG-3'	5	704	Ülbegi-Mohyla et al. (2010b)
<i>plo F</i>	5'-CGATCCCTCTGGTGTACTTGC-3'			
<i>plo R</i>	5'-GCTTGACAAAAATCTGGCGTCC-3'	6	328	Ülbegi-Mohyla et al. (2010b)
<i>cbpA F</i>	5'-CTTGAAATCGAACTTAAGGCTGG-3'			
<i>cbpA R</i>	5'-ATCGCCAGTCACCTTAGACG-3'	7	781	Silva et al. (2008)
<i>nanH F</i>	5'-CGCTAGTGCTGTAGCGTTGTTAAGT-3'			
<i>nanH R</i>	5'-CCGAGGAGTTTTGACTGACTTTGT-3'	7	150	Silva et al. (2008)
<i>nanP F</i>	5'-TTGAGCGTACGCAGCTCTTC-3'			
<i>nanP R</i>	5'-CCACGAAATCGGCCTTATTG-3'	8	605	Silva et al. (2008)
<i>fimA F</i>	5'-CACTACGCTCACCATTCAAG-3'			
<i>fimA R</i>	5'-GCTGTAATCCGCTTTGTCTGTG-3'			

¹PCR program 1: 1 × (95°C, 600 s), 33 × (94°C, 30 s, 39°C, 50 s, 72°C, 60 s), 1 × (72°C, 420 s). PCR program 2: 1 × (94°C, 600 s), 30 × (95°C, 60 s, 37°C, 60 s, 72°C, 60 s), 1 × (72°C, 420 s). PCR program 3: 1 × (94°C, 600 s), 25 × (94°C, 20 s, 62°C, 20 s, 72°C, 30 s), 1 × (72°C, 600 s). PCR program 4: 1 × (95°C, 600 s), 30 × (95°C, 30 s, 64°C, 15 s, 72°C, 30 s), 1 × (72°C, 420 s). PCR program 5: 1 × (95°C, 600 s), 30 × (95°C, 60 s, 62°C, 60 s, 72°C, 60 s), 1 × (72°C, 420 s). PCR program 6: 1 × (94°C, 600 s), 30 × (95°C, 30 s, 58°C, 30 s, 72°C, 60 s), 1 × (72°C, 420 s). PCR program 7: 1 × (94°C, 180 s), 35 × (94°C, 60 s, 60°C, 60 s, 72°C, 180 s), 1 × (72°C, 420 s). PCR program 8: 1 × (94°C, 180 s), 35 × (94°C, 60 s, 57°C, 60 s, 72°C, 180 s), 1 × (72°C, 420 s).

²For *Arcanobacterium bernardiae* DSM 9152^T, *Arcanobacterium hemolyticum* DSM 20595^T, *Arcanobacterium phocae* DSM 10002^T, *Arcanobacterium pluranimalium* DSM 13483^T, and *A. pyogenes* DSM 20630^T.

³For *Arcanobacterium abortusis* DSM 19515^T, *Arcanobacterium bonasi* DSM 17163^T, and *Arcanobacterium hippocoleae* DSM 15539^T.

Figure 1. Dendrogram analysis of gene *sodA* of 8 reference strains of genus *Arcanobacterium* using the Megalign program (Clustal method).

DSM 20594 of bovine origin was negative for *cbpA*. The importance of the distribution of *cbpA*, which occurred more pronounced among *A. pyogenes* isolated from pigs, remains unclear.

According to Jost and Billington (2005), all *A. pyogenes* strains additionally carried *nanH* and 64.2% *nanP*, which both seem to play a role in the adhesion of this organism to host epithelial cells. In the study of Silva et al. (2008), all investigated *A. pyogenes* strains carried *nanH* and *nanP*. In the present study, 53 (87%) of the *A. pyogenes* isolates were positive for *nanH* and 46 (75%) isolates positive for *nanP*. However, some of the *A. pyogenes* isolates of the present study were negative for these putative virulence genes. This negative reaction occurred singly ($n = 8$, negative for *nanH*; $n = 11$, negative for *nanP*) or in combination ($n = 3$, negative for both *nanH* and *nanP*), with no significant differences between the *A. pyogenes* isolates of various origins ($P > 0.05$). However, the *nanH-nanP*-positive or negative isolates of the present study were not investigated for neuraminidase activity phenotypically.

According to Jost and Billington (2005), *fimA* encodes a protein with some similarity to the *Actinomyces naeslundii* type 2 fimbrial subunit and also seems to be involved in adhesion processes. Gene *fimA* could be found by Jost and Billington (2005), comparable to the findings of the present study, with 98% *fimA*-positive isolates, in 94% of the investigated *A. pyogenes* isolates. According to Silva et al. (2008), 100% of the isolates and, according to Santos et al. (2010), 90.9% of the *A. pyogenes* isolated from metritis compared with 64.1% of the *A. pyogenes* isolated from nonmetritic cows, were *fimA* positive. However, the importance of the differences observed by Santos et al. (2010) remains unclear. In contrast to the findings of Silva et al. (2008) and

Santos et al. (2010), *A. pyogenes* DSM 20630^T (ATCC 19411) investigated in the present study appeared to be *fimA* negative. The genetic properties of the *A. pyogenes* isolates of the present study are summarized in Table 2.

The present study gives a detailed genotypic characterization of *A. pyogenes* isolated from bovine mastitis, from nonmastitic origin of bovines, and from several various other animals, indicating that, independent of

Figure 2. Typical amplicons using the *Arcanobacterium pyogenes* *sodA* gene-specific oligonucleotide primer pair. *A. pyogenes* DSM 20630^T (1) and 2 *A. pyogenes* isolates of the present study (2, 3) with a size of approximately 200 bp. Negative reaction of *Arcanobacterium abortus* DSM 19515^T (4). M = GeneRuler 100-bp DNA ladder (Fermentas, St. Leon-Rot, Germany).

Table 3. PCR-mediated amplification of various known and putative virulence factor encoding genes of *Arcanobacterium pyogenes* isolated from bovine mastitis and from various other origins

Origin of the isolates	Virulence factor encoding genes						Number of virulence genes present in all isolates
	n	<i>plo</i> (704 bp) ¹	<i>cbpA</i> ² (327 bp)	<i>nanH</i> (781 bp)	<i>nanP</i> (150 bp)	<i>fimA</i> (605 bp)	
Bovine (mastitis)	15	15 ³	1	10	9	15	2
Bovine (non-mastitis)	14	14	1	13	10	14	2
Pig	13	13	7	13	12	13	3
Small ruminants	5	5	0	4	4	5	2
Wild ruminants	5	5	0	5	4	5	3
Horse	1	1	0	1	1	1	4
Companion animals	4	4	1	4	3	3	2
Rabbit	1	1	0	1	1	1	4
Rat	1	1	0	0	0	1	2
Reptiles	2	2 ⁴	2 ⁴	2	2	2	5
Σ	61	61 (100%)	12 (20%)	53 (87%)	46 (75%)	60 (98%)	
<i>A. pyogenes</i> DSM 20630 ^T		Positive	Positive	Positive	Positive	Negative	
<i>A. pyogenes</i> DSM 20594		Positive	Negative	Positive	Positive	Positive	

¹Expected amplicon size.
²Significant difference between *A. pyogenes* isolated from ruminants and pigs (*P* = 0.0004).
³Number of isolates with the respective property.
⁴Results described previously (Ülbeği-Mohyla et al., 2010b).

the origin of the isolates, the proposed species-specific oligonucleotide primers of the target molecules *sodA*, *ISR*, and *plo* could be used for molecular identification of this species. The additionally investigated potential virulence genes allowed an individual strain characterization and might help to understand the process of infection with this bacterial pathogen.

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