Muaz Hijazin

Phenotypic and genotypic characteristics of bacteria of genera *Arcanobacterium*, *Trueperella* and *Actinomyces*, with the emphasis on *Arcanobacterium* (*Trueperella*) *pyogenes*

INAUGURAL-DISSERTATION zur Erlangung des Grades eines **Dr. med. vet.**
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eingereicht von

Muaz Hijazin
Tierarzt aus Amman-Jordanien

Gießen 2012

Mit Genehmigung des Fachbereichs Veterinärmedizin

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Tag der Disputation: 17.01.2012

Dedicated to my beloved
wife Mervat and my children
Sarah and Karam

Erklärung

Ich habe die vorgelegte Dissertation selbständig und ohne unerlaubte fremde Hilfe und nur mit den Hilfen angefertigt, die ich in der Dissertation angegeben habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten oder nicht veröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht. Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der „Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis“ niedergelegt sind, eingehalten.

Muaz Hijazin

Some results of this work had been published:

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Hijazin, M., Hassan, A. A., Alber, J., Lämmler, C., Timke, M., Kostrzewa, M., Prenger-Berninghoff, E., Zschöck, M., 2012b. Evaluation of matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) for species identification of bacteria of genera *Arcanobacterium* and *Trueperella*. Vet. Microbiol. (in press).

Table of Contents

Frequently used abbreviations and units

A.	*Arcanobacterium*
A.	*Actinomyces*
aqua bidest.	aqua bidestillata
aqua dest.	aqua destillata
bp	base pair
C	cytosine
Da	dalton, unit of molecular mass
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
EDTA	ethylenediaminetetraacetic acid
et al.	et alii (and others)
Fig.	figure
g	gram
G	guanine
gen. n.	genus novus or new genus
h	hour
ILY	Intermedilysin from *Streptococcus intermedius*
ily	*S. intermedius* intermedilysin encoding gene
ISR	16S-23S rDNA intergenic spacer region
kb	kilobase
kDa	kilodalton
kg	kilogram
l	liter
LLO	Listeriolysin O from *Listeria monocytogenes*
llo	*L. monocytogenes* listeriolysin encoding gene
mA	milliampere
MALDI-TOF MS	matrix-assisted laser desorption ionization-time of flight mass spectrometry
mg	milligram
min	minute
mm	millimeter
mmol	millimole
mol	amounts of a chemical substance
MSP	main spectrum (reference spectrum of the peptidic raw spectra)
N	normality
N. L.	new latin, i.e., latinate phrases coined for scientific terms
p	pico (10^{-12})
PCR	polymerase chain reaction
PFO	Perfringolysin O from *Clostridium perfringens*
pfo	*C. perfringens* perfringolysin encoding gene
phl	*Arcanobacterium phocae* phocaelysin encoding gene

pld	*Arcanobacterium haemolyticum* phospholipase D encoding gene
PLO	Pyolysin from *Arcanobacterium* (*Trueperella*) *pyogenes*
plo	*A.* (*T.*) *pyogenes* pyolysin encoding gene
PLY	Pneumolysin from *S. pneumoniae*
ply	*Streptococcus pneumoniae* pneumolysin encoding gene
pmol	picomole
RNA	ribonucleic acid
*rpo*B	beta subunit of the RNA polymerase encoding gene
s	second
SLO	Streptolysin O from *Streptococcus pyogenes*
slo	*S. pyogenes* streptolysin encoding gene
*sod*A	superoxid dismutase A encoding gene
T.	*Trueperella*
Tab.	table
U	unit (international unit)
UV	ultraviolet
V	volt
Vol.	volume
°C	degree Celsius
µg	microgram
µl	microliter
µm	micrometer

1. Introduction

Bacteria of genus *Arcanobacterium* (*A*.) belonging to the family *Actinomycetaceae* are major pathogens for animals and humans. Genus *Arcanobacterium* was first described by Collins et al. (1982b) for *Arcanobacterium haemolyticum*, formerly named *Corynebacterium haemolyticum* (Maclean et al., 1946). In the following years, the mainly animal pathogenic species *Actinomyces pyogenes* and human pathogenic *Actinomyces bernardiae* were reclassified to genus *Arcanobacterium* as *Arcanobacterium pyogenes* and *Arcanobacterium bernardiae*, respectively (Ramos et al., 1997). In addition, six novel species, namely *Arcanobacterium phocae*, *Arcanobacterium pluranimalium*, *Arcanobacterium hippocoleae*, *Arcanobacterium bialowiezense* and *Arcanobacterium bonasi* and *Arcanobacterium abortisuis* were described by Ramos et al. (1997), Lawson et al. (2001), Hoyles et al. (2002), Lehnen et al. (2006) and Azuma et al. (2009), respectively. However, according to a proposal of Yassin et al. (2011), genus *Arcanobacterium* should be restricted to *A. haemolyticum*, *A. hippocoleae, A. phocae* and *A. pluranimalium* and the species *A. abortisuis*, *A. bernardiae*, *A. bialowiezense*, *A. bonasi* and *A. pyogenes* be reclassified into the new genus *Trueperella* (*T*.), as *Trueperella pyogenes*, *Trueperella abortisuis*, *Trueperella bernardiae*, *Trueperella bialowiezensis* and *Trueperella bonasi.*

A. (*T*.) *pyogenes* is a worldwide known pathogen mainly of domestic ruminants and pigs (Lämmler and Hartwigk, 1995; Hirsh and Biberstein, 2004; Moore et al, 2010), *A. haemolyticum* is commonly described in association with pharyngitis and skin infection in humans (Brown, 1990; Tan et al., 2006) and *A*. (*T*.) *bernardiae* causes soft tissue infections (Clarke et al., 2010; Weitzel et al., 2011). However, the information about the six other species of both genera *A*. (*T*.) *abortisuis*, *A*. (*T*.) *bialowiezense*, *A*. (*T*.) *bonasi*, *A. hippocoleae*, *A. phocae* and *A. pluranimalium*, mainly of veterinary importance, is limited.

The aim of the present study was to identify and further characterize *A*. (*T*.) *pyogenes* isolated from various animals, *A*. (*T*.) *abortisuis* isolated from pigs and cows, *A. haemolyticum* isolated from horses and three strains representing a novel species of genus *Actinomyces* with conventional and molecular methods. The bacteria were investigated together with reference strains of genera *Arcanobacterium*, *Trueperella* and *Actinomyces* for cultural characteristics, further phenotypical properties, by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) and by genotypic methods. The latter were performed by investigating various species specific targets and by determination of putative virulence factor encoding genes.

2. Review of the literature

2.1 Genus *Arcanobacterium* and *Trueperella*

2.1.1 History and taxonomy

Genus *Arcanobacterium*, the Latin name is derived from the adjective "*arcanus*"- secretive or mysterious and the word "*bakterion*"- a small rod. Bacteria of genus *Arcanobacterium* belong to family *Actinomycetaceae*. The current classification of genus *Arcanobacterium* obtained from the taxonomy browser of the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/guide/taxonomy/) and the German Collection of Microorganisms and Cell Cultures (http://www.dsmz.de/microorganisms/main.php?_contentleft_id=14) is shown in **Tab. 1**.

Lucet in 1893 first described *A.* (*T.*) *pyogenes* as *Bacillus liquefaciens pyogenes* as one of the most frequently isolated microorganism in suppurative infections in cattle. Several other authors found similar organism in infections of cattle, swine and from cow mastitis (Grips, 1898; Glage, 1903; Künnemann, 1903). In 1903, Glage assumed that the microorganisms isolated by these authors are identical and suggested the name *Bacillus pyogenes*. Based on the similarity to the type species *Corynebacterium diphtheriae* and related animal pathogenic corynebacteria, these bacteria were, according to the 8th edition of Bergey's Manual of Determinative Bacteriology, assigned to genus *Corynebacterium* as *Corynebacterium pyogenes* (Rogosa et al., 1974). However, the taxonomic placement of *Corynebacterium pyogenes* within genus *Corynebacterium* had been questioned by many investigators (Jones 1975; Goodfellow et al., 1976; Minnikin et al., 1978). Results from Collins and Jones (1982) and Reddy et al. (1982), investigating *Corynebacterium pyogenes*, indicated that this species should be reclassified as *Actinomyces pyogenes* to genus *Actinomyces*.

Genus *Arcanobacterium* was first introduced by reclassification of *Corynebacterium haemolyticum* to *Arcanobacterium haemolyticum*. This was based on the cell wall fatty acids and the peptidoglycan components of this microorganism which differed from species of genus *Corynebacterium* (Collins et al., 1982b). In 1997, *Actinomyces pyogenes* and *Actinomyces bernardiae* were reclassified as *Arcanobacterium pyogenes* and *Arcanobacterium bernardiae*, respectively, together with the newly described species *Arcanobacterium phocae* which had been isolated from seals (Ramos et al., 1997).

During the last decade, *Arcanobacterium pluranimalium* isolated from a porpoise and a deer (Lawson et al., 2001), *Arcanobacterium hippocoleae* isolated from the vagina of a horse (Hoyles et al., 2002), *Arcanobacterium bialowiezense* and *Arcanobacterium bonasi* which could be recovered from prepuce of European bison bulls (Lehnen et al., 2006) and *Arcanobacterium abortisuis* isolated from placenta of a sow following an abortion (Azuma et al., 2009) were also classified to genus *Arcanobacterium*.

In 2011, Yassin et al. proposed on the basis of 16S rDNA signature nucleotides, menaquinones and phospholipid composition that genus *Arcanobacterium* should be restricted to *A. haemolyticum*, *A. hippocoleae, A. phocae* and *A. pluranimalium*. The remaining five species of genus *Arcanobacterium*, also including *A. pyogenes,* should be reclassified in the new genus *Trueperella*, as *Trueperella pyogenes, Trueperella abortisuis*, *Trueperella bernardiae*, *Trueperella bialowiezensis* and *Trueperella bonasi*. Genus *Trueperella* was named after Hans Georg Trüper, a German microbiologist. The current classification of genus *Trueperella* is also shown in **Tab.1**.

Tab. 1: Current classification of family *Actinomycetaceae*, genera *Trueperella* and *Arcanobacterium* according to the National Center for Biotechnology Information and the German Collection of Microorganisms and Cell Cultures.

Phylum	*Actinobacteria*
Class	*Actinobacteria*
Subclass	*Actinobacteridae*
Order	*Actinomycetales*
Suborder	*Actinomycineae*
Family	*Actinomycetaceae*
Genus	*Trueperella*
Species	*Trueperella pyogenes*
Species	*Trueperella abortisuis*
Species	*Trueperella bernardiae*
Species	*Trueperella bialowiezensis*
Species	*Trueperella bonasi*
Genus	*Arcanobacterium*
Species	*Arcanobacterium haemolyticum*
Species	*Arcanobacterium hippocoleae*
Species	*Arcanobacterium phocae*
Species	*Arcanobacterium pluranimalium*

2.1.2 Morphology and growth conditions

Bacteria of genus *Arcanobacterium* and the newly described closely related genus *Trueperella* are Gram-positive, irregular rod-shaped bacteria. According to Holt et al. (1994) bacteria of genus *Arcanobacterium* are 0.3-0.8 x 1.0-5.0 µm in young cultures, non motile, not acid fast and non spore forming. The cell shape is not uniform. They are pleomorphic, meaning that they show rod- or coccoid-like shape and that they usually are arranged in V- or T-shape (Hirsh and Biberstein, 2004; Moore et al., 2010; Yassin et al., 2011). Bacteria of genus *Arcanobacterium* are facultatively anaerobic and their growth is enhanced in CO_2-enriched atmosphere after 24 to 48 h incubation at 37 °C under microaerobic conditions in a candle jar. Growth of the bacteria occurred on ordinary media but enhanced on blood or serum containing media. A complete zone of hemolysis could be observed on blood agar for all nine species. Bacteria of genus *Arcanobacterium* are inactivated at a temperature of 60 °C in 15 min (Collins et al., 1982b).

2.2 Species of genus *Arcanobacterium* and genus *Trueperella*

2.2.1 *Arcanobacterium* (*Trueperella*) *pyogenes*

The species name of *Arcanobacterium* (*Trueperella*) *pyogenes* was derived from the Latin word "*pyum*"- pus and the word "*gennaio*"- produce to describe the pus producing bacterium. The taxonomy of *A.* (*T.*) *pyogenes* was not clear for many years. In 1893, Lucet described *Bacillus liquefaciens pyogenes bovis* isolated from cases of suppuration, abscesses and septicemia in cows. Grips (1898), found *Bacillus pyogenes suis* commonly present in cases of swine pleuritis and peritonitis. In 1903, Künnemann detected in 90 % of suppurations in cattle similar organism and named it *Bacillus pyogenes bovis*. Glage (1903) investigated *B. pyogenes suis* (Grips, 1898) and *B. pyogenes bovis* (Künnemann, 1903) and concluded both isolates from pigs and bovines to be identical and suggested the name *B. pyogenes*. In the 8th edition of Bergey's Manual of Determinative Bacteriology this species, as well as the closely related species *Corynebacterium haemolyticum*, has been assigned to genus *Corynebacterium* as *Corynebacterium pyogenes* (Rogosa et al., 1974). Barksdale et al. (1957) also suggested, according to phenotypic and cell wall structure analysis for both species, that *C. haemolyticum* is a mutant form of *C. pyogenes.* Many investigators had long been recognized

that *C. pyogenes* has phenotypic and cell wall structure similarities to *C. haemolyticum* and that taxonomically both species should belong to genus *Streptococcus* (Cummins and Harris, 1956; Barksdale et al., 1957). However, in the following years, based on numerical taxonomic studies, *C. haemolyticum* and *C. pyogenes* were clearly distinguished from each other. *C. pyogenes* seemed to be taxonomically associated to *Actinomyces bovis* and should be reclassified, according to Schofield and Schaal (1981) and Collins et al. (1982a), to genus *Actinomyces* as *Actinomyces pyogenes*. In 1982, *C. pyogenes* was finally reclassified to genus *Actinomyces* as *Actinomyces pyogenes* by Collins and Jones, (1982) and Reddy et al. (1982). The assignment to genus *Actinomyces* was based on numerical phenotypic and chemical studies indicating, as already found by Schofield and Schaal, (1981) and Collins et al. (1982a), a close relationship between *C. pyogenes* and the species *A. bovis* (Collins and Jones, 1982; Reddy et al., 1982). In 1997, Ramos et al. (1997) suggested by 16S rDNA sequencing, a taxonomic revision and gave the final taxonomic destination of *A. pyogenes* to genus *Arcanobacterium* as *Arcanobacterium pyogenes*. The 16S rDNA sequence analysis of *A. pyogenes* yielded a phylogenetic proximity to the species *A. bernardiae* with a 16S rDNA sequence homology of 98.3 % (Ramos et al., 1997). However, as mentioned before (2.1.1), Yassin and coauthors (2011) proposed that *A. pyogenes* should be reclassified into the new genus *Trueperella*, as *Trueperella pyogenes*.

A. (*T.*) *pyogenes* appears to be an opportunistic pathogen of veterinary importance. *A.* (*T.*) *pyogenes* was an obligate inhabitant of the mucous membrane of domestic animals and was found to be associated with infections in a variety of organs, including skin, joints and visceral organs in a large number of various animal species (Schaal, 1986; Nattermann and Horsch, 1977; Lämmler and Hartwigk, 1995; Narayanan et al., 1998; Trinh et al, 2002; Jost and Billington, 2005).

In cattle, *A.* (*T.*) *pyogenes* is commonly associated with abscessation (Narayanan et al., 1998; Ertaş et al., 2005; Lin et al., 2010), endometritis (Kaneko et al., 1997; Huszenicza et al., 1999; Silva et al., 2008) and pneumonia (Gagea et al., 2006). Narayanan et al. (1998) found that *A.* (*T.*) *pyogenes* could be recovered from bovine ruminal mucosa (n = 59). In 2009, Petit et al. isolated *A.* (*T.*) *pyogenes* from 41 % of cows with endometritis and from 3.5 % of cows without endometritis. These findings supported previous studies that *A.* (*T.*) *pyogenes* considers as major cause of endometritis (Kaneko et al., 1997; Huszenicza et al., 1999; Silva et al., 2008). Zambrano-Nava et al. (2011) showed that *A.* (*T.*) *pyogenes* was

isolated from 23 % of healthy Criollo Limonero cows, yielding the assumption that this bacterium is also part of the vaginal flora and should be considered as opportunistic pathogen. *A.* (*T.*) *pyogenes* could also be recovered from mastitic cultures, particularly in summer mastitis cases which most commonly lead to a loss of the mammary gland function (Quinn et al., 2002; Gröhn et al., 2004). Summer mastitis characterized by a foul-smelling and being purulent is most prevalent in non-lactating pregnant cows (Hillerton, 1987). However, in 2009, Vasil' isolated from milk and udder secretion of lactating dairy cows with mastitis *A.* (*T.*) *pyogenes* together with *Streptococcus* sp. and coagulase-negative staphylococci as one of the main etiological agents.

In pigs, *A.* (*T.*) *pyogenes* seems to be associated with suppurative pneumonia (Høie et al., 1991), polyarthritis (Hariharan et al., 1992) and abscessation (Ohba et al., 2007). Comparable to the previously described isolation of *A.* (*T.*) *pyogenes* from bovine ruminal mucosa (Narayanan et al., 1998), this opportunistic inhabitant was found in 39 % of porcine stomachs investigated (Jost et al., 2002a). Moreover, *A.* (*T.*) *pyogenes* had been documented to be responsible on direct economical losses caused by carcass condemnation of pigs (Martínez et al., 2007).

In addition, *A.* (*T.*) *pyogenes* also seems to be associated with infections in a large number of various other animal species, including antelopes, bison, camels, cats, chicken, deer, dogs, elephants, gazelles, horses, macaws, reindeer, turkeys and wildebeest (Jost and Billington, 2005). The isolation of *A.* (*T.*) *pyogenes* in high density growth from metritis and hydrosalpinx in Iraqi buffaloes was recognized by Azawi et al. (2007; 2010). In both studies *A.* (*T.*) *pyogenes* was recovered together with various other Gram-positive and Gram-negative bacteria and the pathogenic importance of this species was not clear. In camels, *A.* (*T.*) *pyogenes* was isolated by Al-Tarazi, 2001 and Bani Ismail et al., 2007 from pneumonia and arthritis.

In addition, this opportunistic pathogen was involved in several infections in wild-living animals such as a thoracic abscess in an alpaca (Adolf et al., 2001), foot infections in fallow deer (Lavin et al., 2004), septicemic infections involving skin, joints and visceral organs in captive blackbucks (Portas and Bryant, 2005), an intracranial abscessation in a white-tailed deer (Karns et al., 2009) and in septicemia in a southern pudu following uterine prolapse (Twomey et al., 2010). In 2010, Ülbegi characterized two *A.* (*T.*) *pyogenes* strains isolated by post mortem examinations from specimens of a bearded dragon and a gecko. In this study the *A.* (*T.*) *pyogenes* strains were isolated in both animals in large numbers together with several

other bacterial species from various organs, indicating a diagnosis of septicemia, with *A.* (*T.*) *pyogenes* as major causative bacterial agent.

In 2002a, Billington and coauthors described a case of feline otitis externa, where *A.* (*T.*) *pyogenes* was isolated in pure cultures and a second case from a dog with urinary tract infection in which *A.* (*T.*) *pyogenes* was isolated from urine as predominant bacterial species.

The finding of *A.* (*T.*) *pyogenes* had also been reported in co-infections with Gram-negative anaerobic bacteria. Several studies suggested a possible pathogenic synergism between *A.* (*T.*) *pyogenes* and *Fusobacterium necrophorum* which increased the virulence and resulted in severe infections (Roberts, 1967; Ruder et al., 1981; Roeder et al., 1989; Seimiya et al., 2004; Williams et al., 2005). The synergistic effect is thought to be caused by a leucocidal toxin produced by *F. necrophorum* that inhibited phagocytosis and in turn enhanced the growth of *A.* (*T.*) *pyogenes*. The latter, according to Ruder et al. (1981), provides catalase and growth factors which were necessary for the growth of *F. necrophorum*.

A. (*T.*) *pyogenes* rarely causes diseases in humans. Gahrn-Hansen and Frederiksen 1992 reported about the isolation of *A.* (*T.*) *pyogenes* from 11 Danish patients from the year 1986 to 1992 with no data about possible animal contact. These 11 isolates were mainly recovered in pure culture from different soft tissue infections, abscessation and septicemia. According to Gahrn-Hansen and Frederiksen (1992), eight out of the 11 isolates were phenotypically identified as *A.* (*T.*) *pyogenes*. In the following years, only few studies described the isolation of *A.* (*T.*) *pyogenes* in infections in humans. Lynch et al., 1998 reported about a case of septic arthritis and osteomyelitis in a diabetic farmer, Ide et al. (2006) about a case of spondylodiscitis in a veterinarian. A first description of an endocarditis in a man without animal contact was reported by Plamondon et al., 2007. Recently, a sepsis case of a farmer in Brazil caused by *A.* (*T.*) *pyogenes* was described by Levy et al., 2009 and wound infections associated with *A.* (*T.*) *pyogenes* by Kavitha et al., 2010.

A. (*T.*) *pyogenes* was reported to be a 1 to 2 µm by 0.2 to 0.5 µm, small, non-motile and non-spore-forming bacterium (Lämmler and Hartwigk, 1995). This species was described as facultatively anaerobic (Collins and Jones, 1982; Reddy et al, 1982; Schaal, 1986; Lämmler and Hartwigk, 1995; Narayanan et al, 1998). *A.* (*T.*) *pyogenes* was Gram-positive after staining from fresh cultures, cells from older cultures were Gram-variable. It occurred singly or in pairs arranged in V- or T-shapes (Reddy et al., 1982). Macroscopically, colonies of *A.* (*T.*) *pyogenes* grown on sheep blood agar were described as pinpoint, convex, slightly translucent and circular colonies that were surrounded by a β-hemolysis (Schaal,

1986; Lämmler and Hartwigk, 1995). According to Collins and Jones (1982), *A.* (*T.*) *pyogenes* could be inactivated at 60 °C within 15 min. The peptidoglycan of *A.* (*T.*) *pyogenes* cell wall is based on lysine, fatty acid analyses revealed a straight chain saturated and monounsaturated acid (Reddy et al., 1982). *A.* (*T.*) *pyogenes* has also proteolytic properties which could be demonstrated by serolysis on Loeffler medium (Hartwigk and Marcus, 1962; Lämmler and Hartwigk, 1995). Moreover, the ability to hydrolyze gelatin had been observed by many investigators (Gahrn-Hansen and Frederiksen, 1992; Narayanan et al., 1998; Goyal et al., 2005 and Plamondon et al., 2007). *A.* (*T.*) *pyogenes* had been shown to cross react with streptococcal serogroup G specific antiserum. This property could be used for serological species identification (Lämmler and Blobel, 1988; Lämmler and Hartwigk, 1995; Lynch et al., 1998; Ide et al., 2006). The genomic DNA of *A.* (*T.*) *pyogenes* contained between 56 to 58 mol % Guanine and Cytosine (G + C) (Collins and Jones, 1982; Reddy et al, 1982).

2.2.1.1 Putative virulence factors of *A.* (*T.*) *pyogenes*

2.2.1.1.1 Pyolysin

A. (*T.*) *pyogenes* primary virulence factor pyolysin (PLO) is a well known and potent extracellular toxin (Ding and Lämmler, 1996; Billington et al., 1997; Jost et al., 1999). This hemolysin was characterized preliminary by Ding and Lämmler (1996) and Funk et al. (1996) as heat-labile, oxygen-stable and sensitive to be treated with protease, trypsin and amylase. Its activity was not affected by treatment with oxidizing agents (Funk et al., 1996).

Beside the first identification as a hemolysin for erythrocytes of different animal species (Funk et al., 1996), PLO displayed cytotoxic effects on a varying number of host cells such as polymorphonuclear leucocytes (PMNs) and macrophages (Ding and Lämmler, 1996; Billington et al., 1997; Jost et al., 1999). According to Billington et al. (1997) gene *plo* has a nucleotide sequence of 1,605 bp encoding protein PLO of 57.9 kDa with 30 to 40 % identity to other thiol-activated cytolysins (TACYs) of a number of Gram-positive bacteria such as intermedilysin (ILY), listeriolysin O (LLO), perfringolysin O (PFO), pneumolysin (PLY) and streptolysin O (SLO). Thiol-activated cytolysins are cholesterol-dependent cytolysins (CDCs) indicating that their cytolytic effect through pore formation in eukaryotic cell membranes is restricted to cholesterol containing target membranes (Giddings et al., 2003). However, according to Funk et al. (1996) cholesterol had no effect on PLO activity. All CDCs share a

three dimensional structure, composed of four β-sheet rich domains. Domain 4 was found to contain a characteristic undecapeptide sequence and is thought to be involved in interaction with host membrane cholesterol **(Fig. 1)** (Michel et al., 1990; Boulnois et al., 1991; Sekino-Suzuki et al., 1996; Jacobs et al., 1999; Heuck et al., 2000; Nagamune et al., 2004; Ramachandran et al., 2002). According to Billington et al. (2002a, b) the three tryptophan residues within the undecapeptide region of PLO are important for hemolytic and cholesterol binding activities. Although PLO was classified to the TACYs family its hemolytic activity is not thiol-activated because the cysteine residue at position two of the undecapeptide region was replaced by alanine **(Fig. 1)**. The side chain thiol on cysteine residue is responsible for the oxygen-labile nature of the TACYs family (Billington et al., 1997). A site-directed cysteine-to-alanine mutation at the undecapeptide in PLY, SLO and LLO resulted in oxygen-stable cytolysins (Pinkney et al., 1989; Saunders et al., 1989; Michel et al., 1990). These results come in line with a study that the variant nature of PLO undecapeptide is required for its full cytolytic activity (Billington et al., 2002b).

Partially purified PLO was found to induce dermonecrotic and lethal effects in laboratory animals (Lovell, 1944). An additional study involved the use of specific antibodies against purified PLO. These antibodies completely neutralized the hemolysin activity of *A.* (*T.*) *pyogenes* and protected mice from death after infection with *A.* (*T.*) *pyogenes*, suggesting the importance of PLO, like that of other TACYs cytolysins, in the pathogenesis process (Billington et al., 1997). PLO like other TACYs is also cytolytic for phagocytic cells and neutrophils (Jost et al., 1999). Insertional inactivation of *plo* gene resulted in a loss of PLO expression and a loss in cytolytic activities, indicating the importance of PLO for in-vivo survival of *A.* (*T.*) *pyogenes*, possibly protecting the bacteria during the early stage of infection from host immune defense (Jost et al., 1999). These authors also showed that this *A.* (*T.*) *pyogenes plo* mutant at dose 10^8 bacteria was unable to establish intraperitoneal infection in mice, whereas 7 of 8 mice that received the wildtype strain had infection and *A.* (*T.*) *pyogenes* was isolated in large numbers from their liver and peritoneal fluid. According to Jost et al. (1999) the *plo* mutant *A.* (*T.*) *pyogenes* strain could restore its full virulence by providing *plo* gene *in trans* from a wild-type *A.* (*T.*) *pyogenes*, confirming that the reduction in the virulence observed with the PLO mutant was due to a defect in PLO production. These results underline the role *A.* (*T.*) *pyogenes* PLO, like other TACYs of other Gram-positive bacteria, plays in the pathogenesis of infection. According to Ding and Lämmler (1996), Billington et al. (1997), Jost et al. (1999) and Silva et al. (2008) PLO is

produced by all *A.* (*T.*) *pyogenes* strains examined to date, and its expression is restricted to stationary phase cultures (Ding and Lämmler, 1996). In a study designed to investigate the PLO expression under in vitro conditions in broth culture, it was shown that PLO production was induced during the early stationary phase and that this correlated with an increase in *plo*-specific mRNA (Rudnick et al., 2008).

PLO	**491**	**E**	**A**	**T**	**G**	**L**	**A**	**W**	**D**	**P**	**W**	**W**	**-**	**501**
LLO	**483**	**E**	**C**	**T**	**G**	**L**	**A**	**W**	**E**	**-**	**W**	**W**	**R**	**493**
PFO	**458**	**E**	**C**	**T**	**G**	**L**	**A**	**W**	**E**	**-**	**W**	**W**	**R**	**468**
PLY	**427**	**E**	**C**	**T**	**G**	**L**	**A**	**W**	**E**	**-**	**W**	**W**	**R**	**437**
SLO	**532**	**E**	**C**	**T**	**G**	**L**	**A**	**W**	**E**	**-**	**W**	**W**	**R**	**542**
ILY	**485**	**G**	**A**	**T**	**G**	**L**	**A**	**W**	**E**	**P**	**-**	**W**	**R**	**495**

Fig. 1: Amino acid alignment of the undecapeptide sequence of TACYs (CDCs). Amino acids in ILY and PLO which diverge from the consensus undecapeptide sequence are marked. Amino acid numbers are shown at each end of the sequence (according to Jost and Billington, 2005).

2.2.1.1.2 Extracellular matrix-binding proteins

The first critical step for infection is the adhesion of microorganisms to host tissues, leading to colonization, proliferation and invasion which subsequently lead to infection. The extracellular matrixes (ECM) are host structural macromolecules composed of glycoproteins, such as collagen, fibronectin, fibrinogen, laminin and elastin (Jost and Billington, 2005). According to Patii et al., 1994; Esmay et al., 2003; Jost and Billington, 2005 and Pietrocola et al., 2007, these extracellular matrixes surround eukaryotic epithelial cells and serve as cellular ´glue`.

It had been described by many investigators that ECM can be recognized by microbial surface components recognizing adhesive matrix molecules (MSCRAMM). This mediated microbial adhesion to host tissues (Bodén and Flock, 1989; Signäs et al., 1989; Świtalski et al., 1989; Park et al., 1991; Hubble et al., 2003). The role of MSCRAMMs binding fibrinogen, fibronectin and collagen as virulence factor was investigated in several studies (Bodén et al., 1989; Signäs et al., 1989; Esmay et al., 2003; Hubble et al., 2003). A single MSCRAMM might bind several ECM components. This was shown for the plasmid-encoded virulence factor *Yersinia* adhesion A (YadA) which was associated to the pathogenicity of *Yersinia*

enterocolitica. YadA appeared to be a collagen-binding protein (Emödy et al., 1989) which can also bind fibronectin (Tertti et al., 1992). Moreover, microorganisms can express several MSCRAMMs that recognize the same matrix molecule. For example, *Staphylococcus aureus* appears to express several fibrinogen-binding proteins (Bodén et al., 1989). This type of variation in the interactions between bacterial MSCRAMMs and host ECM components suggests its pathogenesis importance (Patti et al., 1994).

Collagen, a major target site for many microorganisms, represents the most abundant protein in mammals (Hay, 1991). *A.* (*T.*) *pyogenes* expresses, as described by Esmay et al. (2003), a collagen-binding protein (CbpA). According to these authors a *cbp*A mutant of *A. pyogenes* exhibited a reduced adhesion to both HeLa (epithelial) and 3T6 (fibroblast) cell lines, as the introduction of *cbp*A into a naturally *cbp*A negative isolate enhanced the adhesion to both cell lines. This underlines the importance of CbpA as virulence factor which promotes adhesion and subsequently the colonization of *A.* (*T.*) *pyogenes* to collagen rich tissues. The binding specificity of *A.* (*T.*) *pyogenes* CbpA was investigated by Esmay et al. (2003). Purified CbpA bound collagen type I, II and IV but not fibronectin. CbpA recognized the triple helical structure of collagen but was not able to bind to denatured collagen, indicating, according to Esmay et al. (2003), the specificity of CbpA for collagen. In 2007, Pietrocola et al. investigated functional and structural properties of *A.* (*T.*) *pyogenes* CbpA. These authors showed that CbpA bound to almost all collagen types including type I, II, III, IV, V, IX and XI, and that CbpA shows a second binding activity for fibronectin and to a lesser extent for fibrinogen. The results from Pietrocola et al., 2007 come in line with other studies investigating several bacterial surface proteins which bind different host ECM proteins (Roche et al., 2004; Walsh et al., 2004). In 2007, Pietrocola et al., also found that antibodies against CbpA inhibited the attachment of *A.* (*T.*) *pyogenes* to collagen. These findings might highlight the fact that CbpA could be used as vaccine against *A.* (*T.*) *pyogenes* infections.

According to DNA dot blot results of Esmay et al. (2003) *cbp*A was found in *A.* (*T.*) *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 *cbp*A in all 57 investigated *A.* (*T.*) *pyogenes* isolated from cows with uterine infections. However, Santos et al. (2010) found *cbp*A only in 1.4 % of *A.* (*T.*) *pyogenes* isolated from bovine origin. According to the results from Silva et al. (2008) and Santos et al. (2010) reference strain *A.* (*T.*) *pyogenes* DSM 20630^{T} (ATCC 19411), originally isolated from a pig, did not carry gene *cbp*A.

Several microorganisms bind fibronectin. In 1978, Kuusela first reported about the binding of fibronectin to *S. aureus*. Subsequently many investigators identified and further characterized corresponding fibronectin-binding proteins and genes in several Gram-positive and Gram-negative bacteria (reviewed by Joh et al., 1999). According to Jost and Billington (2005), *A.* (*T.*) *pyogenes* expresses a 20 kDa cell wall fibronectin-binding protein. However, the fibronectin-binding protein from *A.* (*T.*) *pyogenes* was not further characterized and it was assumed that it plays a role in host cell adhesion as it was described for various other bacteria (Jost and Billington, 2005).

Expression of cell surface proteins by microorganisms binding fibrinogen had been observed for a long time. *S. aureus* is known to express clumping factor, which is a fibrinogen-binding protein associated with clump formation in the presence of blood plasma in vitro (Much, 1908). According to Lämmler (1994), 72 % of the *A. haemolyticum* cultures investigated displayed binding properties for human fibrinogen. In addition, *A.* (*T.*) *pyogenes* was found to have fibrinogen-binding properties (Lämmler and Ding, 1994). In the study of these authors, heat treatment (60 °C, 1 h) and treatment of the *A.* (*T.*) *pyogenes* with guanidinium chloride significantly enhanced the binding of human fibrinogen, possibly suggesting the removal of slime-like material covering the binding molecule (Lämmler and Ding, 1994). This come in line with a study showing that after guanidinium chloride treatment of group-B streptococci the fibrinogen binding activity was increased (Chhatwal et al., 1984). Heating at 95 °C and proteolytic treatment of the *A.* (*T.*) *pyogenes* cultures reduced fibrinogen binding activity, indicating the protein nature of the binding site (Lämmler and Ding, 1994).

Lämmler et al. (1985) investigated binding properties of *A.* (*T.*) *pyogenes* for human α_2-macroglobulin (α_2-M) and haptoglobin (Hp). In this study it was shown that *A.* (*T.*) *pyogenes* bound α_2-M and Hp and that this binding activity was saturable and could be completely inhibited by the respective plasma protein (Lämmler et al., 1985). In addition, binding of Hp to *A.* (*T.*) *pyogenes* was blocked by α_2-M while the binding of α_2-M to *A.* (*T.*) *pyogenes* was not inhibited by Hp (Lämmler et al., 1985). The α_2-M and Hp binding properties could be reduced by proteolytic enzymes and by heat treatment of the bacteria, indicating again that the binding sites were of protein nature (Lämmler et al., 1985; Ding and Lämmler, 1997). However, at present no further studies exist characterizing the *A.* (*T.*) *pyogenes* MSCRAMMs for fibronectin, fibrinogen, α_2–macroglobulin and haptoglobin and elucidating their possible role in bacterial virulence.

2.2.1.1.3 Exoenzymes

2.2.1.1.3.1 Neuraminidases

Neuraminidase (sialidase) is an enzyme that cleaves sialic acid residues from carbohydrates and glycoproteins and makes them available as carbon source for bacteria. Neuraminidases are enzymes described to be produced by viruses, bacteria, protozoa, parasites, fungi and vertebrates (Gottschalk and Bhargava, 1971). Bacterial neuraminidases play an important role in promoting adhesion to epithelial cells especially for bacteria that inhabit mucus membranes (Galen et al., 1992; Giebink, 1999; Tong et al., 2000). In 1989, an *A.* (*T.*) *pyogenes* neuraminidase was first reported for two of 42 *A.* (*T.*) *pyogenes* cultures by Schaufuss and Lämmler. Jost et al. (2001) characterized the neuraminidase and the encoding gene *nan*H (3,009 bp) from an *A.* (*T.*) *pyogenes* strain isolated from a bovine abscess. In this study, a total of 53 *A.* (*T.*) *pyogenes* strains isolated from bovine, pig and avian origin were positive for neuraminidase activity, showing that this enzyme is probably expressed by all *A.* (*T.*) *pyogenes* (Jost et al., 2001). Construction of a *nan*H mutant of *A. pyogenes* exhibited 80 % of wildtype neuraminidase activity, indicating that *A.* (*T.*) *pyogenes* seems to express more than one neuraminidase (Jost et al., 2001). This led to the identification of a second neuraminidase and the encoding gene *nan*P (5,112 bp) which might also be involved in host epithelial cell adherence (Jost et al., 2002b). Unlike *nan*H, which was present in all investigated *A.* (*T.*) *pyogenes* (n = 53), gene *nan*P was only present in 64.2 % of the 53 strains investigated by Jost et al. (2002b). A *nan*H-*nan*P double mutant of *A.* (*T.*) *pyogenes* completely lacked neuraminidase expression and impaired the ability to adhere to HeLa cells, indicating the importance of neuraminidase as virulence factor in adhesion to host epithelial cells (Jost et al., 2002b).

2.2.1.1.3.2 Proteases

An extracellular protease from *A. pyogenes* was first described by Schaufuss et al. (1989). In 1995, Takeuchi et al. detected five proteases with calcium-dependent proteolytic activity produced by *A.* (*T.*) *pyogenes* originated from cows and pigs. On SDS-PAGE gels *A.* (*T.*) *pyogenes* proteases had gelatinase and caseinase activity (Takeuchi et al., 1995). *A.* (*T.*) *pyogenes* proteases were inhibited by phenylmethylsulfonyl fluoride (PMSF) and diisopropyl

fluorophosphate (DFP), but not by other protease inhibitors investigated, suggesting that the protein produced could be classified as serine protease (Takeuchi et al., 1995). In a former study performed by Takeuchi et al. (1979), ninety three percent of pigs carrying abscesses from which *A.* (*T.*) *pyogenes* were isolated had antibodies to proteases. According to Jost and Billington (2005), *A.* (*T.*) *pyogenes* proteases degraded host proteins making amino acids available as nutrients. The proteases may also degrade immunoglobulin A (IgA) which is involved in host defense, indicating that these extracellular proteases may have a role in *A.* (*T.*) *pyogenes* pathogenesis. The findings from Jost and Billington (2005) come in line with results about several other proteolytic enzymes of bacteria involved in host tissue damage and immune defense inhibition (Travis et al., 1995). At present no further studies about *A.* (*T.*) *pyogenes* proteases exist.

2.2.1.1.3.3 DNase

A. (*T.*) *pyogenes* additionally produces DNase (Sneath et al., 1986; Lämmler and Blobel, 1988; Hirsh and Biberstein, 2004; Moore et al., 2010). According to Jost and Billington (2005) and Moore et al. (2010), DNase is thought to aid in the release of bacteria through depolymerization of highly viscous DNA released from host cells in inflammatory lesions and to make nucleotides available for bacterial utilization. However, the actual role of DNase for *A.* (*T.*) *pyogenes* pathogenesis is still unknown.

2.2.1.1.4 Fimbriae

Prokaryotic fimbriae which are proteinaceous appendages that emerge from bacterial cell surface serve for adherence of numerous bacterial pathogens. They are well known in Gram-negative bacteria mediating the adherence to host tissue (Beachey, 1981; Clegg and Gerlach; 1987). Only few Gram-positive bacteria had been described to produce fimbriae. These include some oral streptococci, *Actinomyces* spp. (Cisar and Vatter, 1979; Wu and Fives-Taylor, 2001) and several *Corynebacterium* spp. (Yanagawa and Honda, 1976; Ton-That et al, 2004). Fimbriae in Gram-positive bacteria are morphologically similar to those of Gram-negative bacteria with 200 to 700 nm in length and 2.5 to 4.5 nm in width (Yanagawa and Honda, 1976; Cisar and Vatter, 1979, Ton-That et al., 2004).

Jost and Billington (2005) preliminarily characterized fimbriae from *A.* (*T.*) *pyogenes*. Like other bacterial fimbriae they might be involved in adhesion of *A.* (*T.*) *pyogenes* to host tissue. In 2008, Silva et al. evaluated the presence of eight putative virulence factors of *A.* (*T.*) *pyogenes* isolated from bovines. Among these eight factors, the authors investigated the four fimbrial genes *fim*A, *fim*C, *fim*E and *fim*G encoding the major fimbrial subunit of four different fimbriae (Silva et al., 2008). In the study of these authors *fim*A was found in all *A.* (*T.*) *pyogenes* (n = 57), *fim*E in 98 % and the gene *fim*C and *fim*G in 67 %, respectively, of the *A.* (*T.*) *pyogenes* investigated. More recently, Santos et al. (2010) studied the occurrence of the genes *fim*A and *fim*G in *A.* (*T.*) *pyogenes.* The cultures represented isolates from uterine samples of metritic as well as healthy cows. The two fimbrial genes *fim*A and *fim*G were identified in 76.4 % and 50 % of the *A.* (*T.*) *pyogenes* isolates, respectively. Gene *fimA* was present in higher ratio in *A.* (*T.*) *pyogenes* isolated from metritic cows compared to healthy cows (P □ 0.01). According to Santos et al. (2010) *A.* (*T.*) *pyogenes* DSM 20630^T (ATCC 19411^T), originally isolated from a pig, carried *fim*A and *fim*G.

2.2.2 *Arcanobacterium* (*Trueperella*) *abortisuis*

The first description of *A.* (*T.*) *abortisuis* was given by Azuma et al. (2009). The Latin name was derived from the word "*abortus*"- abortion and the word "*suis*"- pig that refers to the type strain which was isolated from an abortion of a pig. However, according to a proposal from Yassin and coauthors (2011) this species should be reclassified into the new genus *Trueperella*, as *Trueperella abortisuis*.

The primary isolation of *A.* (*T.*) *abortisuis* was from a suppurative placentitis of a pig after abortion. Further bacteriological examinations of three aborted fetuses led to the isolation of similar microorganisms. However, these strains were not further characterized. The pig was originally from a farm with 600 animals in which abortions occurred intermittently.

The classification of this novel species to genus *Arcanobacterium* was mainly based on analysis of the 16S rDNA. The reference strain *A.* (*T.*) *abortisuis* DSM 19515^T was named after one of the authors as *A.* (*T.*) *abortisuis* strain Murakami. *A.* (*T.*) *abortisuis* was described as Gram-positive, strictly anaerobic, short and diphtheroid-shaped bacterium which was arranged in a V-shape (Azuma et al., 2009). The genomic DNA of *A.* (*T.*) *abortisuis* DSM 19515^T contained 63.8 mol % G and C (Azuma et al., 2009).

In an additional study to elucidate the role *A.* (*T.*) *abortisuis* plays in abortion in pigs, Murakami and coauthors could demonstrate by pathological and immunohistochemical investigations the association of *A.* (*T.*) *abortisuis* with pulmonary and placental lesions of aborted fetuses and the pig, respectively (Murakami et al., 2011). At present no information exists about the occurrence of *A.* (*T.*) *abortisuis* in infections of other animals or humans.

2.2.3 *Arcanobacterium* (*Trueperella*) *bernardiae*

The original isolation of *A.* (*T.*) *bernardiae* was performed in 1987 with 11 strains described as coryneform group 2 bacteria. These strains were isolated from blood, wounds and the urinary tract of humans in the Centers for Disease Control and Prevention (CDC, Atlanta, Georgia, USA) (Na'was et al., 1987). In 1995, based on phenotypic, chemotaxonomic and molecular results, Funke and coauthors gave a first description of these strains, previously named coryneform group 2 bacteria, as *Actinomyces bernardiae*, classified to genus *Actinomyces* with the type strain *Actinomyces bernardiae* DSM 9152^T which was originally isolated from a human blood culture. 16S rDNA analysis revealed a relation of *A. bernardiae* to *Actinomyces pyogenes* (98.1 %, 16S rDNA sequence similarity) (Funke et al., 1995). The species name was given in honor of the Canadian microbiologist Kathryn A. Bernard, for her contributions to the study of Gram-positive, non-spore-forming rod-shaped bacteria. Ramos et al. finally reclassified *Actinomyces bernardiae* in 1997 to genus *Arcanobacterium* as *Arcanobacterium bernardiae.* This was based on 16S rDNA sequencing results. In 2011, Yassin and coauthors proposed that *A. bernardiae* should be reclassified into the new genus *Trueperella*, as *Trueperella bernardiae*.

The isolation of *A.* (*T.*) *bernardiae* had been reported from blood culture, as already described, and also from abscesses of humans (Funke et al., 1995), from urinary tract infections (Ieven et al., 1996; Lepargneur et al., 1998), from septic arthritis (Adderson et al., 1998), from an infection of a prosthetic joint (Loïez et al., 2009), from osteitis (Bemer et al., 2009), from necrotizing fasciitis (Clarke et al., 2010) and from a case of bacteremia in a patient with deep soft tissue infection (Weitzel et al., 2011).

A. (*T.*) *bernardiae* was described as Gram-positive, facultatively anaerobic, non-motile, non-spore-forming rod with coccobacilli shape (Funke et al, 1995; Bemer et al, 2009; Loïez et al, 2009). According to Funke et al. (1995), the G + C content of *A.* (*T.*) *bernardiae* genomic

DNA was between 63-66 mol %. At present no information exists about the occurrence of *A.* (*T.*) *bernardiae* in infections of animals.

2.2.4 *Arcanobacterium* (*Trueperella*) *bialowiezense* and *Arcanobacterium* (*Trueperella*) *bonasi*

A. (*T.*) *bialowiezense* and *A.* (*T.*) *bonasi* were first described as two novel species of genus *Arcanobacterium* by Lehnen et al. (2006). Strains of these two species were isolated from preputial swabs of European bison bulls. According to Kita et al. (1994), a chronic disease of the external genital organs of male European bison bulls had already been observed since 1980 on the Polish side of Bialowieza National Park. Characterization of bacteria isolated from this balanoposthitis of the European Bison by a combination of genomic, physiological and chemotaxonomic methods revealed the two novel species *Arcanobacterium bialowiezense* and *Arcanobacterium bonasi* belonging to genus *Arcanobacterium* (Lehnen et al., 2006). The Latin word "*bialowiezense*" is derived from this Bialowieza National Park in Poland, where the type strain was isolated. The second species name "*bonasi*" is derived from the European bison bulls (*Bison bonasus*), from which the type strain was isolated.

According to 16S rDNA sequencing results *A.* (*T.*) *bialowiezense* and *A.* (*T.*) *bonasi* shared 97.2 % sequence homology to each other, 96.1 % and 96.4 % sequence homology to 16S rDNA of *A. pyogenes* DSM 20630^T, respectively and 95.5 % and 95.8 % sequence homology to 16S rDNA of *A.* (*T.*) *bernardiae* DSM 9152^T, respectively (Lehnen et al., 2006). As mentioned before for *A.* (*T.*) *pyogenes*, *A.* (*T.*) *abortisuis* and *A.* (*T.*) *bernardiae*, Yassin and coauthors also proposed that *A. bialowiezense* and *A. bonasi* should be reclassified into the new genus *Trueperella*, as *Trueperella bialowiezensis* and *Trueperella bonasi*.

A. (*T.*) *bialowiezense* and *A.* (*T.*) *bonasi* were described as Gram-positive, facultatively anaerobic, short pleomorphic rod-shaped and non-motile bacteria. After cultivation on sheep blood agar colonies of both species were translucent, convex, approximately 0.5 mm in diameter and surrounded by a narrow zone of β-hemolysis. According to Lehnen et al. (2006), both species have an optimum growth on sheep blood agar at 37 °C under aerobic conditions, less pronounced under CO_2-enriched or anaerobic atmosphere conditions.

The *A.* (*T.*) *bialowiezense* and *A.* (*T.*) *bonasi* strains described by Lehnen et al., 2006 were isolated from balanoposthitis of bison bulls together with various other Gram-positive and

Gram-negative bacteria, indicating that the pathological significance of these two new species is unclear.

In 2008, Hassan et al. confirmed the species identity of three *A.* (*T.*) *bialowiezense* and seven *A.* (*T.*) *bonasi* strains by PCR-mediated amplification of species specific parts of 16S-23S rDNA intergenic spacer region (ISR) from both species. At present no information exists about the occurrence of *A.* (*T.*) *bialowiezense* and *A.* (*T.*) *bonasi* in infections of other animals or humans.

2.2.5 *Arcanobacterium haemolyticum*

A. haemolyticum, formerly known as *Corynebacterium haemolyticum* was first described in 1946 as cause of nasopharynx and skin infections in humans (MacLean et al., 1946). The Latin name was derived from the word "*haema*"-blood and the adjective "*lyticus*"-dissolving that referred to the hemolytic properties of this species.

According to MacLean et al. (1946), *C. haemolyticum* resembles characteristics to the animal pathogens *C. pyogenes* and *Corynebacterium ovis*. However, the taxonomic placement of *C. haemolyticum* as well as *C. pyogenes* within genus *Corynebacterium* had been questioned by many investigators (Cummins and Harris, 1956; Barksdale et al., 1957; Rogosa et al., 1974; Jones, 1975; Schofield and Schaal, 1981; Collins et al., 1982a).

The chemical composition of the cell wall of *C. haemolyticum and C. pyogenes* was investigated by Cummins and Harris (1956) and Barksdale et al. (1957). These authors found that the cell wall composition of the two species is similar to each other and that taxonomically both species belong to genus *Streptococcus*. Barksdale et al. (1957) also suggested according to cell wall analysis and biochemical results for both species that *C. haemolyticum* is a mutant form of *C. pyogenes*. Other authors also investigated the taxonomic position of *C. haemolyticum* and *C. pyogenes*. According to Collins et al. (1982a) *C. haemolyticum* was considered to be quite distinct from bacteria of genus *Corynebacterium* and it may represent a new genus. Schofield and Schaal (1981) and Collins et al. (1982a), based on numerical taxonomic studies, distinguished *C. haemolyticum* and *C. pyogenes* from each other and showed that *C. pyogenes* is taxonomically associated to *Actinomyces bovis* and should be reclassified to genus *Actinomyces* as *Actinomyces pyogenes.*

In 1982, *C. haemolyticum* was reclassified as *Arcanobacterium haemolytic*um, the first species in the newly proposed genus *Arcanobacterium* (Collins et al., 1982b). In 2011, based

on phylogenetic studies, Yassin and coauthors rearranged the nine species of genus *Arcanobacterium* into the two genera *Arcanobacterium* and *Trueperella*. These authors had assigned *A. haemolyticum* into genus *Arcanobacterium*, as *Arcanobacterium haemolyticum.*

A. haemolyticum generally is a human pathogenic organism and most commonly associated with pharyngitis and skin infections (Banck and Nyman, 1986; Waagner, 1991; Esteban et al., 1994). However, *A. haemolyticum* rarely can disseminate from the primary infection site and cause septicemia and soft tissue infection (Jobanputra and Swain, 1975; Skov et al., 1998; Tan et al., 2006), endocarditis (Al□s et al., 1995), arthritis and osteomyelitis (Goyal et al., 2005), pyothorax (Parija et al., 2005) and brain abscess (Vargas et al., 2006). Younus et al. (2002) reported about a deep tissue abscessation in a human caused by a co-infection of *A. haemolyticum* and *Fusobacterium necrophorum*. A necrotizing fasciitis case was reported by Lee et al. (2008) in a dog bitten patient where *A. haemolyticum* was recovered together with *Streptococcus agalactiae* and *Finegoldia magna* from the site of infection. According to Lee et al. (2008), the transmission of *A. haemolyticum* to the patient's wound from the dog bite was not clear. In 2009, Fernández-Suárez et al., recovered *A. haemolyticum* from case of Lemierre syndrome and septicemia in an immunocompetent patient.

The occurrence of *A. haemolyticum* in animals was first described from bovine semen and from pneumonia of sheep (Richardson and Smith, 1968; Roberts, 1969). Tyrrell et al. (2002) discussed the etiological role of a single *A. haemolyticum* strain isolated from a periodontal infection of a rabbit. In 2009, Hassan et al. characterized phenotypically and genotypically seven *A. haemolyticum* isolated from infections of six horses. However, no data were given about the route of infection and about the zoonotic importance of these strains (Hassan et al., 2009). This was the first report about the isolation of *A. haemolyticum* from horses. More recently, Bancroft-Hunt et al. (2010) characterized an *A. haemolyticum* strain isolated in pure culture from a necrotic fasciitis in a bull. This was the first report about the isolation of *A. haemolyticum* as a primary causative bacterium for necrotic fasciitis in animals.

Sequencing of *A. haemolyticum* 16S rDNA was carried out by Stubbs and Collins (1993). According to Ramos et al. (1997), the 16S rDNA sequence of *A. haemolyticum* showed a phylogenetic proximity to the species *A. phocae*.

A. haemolyticum was described as Gram-positive, facultatively anaerobic, irregularly shaped, non-motile, non-spore-forming and non-acid-fast bacterium (Jobanputra and Swain, 1975; Collins et al, 1982b; Lämmler and Hartwigk, 1995; Almuzara et al, 2002). Colonies of *A. haemolyticum* were small (about 0.75 mm in size) after 24 h incubation on

blood agar, becoming larger (1.5-2.5 mm) after prolonged incubation and were surrounded on sheep blood agar by a zone of complete hemolysis (Collins et al, 1982b; Lämmler and Hartwigk, 1995; Goyal et al., 2005; Tan et al., 2006). The hemolysis of *A. haemolyticum* was significantly increased after cultivation on rabbit and human blood agar compared to cultivation on sheep blood agar (Lämmler and Blobel, 1988; Cummings et al, 1993; Lämmler, 1994; Ülbegi-Mohyla et al., 2009).

A. haemolyticum had been found by Fraser et al. (1964) to inhibit staphylococcal β-hemolysin leading to a reverse CAMP reaction. This antagonistic hemolytic reaction was also confirmed by many investigators (Lämmler and Blobel, 1988; Lämmler, 1994; Linder, 1997; Lämmler and Hartwigk, 1995; Almuzara et al, 2002; Goyal et al, 2005; Parija et al, 2005; Tan et al, 2006; Ülbegi-Mohyla et al., 2009).

The extracellular product of *A. haemolyticum* responsible for the inhibition of staphylococcal β-hemolysin was identified as phospholipase D (Souckova and Soucek, 1972), which also had dermonecrotic activity. According to Linder (1997), the phospholipase D which affected animal and human mammalian cell membranes interacted with sphingomyelin which represent the major part of the cell phospholipids. This interaction generated ceramide phosphate in the target cell membrane. The phospholipase D encoding gene *pld* from *A. haemolyticum* was cloned and sequenced and showed DNA sequence homology of 65 % to the corresponding genes from both *Corynebacterium pseudotuberculosis* and *Corynebacterium ulcerans* (Cuevas and Songer, 1993; McNamara et al, 1995). Hassan et al., 2009 confirmed the species identity of seven *A. haemolyticum* strains isolated from horses by PCR-mediated amplification of species-specific regions of gene *pld*. More recently, Lucas et al. (2010) had characterized the effects of *A. haemolyticum pld* gene product on host cells. This study revealed that *pld* gene is found on a genome region of reduced G + C content, suggesting the recent horizontal acquisition. Lucas et al. (2010) also characterized the role of PLD for disease pathogenesis via enhancing bacterial adhesion and promoting the host cell necrosis following invasion.

According to Collins et al. (1982b), the cell wall peptidoglycan of *A. haemolyticum* is based on lysine, and the G and C contents were determined between 50 and 52 mol %.

In 2010 the complete genome of *A. haemolyticum* DSM 20595^T (=ATCC 9345^T) had been submitted to the National Center for Biotechnology Information (NCBI Reference Sequence: NC_014218) (Yasawong et al., 2010).

2.2.6 *Arcanobacterium hippocoleae*

The first description of *A. hippocoleae* was given by Hoyles and coauthors in 2002. The species epithet *A. hippocoleae* was derived from the Latin word "*hippos*"- horse and the word "*colea*"- vagina, indicating the isolation of this species from the vagina of a horse. *A. hippocoleae* was isolated from the vaginal discharge of a horse with clinical vaginitis together with unidentified *Corynebacterium* species and coagulase-negative staphylococci, indicating the indefinite pathological significance of this species. The classification of *A. hippocoleae* was mainly based on a polyphasic taxonomic study also including 16S rDNA sequencing results (Hoyles et al., 2002). The 16S rDNA sequence of this species with a continuous stretch of 1492 bp revealed a sequence similarity to 16S rDNA sequences of five at that time described *Arcanobacterium* species from 94.8 % to 95.7 %. The highest phylogenetic proximity was to *A. phocae* (95.7 %). More recently, Yassin and coauthors (2011) rearranged the nine species of genus *Arcanobacterium* into the two genera *Arcanobacterium* and *Trueperella*. According to these authors *A. hippocoleae* was assigned into genus *Arcanobacterium.*

The isolation of *A. hippocoleae* from horse urine was described by Cai et al. (2003). In this study *A. hippocoleae* was isolated in pure culture, but the clinical significance of this species was not further investigated. A third isolation of *A. hippocoleae* was from stomach contents of a stillborn foal (Bemis et al., 2008). *A. hippocoleae* was also isolated from the lung of the foal carcass as well as from the placenta of the mare showing clinical placentitis. Because additional microorganisms were isolated from the placenta of the mare, the role *A. hippocoleae* played remained unclear (Bemis et al., 2008).

In a study designed to investigate the synergistic and antagonistic hemolytic activities of bacteria of genus *Arcanobacterium*, *A. hippocoleae* reference strain DSM 15539^T displayed an enhanced hemolysis on rabbit blood agar compared to sheep blood agar after cultivation under microaerobic conditions in a candle jar (Ülbegi-Mohyla et al., 2009). In addition, *A. hippocoleae* displayed CAMP-like hemolytic reactions with β-hemolytic *S. aureus* and *A. haemolyticum* DSM 20595^T as indicator strains.

According to Hoyles and coauthors (2002), *A. hippocoleae* was found as Gram-positive, non-acid-fast and non-motile, facultatively anaerobic, irregular-shaped and non-branching rod. *A. hippocoleae* produced a weak hemolysis on blood agar. At present no information exists about the occurrence of *A. hippocoleae* in infections of other animals or humans.

2.2.7 *Arcanobacterium phocae*

The first description of *Arcanobacterium phocae* was given in 1997 by Ramos and coauthors. The bacteria were isolated from the peritoneal fluid of a common seal. The species name *A. phocae* is derived from the Latin name for the common seal *Phoca vitulina*. In 2011, the nine species of genus *Arcanobacterium* were rearranged into the two genera *Arcanobacterium* and *Trueperella* (Yassin et al., 2011). These authors had assigned *A. phocae* into genus *Arcanobacterium.*

Ramos et al. (1997) isolated primarily six *A. phocae* strains from various tissues and fluids including lung, spleen, intestine, lymph nodes, mouth lesions, nasal swabs and peritoneal fluid of common seals and grey seals from the coastal waters around Scotland. These *A. phocae* strains were recovered in mixed cultures with other bacteria from pneumonic and septicemic seals, indicating the indefinite pathological significance of this novel species of genus *Arcanobacterium* (Ramos et al., 1997).

Johnson et al. (2003) described the isolation of *A. phocae* from samples obtained from marine mammals along the central California coast. This included isolates recovered from California sea lions (*Zalophus californianus*), Pacific harbor seals (*Phoca vitulina richardii*), northern elephant seals (*Mirounga angustirostris*), southern sea otters (*Enhydra lutris nereis*) and one common dolphin (*Delphinus delphis*).

Of the 77 *A. phocae* recovered from live animals, most isolates were from abscesses, wound infections and from nasal, tracheal and ocular discharge (Johnson et al., 2003). During postmortem examinations Johnson et al. (2003) isolated 84 *A. phocae* from inflammation sites and tissue samples including abscesses, wound infections, bones, and from internal organs like liver, lung, kidney, spleen and brain and also from samples like peritoneal and pleural fluids and lymph nodes. According to Johnson et al. (2003), other microorganisms like *Escherichia coli*, β-hemolytic streptococci, *Enterococcus* spp., *Proteus* spp., *Pseudomonas* spp., *S. aureus* and other Gram-positive and Gram-negative bacteria were also isolated together with *A. phocae,* so that the pathogenic importance of *A. phocae*, as already described by Ramos et al. (1997), remains unclear.

In 1998 Thornton et al., identified 108 strains isolated from stranded marine mammals from 1994 to 1998 as *Listeria ivanovii*. According to Johnson et al. (2003), these *L. ivanovii* isolates had been misidentified because of limitations in the phenotypic tests and possibly are all *A. phocae.*

The classification of *A. phocae* to genus *Arcanobacterium* was based on 16S rDNA sequencing results (Ramos et al., 1997). The 16S rDNA analysis revealed a phylogenetic proximity to the species *A. haemolyticum* with a sequence similarity value of 97.3 %. *A. phocae* was found to be a Gram-positive, non-motile, non-spore-forming, non-acid-fast coccobacilli and short rod. The bacteria grew facultative-anaerobic and displayed after 24 to 48 h growth on blood agar colonies with β-hemolysis (Ramos et al., 1997; Johnson et al., 2003). According to Johnson et al. (2003), a reverse CAMP-reaction in the zone of staphylococcal β-hemolysin and a positive CAMP-like reaction with *Rhodococcus equi* appeared to be a characteristic property of *A. phocae.* In 2009, Ülbegi-Mohyla et al. showed, comparably as previously described by Johnson et al. (2003), that six *A. phocae* strains displayed a synergistic CAMP-like hemolytic reactions with *Streptococcus agalactiae*, *R. equi*, *Psychrobacter phenylpyruvicus* as indicator strains and an antagonistic CAMP-like hemolysis in the zone of staphylococcal β-hemolysin. At present no information exists about the occurrence of *A. phocae* in infections of other animals or humans.

2.2.8 *Arcanobacterium pluranimalium*

The first description of *A. pluranimalium* was given in 2001 by Lawson and coauthors. The Latin name was derived from the word "*pluris*"- many and the word "*animalium*"- animals that refers to the strains isolation from various animals. *A. pluranimalium* was originally described with two isolates, one from a dead harbour seal and the second one from a dead sallow deer showing an identical 16S rDNA sequence with 100 % sequence homology to each other. The classification of the two strains to genus *Arcanobacterium* was performed after 16S rDNA sequence analysis. According to Lawson et al. (2001), the *A. pluranimalium* 16S rDNA sequence showed a similarity in the range from 93.9 % to 96.5 % to four other species of genus *Arcanobacterium* with the highest similarity to *A. phocae* (96.5 % similarity). As mentioned before for *A. haemolyticum*, *A. hippocoleae* and *A. phocae* the species *A. pluranimalium* had been assigned to genus *Arcanobacterium* as monophyletic genus distinct from the newly proposed genus *Trueperella* (Yassin et al., 2011).

A. pluranimalium was described as Gram-positive, straight to slightly curved, non-branching slender rod and as non-motile, non-acid-fast and non-spore-forming bacterium. *A. pluranimalium* was considered as facultatively anaerobic growing bacterium showing α-hemolysis on sheep blood agar (Lawson et al., 2001). The G + C content of *A. pluranimalium*

DSM 13483^T genomic DNA was 57 mol %. However, according to Lawson et al. (2001) the importance of *A. pluranimalium* as causative agent of the diseases in the harbor porpoise and the deer was not clear. According to Ülbegi-Mohyla et al., 2009, *A. pluranimalium* reference strain DSM 13483^T displayed an enhanced hemolysis on rabbit blood agar compared to sheep blood agar after cultivation under microaerobic conditions in a candle jar and CAMP-like hemolytic reactions with β-hemolytic *S. aureus*, *S. agalactiae* and *A. haemolyticum* DSM 20595^T as indicator strains.

More recently, Ülbegi-Mohyla et al. (2010) characterized an *A. pluranimalium* strain isolated from pyoderma of a dog. This strain was isolated together with other microorganisms, indicating that the pathogenic importance of *A. pluranimalium* for animal infections, as previously described by Lawson et al. (2001), remains unclear. At present no information exists about the occurrence of *A. pluranimalium* in infections of humans.

2.3 Matrix-assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) for bacterial identification

Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) fingerprinting is a method for bacterial identification to genus and species level and for some instances to subspecies or strain level (Lynn et al., 1999; Bernardo et al., 2002; Ruelle et al., 2004; Rupf et al., 2005; Murray, 2010; Bizzini et al., 2011). MALDI-TOF MS allowed a bacterial identification through generating protein mass spectral fingerprints in the mass range from e.g. 2 to 20 kDa from a bacterial cell. This could be conducted with little or no sample pretreatment in few minutes. According to Suh et al. (2005) and Maier and Kostrzewa (2007) the protein mass spectra obtained from whole bacterial cells were found to be mainly of ribosomal proteins.

During the last two decades, MALDI-TOF MS had been increasingly investigated and used for the identification of microorganisms. In 1996, Krishnamurthy et al. described specific biomarkers obtained from protein extracts of individual bacterial cells that could be used to detect pathogenic and non-pathogenic bacteria. Another study reported about the usefulness of MALDI-TOF MS for bacterial identification using a single colony directly from the sample plate (Claydon et al., 1996). Furthermore, Holland et al. reported also in 1996 about a chemotaxonomic approach using MALDI-TOF MS analysis of bacterial proteins obtained directly from intact bacterial cells. In these studies, the identification was done through the

comparison of the obtained unknown spectra with archived reference spectra or by co-analysis with spectra of known bacteria. The bacterial identification by MALDI-TOF MS is based on matching of spectral profiles or peaks derived from unknown bacteria against a database established in advance. For this, reproducible spectra are important and several pre-analysis perspectives should be taken into consideration. However, several investigators reported about some difficulties associated with the spectra reproducibility involved in MALDI-TOF MS analysis. Bacterial storage, handling, or culturing over different time intervals prior to MALDI-TOF MS analysis resulted into non-identical spectra (Lay Jr, 2001). In some instances the bacterial growth cycle could result in MALDI-TOF MS spectrum change (Vargha et al., 2006). Arnold et al. (1999) showed that non-identical MALDI-TOF MS spectra varied in the peaks and intensities which were caused by different time intervals associated with removing and analyzing of the bacterial cells. In another study, Welham et al. (1998) reported about spectral variations resulted from different amounts of loaded samples and from different incubation periods before analysis. However, in this study the authors observed that the protein spectra contained a number of relatively reproducible peaks. This comes in line with the results from Wang et al. (1998) who reported about conserved peaks in the spectra obtained under different experimental parameters. Generally, different culture conditions such as cultural media and temperature had little effect on MALDI-TOF MS fingerprinting (Valentine et al., 2005).

For sample preparation, several studies have shown the possibility to obtain peptidic spectra either from protein extracts (Cain et al., 1994; Krishnamurthy et al., 1996) or from whole bacterial cells (Holland et al., 1996; Krishnamurthy and Ross, 1996) incorporated with the MALDI matrix. In general, biological material from single colonies could be smeared directly on the steel MALDI target plate and overlaid with the MALDI matrix. According to Maier and Kostrzewa (2007) spectra obtained by different sample preparation protocols resulted in a very similar peak patterns. Different matrices have been described to be useful for MALDI-TOF MS fingerprinting. Of these matrices, α-cyano-hydroxycinnamic acid (HCCA) exhibited a significant advantage for facilitating the measurement automation (Lay Jr, 2001; Maier and Kostrzewa, 2007). The analysis of the sample is performed by hitting the loaded sample/matrix complex on the MALDI target plate with a laser beam (337 nm). This resulted into evaporation and in parallel ionizing of the analyte molecules. The analyte molecules are accelerated by an electric field into the flight tube of the mass spectrometer where they are

separated according to their different velocity (i.e. a function of ion mass and charge) in a field free drift region and the more light ions are faster to reach the detector **(Fig. 2)**.

$$tof = \alpha \times \sqrt[2]{m / z}$$

Fig. 2: Time of flight (TOF) equation where α = a constant multiplied by the square root of the mass (m) divided by charge (z) (modified according to http://de.wikipedia.org/wiki/MALDI-TOF).

More recently, MALDI-TOF MS had been introduced in microbiological diagnostic laboratories with a clear success to identify several Gram-positive and Gram-negative pathogenic bacteria obtained from humans such as *Salmonella* spp. (Leuschner et al., 2004), *Campylobacter* spp. (Mandrell et al., 2005), *Listeria* spp. (Barbuddhe et al., 2008), *Clostridium* spp. (Grosse-Herrenthey et al., 2008), *Corynebacterium* spp. (Konard et al., 2010), 32 genera obtained from blood cultures (Stevenson et al., 2010) and for *Legionella* spp. as respiratory human pathogen (Gaia et al., 2011). In veterinary microbiology MALDI-TOF MS was used to identify subclinical mastitis pathogens obtained from milk samples (Barreiro et al., 2010), the *Staphylococcus intermedius* group (SIG) (Decristophoris et al., 2011) and for *Streptococcus* spp. (Hinse et al., 2011). In addition, in environmental microbiology, MALDI-TOF MS allowed the identification of a variety of bacterial species (Ruelle et al., 2004; Munoz et al., 2011).

2.4 Molecular targets for PCR-mediated identification

2.4.1 Ribonucleic acid (RNA)

2.4.1.1 16S rRNA gene

From the early history of microbiology different phylogeny models had emerged microorganisms identification and classification, including morphological and structural, physiological and chemotaxonomic properties. Since 1980s, the introduction of ribosomal ribonucleic acid (16S rRNA) sequence analysis had significantly changed the history of

microbiology. The rRNA is the RNA component of the ribosome. The latter represents the site of protein synthesis in all living cells. The bacterial ribosome (70S) consists of two dissimilar subunits, the small 30S subunit (small ribosomal subunit RNA, SSU) and the large 50S subunit (large ribosomal subunit RNA, LSU). The unit S measures the sedimentation rate after centrifugation, which refers to the molecular weight of each part. The small subunit contains the 16S rRNA and an intergenic spacer region, the large subunit the 23S rRNA, another intergenic spacer region and the 5S rRNA **(Fig. 3)** (Gürtler and Stanisich, 1996). Because 16S rDNA is a universal target among bacteria and large enough (1,500 bp) for informatic purposes, as well as containing species specific variable regions, it has emerged as rDNA-based molecular identification tool for bacterial phylogeny (Weisburg et al., 1991; Patel, 2001; Cai et al., 2003). No universal definition for bacterial species identification using 16S rDNA sequencing exists, but several investigators had proposed acceptable criteria for establishing a species and genus identification cutoff. In 2000, Drancourt et al. proposed cutoff values of 16S rDNA-based bacterial identification. According to these authors a value of ≥ 99 % similarity of 16S rDNA sequence was assigned as suitable cutoff for bacterial species identification and ≥ 97 % for bacterial identification at genus level. Results from Cai et al. (2003) showed that full 16S rDNA sequencing (about 1,540 bp) gives more precise species identification of clinical bacteria of veterinary origin. While the costs are important in some highly throughput microbiological laboratories, species-specific variable regions within this gene could allow a tentative identification to genus level similar to that of the full 16S rDNA sequencing result (Cai et al., 2003).

Several authors had evaluated the significance of using 16S rRNA as single gene based bacterial phylogeny compared to global molecular targets such as RNA polymerase subunits and heat shock protein (Goh et al., 1996; Ghebremedhin et al., 2008). These authors had shown that the phylogenetic trees drawn from the protein sequences of these target genes demonstrate a remarkable similarity to that derived from 16S rDNA sequencing results. However, Ludwig (2010) had concluded that 16S rDNA is among the most informative targets and considered to be the most fundamental molecular approach to ascertain the degree of genetic relatedness of different microorganisms.

Fig. 3: Schematic representation of ribosomal RNA (modified according to Gürtler and Stanisich, 1996).

Referring to the bacterial subjects of this study, except *A. haemolyticum* as first species assigned to genus *Arcanobacterium* by Collins et al. (1982b), all other eight species of genus *Arcanobacterium* and the newly described closely related genus *Trueperella* were characterized by 16S rDNA sequencing. However, the bacterial assignment to genera *Arcanobacterium* and *Trueperella* was not only based on 16S rDNA sequencing but also on various other phenotypic and chemotaxonomic results (Ramos et al., 1997; Lawson et al., 2001; Hoyles et al., 2002; Lehnen et al., 2006 and Azuma et al., 2009; Yassin et al., 2011).

2.4.1.2 16S-23S rDNA intergenic spacer region (ISR)

The ribosomal intergenic spacer region (ISR) is a stretch of DNA that lies between the 16S and the 23S rRNA genes. The ISR contains various different transfer RNA (tRNA) genes as functional units. Loughney et al. (1982) described that the ISR of *Bacillus subtilis* contained both $tRNA^{Ala}$ (alanine) and $tRNA^{Ile}$ (isoleucine) genes. In 1991, a study performed by Bacot et al. investigating the ISR of *Streptococcus pneumoniae* showed that $tRNA^{Ala}$ but not $tRNA^{Ile}$ was found within the ISR. In Gram-negative bacteria like *E. coli* the ISR contained several different tRNA genes including $tRNA^{Ala}$, $tRNA^{Ile}$ and $tRNA^{Glu}$ (Glutamine) (Condon et al., 1995).

The conserved regions within the 16S rRNA and the 23S rRNA genes could be used for designing primers for amplifying the ISR (Whiley et al., 1995). Kostman et al. (1995) described a universal primer pair from conserved regions of the 16S and 23S ribosomal RNA genes amplifying the ISR of bacterial pathogens including *S. aureus*, *Enterococcus faecium*, *E. coli* and *Enterobacter* spp. The primer pair described by Kostman et al. (1995) could also be used to amplify the ISR of several bacterial species of genus *Streptococcus* and genus *Arcanobacterium* (Chanter et al., 1997; Hassan et al., 2008; Ülbegi, 2010). It had long been recognized that the ISR sequences of various bacterial species are significantly heterogeneous on the sequence level and variable in length on agarose gels, suggesting that the ISR is a

candidate region from which species-specific primers could be designed for bacterial identification (Barry et al, 1991; Tilsala-Timisjärvi and Alatossava, 1997).

The ISR genome segment of nine species of genera *Arcanobacterium* and *Trueperella* was amplified by Hassan et al. (2008) and Ülbegi (2010) using the primer pair described by Kostman et al. (1995) and Chanter et al. (1997), resulting in a clear separation of all nine species. Sequencing ISR also allowed a molecular identification of seven *A. haemolyticum* isolated from infections of horses (Hassan et al., 2009) and *A. pluranimalium* isolated from pyoderma of a dog (Ülbegi-Mohyla et al., 2010). The design of ISR species-specific oligonucleotide primers could be used for PCR-mediated identification of *A.* (*T.*) *pyogenes*, *A.* (*T.*) *bernardiae*, *A.* (*T.*) *bialowiezense*, *A.* (*T.*) *bonasi*, *A. haemolyticum* and *A. hippocoleae* (Hassan et al., 2008; Ülbegi, 2010).

2.4.1.3 23S rRNA gene

In prokaryotes, the 23S rRNA gene is 2,904 bp in length and contains a larger number of sequence variations compared to the 16S rRNA gene (Lewin, 1998). Comparative phylogenetic studies showed variable regions of the 23S rRNA gene which could be used for bacterial identification. These variable areas of the 23S rRNA gene allowed the design of species-specific primers which could be used to discriminate among four *Campylobacter* spp. (Eyers et al., 1993) and *Pasteurella multocida* (Miflin and Blackall, 2001). Moreover, Jost et al. (2004) described ribosomal mutations within the 23S rRNA gene which were associated with macrolides resistance in *A.* (*T.*) *pyogenes.*

For genera *Arcanobacterium* and *Trueperella*, sequencing the 23S rRNA gene allowed a molecular identification of *A. pluranimalium* isolated from pyoderma of a dog and a clear separation of all nine species (Ülbegi-Mohyla et al., 2010).

2.4.2 Superoxide dismutase A encoding gene *sod*A

Superoxide dismutase catalyzes the dismutation of superoxide free radical (O^-) to hydrogen peroxide and oxygen. The superoxide is formed when oxygen (O_2) becomes partially reduced (Fridovich, 1978). According to Imlay and Linn (1988) superoxide results in damage to lipids, proteins and DNA. However, microorganisms which live within highly enriched aerobic environment had developed superoxide dismutases (SODs) to detoxify superoxide into

oxygen and hydrogen peroxide (H_2O_2) ($2\ O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$). H_2O_2 could be broken down into water by the enzyme catalase. McCord and Fridovich (1969) described superoxide dismutase purified from bovine erythrocytes that catalyzes the dismutation of superoxide. These authors suggested that this enzyme has a vital role in protecting organisms against damaging effects of superoxide.

Depending on the metal ion cofactor required for activity, superoxide dismutases could be classified into the Cu/Zn-SOD type (binds both copper and zinc), Fe-SOD (binds iron), Mn-SOD (binds manganese) and Ni-SOD (binds nickel) (Clements et al., 1999). Mn-SOD and Fe-SOD are synthesized typically in prokaryotes (Martin et al., 1986). Several authors studied the usefulness of manganese-dependent superoxide dismutase encoding gene *sod*A in identification and differentiating closely related bacterial species. Gene *sod*A had discriminating power to differentiate *Streptococcus* spp. (Poyart et al., 1998), *Enterococcus* spp. (Poyart et al., 2000), *Staphylococcus* spp. (Poyart et al., 2001) and *Mycobacterium* spp. (Zolg and Philippi-Schulz, 1994). Using the universal oligonucleotide primer pair from Zolg and Philippi-Schulz (1994) and a new primer pair designed by Ülbegi (2010) allowed the amplification and sequencing of gene *sod*A of eight species of genera *Arcanobacterium* and *Trueperella* (excluding *A.* (*T.*) *bialowiezense*). This allowed a clear separation of these eight species of genus *Arcanobacterium* and *Trueperella*.

2.4.3 RNA polymerase encoding gene *rpo*B

The RNA polymerase (RNAP) is an important enzyme in all living organisms and is needed for construction of RNA in the process of transcription (mRNA, tRNA and rRNA) from DNA templates (Adékambi et al., 2009). This enzyme consists of five subunits, with the β-subunit which is encoded by *rpo*B which represents the majority of catalytic functions of RNAP (Adékambi et al., 2009; Yang and Price, 1995).

Gene *rpo*B is a target for some antibiotics such as rifampicin, streptolydigin and lipiarmycin, because RNA chain polymerization is also important for bacterial growth (Yang and Price, 1995; Kurabachew et al., 2008). Yang and Price (1995) showed that resistances to rifampicin in *E. coli* and in *B. subtilis* were associated with mutations in the region of the *rpo*B gene. Moreover, mutations linked to lipiarmycin resistant *Mycobacterium tuberculosis* were also associated with gene *rpo*B (Kurabachew et al., 2008). Based on *rpo*B sequencing results of species of genus *Corynebacterium*, Khamis et al. (2004) showed that this gene is significantly

more discriminative than the 16S rRNA gene and could be use as additional target for phylogenetic studies. According to Rowland et al. (1993) sequencing of the *rpo*B gene enabled a robust and accurate molecular identification of bacteria. The hyper variable regions of gene *rpo*B which lay between the bases 2300 and 3300 also allowed the identification of bacteria to the species and subspecies level (Adékambi et al., 2003; Khamis et al, 2004, 2005). Gene *rpo*B was also used to study the taxonomic relationship of *S. aureus* (Rowland et al., 1993), *Enterobacteriaceae* (Mollet et al., 1997) and for identifying novel species of genus *Mycobacterium* (Adékambi et al., 2006). Using the universal oligonucleotide primer pair described by Khamis et al. (2004) gene *rpo*B of nine reference strains of nine species of genera *Arcanobacterium* and *Trueperella* and two additionally investigated *A*. (*T*.) *pyogenes* strains isolated by post mortem examinations of a bearded dragon and a gecko could be amplified and sequenced. The gene sequencing results clearly separated all nine species of genera *Arcanobacterium* and *Trueperella* and allowed a molecular identification of the two *A*. (*T*.) *pyogenes* strains isolated from reptiles (Ülbegi (2010). More recently, a PCR-restriction fragment length polymorphism (PCR-RFLP) technique based on the hyper variable region of gene *rpo*B was used to differentiate *C. pseudotuberculosis* from *A*. (*T*.) *pyogenes* (Pavan et al., 2011).

2.4.4 Heat shock protein or chaperonin CPN60 encoding gene *cpn*60

Gene *hsp*60 or *cpn*60 encoding the 60 kDa chaperonin protein or heat shock protein (HSP60), (also known as GroEL) seems to be present in all bacteria (Goh, et al., 1996; Hill et al., 2004). Sequence analysis of gene *cpn*60 from a variety of bacterial species resulted in the design of universal degenerate primers that could amplify a PCR product of a 549-567 bp region of this gene from various Gram-positive and Gram-negative bacteria (Goh et al., 1996). This universal nature of gene *cpn*60 which proved to be used for identification of microorganisms as well as for phylogenetic studies, resulted in the introduction of a *cpn*60 database (cpnDB, www.cpndb.ca) (Hill et al., 2004). This database is mostly based on partial gene sequences and represents the largest collection of a protein-encoding gene.

Furthermore, according to Goh et al. (1996) gene *cpn*60 showed species-specific sequence variations suggesting that this gene could be used as alternative DNA target for species identification of *Staphylococcus* spp. and possibly other bacteria. In 2004, Alber et al. described a species-specific oligonucleotide primer of gene *cpn*60 that could be used for the

identification of *Streptococcus phocae.* More recently, *cpn*60 gene sequencing results were shown to be useful for identification and phylogenetic studies of *Lactobacillus* spp. (Blaiotta et al., 2008), *Aeromonas* spp. (Miñana-Galbis et al., 2009) and several other bacterial species (Schellenberg et al., 2011; Verbeke et al., 2011).

3 Materials and methods

3.1 Bacterial cultures

A total of 93 bacterial cultures were investigated in the present study. The cultures included reference strains of genera *Arcanobacterium*, *Trueperella* and *Actinomyces* (*A.* (*T.*) *pyogenes* DSM 20630^T, *A.* (*T.*) *pyogenes* DSM 20594, *A.* (*T.*) *abortisuis* DSM 19515^T, *A.* (*T.*) *bernardiae* DSM 9152^T, *A.* (*T.*) *bialowiezense* DSM 17162^T, *A.* (*T.*) *bonasi* DSM 17163^T, *A. haemolyticum* DSM 20595^T, *A. hippocoleae* DSM 15539^T, *A. phocae* DSM 10002^T, *A. phocae* DSM 10003, *A. pluranimalium* DSM 13483^T, *Actinomyces bovis* DSM 43014^T and *Actinomyces canis* DSM 15536^T), 51 *A.* (*T.*) *pyogenes* isolated from various animal origins, 23 *A.* (*T.*) *abortisuis* isolated from pigs and cows, three *A. haemolyticum* isolated from horses and three cultures isolated from dogs, representing a novel species of genus *Actinomyces*. All cultures were identified and further characterized phenotypically, by use of MALDI-TOF MS and by genotypic methods.

The 51 *A.* (*T.*) *pyogenes* strains were isolated during routine microbiological diagnostics from samples of various animal species. The *A.* (*T.*) *pyogenes* were obtained from bovine mastitis (n = 5), non mastitic origin of bovines (n = 14), pigs (n = 13), small ruminants including sheep and goat (n = 5), wild ruminants including mountain reedbuck, elk, yak and deer (n = 5), from a horse (n = 1), from dogs (n = 2) and cats (n = 2), from a rabbit (n = 1), a rat (n = 1) and from reptiles (n = 2). The 23 *A.* (*T.*) *abortisuis* isolates were collected in a period of 12 years from 1999 to 2010 from samples of pigs (n = 21) and cows (n = 2). The strain designation, the origin, additional information regarding the clinical findings of the diseased animals and the additionally isolated microorganisms from the various samples, respectively are summarized in **Tab. 1 and 2**. The origin of the three *A. haemolyticum* isolated from three horses, and the three strains isolated from three dogs, representing a novel species of genus *Actinomyces*, are summarized in **Tab. 3 and 4**.

Tab. 1: Origin of 51 *A.* (*T.*) *pyogenes* isolated from various animal species

No.	Strain/year of isolation	Animal	Specimen	Additional information	Additionally isolated microorganisms
1	1886/07	Cow	Milk	Mastitis	*Staphylococcus aureus, Streptococcus dysgalactiae*
2	1908/07	Cow	Milk	Mastitis	α-hemolytic streptococci, *Escherichia coli*
3	1947/07	Cow	Milk	Mastitis	*E. coli*
4	2495/07	Cow	Milk	Mastitis	*S. aureus*, *E. coli*
5	4804/07	Cow	Milk	Mastitis	*A.* (*T.*) *pyogenes* in pure culture
6	5852/02	Cow	Inguinal region	n.d	n.d.
7	1873/07	Cow	Kidney	Septicemia	*E. coli*
8	2318/07	Cow	Placenta	Septicemia	*Lactobacillus* spp., *Aeromonas* spp., *E. coli*
9	3197/07	Cow	Lung	Septicemia	*Corynebacterium* spp, *Klebsiella* spp., *E. coli*
10	3201/07	Cow	Uterus	Endometritis	*Klebsiella* spp., *E. coli*
11	4161/07	Cow	Uterus	n.d	α-hemolytic streptococci, *Fusobacterium necrophorum, E. coli*
12	6157/08	Calf	Feces	Septicemia	*Corynebacterium* spp., *F. necrophorum, E. coli*
13	280/09	Cow	Urine	Pyelonephritis	α-hemolytic streptococci, *Corynebacterium* spp., *Acinetobacter* spp., *E. coli*
14	371/09	Cow	Lung	Bronchopneumonia and pleuropneumonia	γ-hemolytic streptococci, *Proteus* spp., *Pasteurella multocida, E.coli*
15	799/09	Cow	Uterus	Fertility problem in the farm domain	*Micrococcus* spp.
16	914/09	Cow	Lung	Bronchopneumonia	γ-hemolytic streptococci, *E. coli*
17	1440/09	Cow	Nostril abscess	Bronchopneumonia and respiratory disorder in the farm domain	*P. multocida*
18	1489/09	Cow	Dermoid cyst	Abscessation	*A.* (*T.*) *pyogenes* in pure culture
19	P2346/09	Cow	Cervix	Vaginal discharge and fertility problem in the farm domain	*Proteus* spp.
20	1848/07	Pig	Hind leg	Abscessation	*P. multocida, Pseudomonas aeruginosa, Klebsiella pneumoniae, Prevotella* spp.
21	1928/07	Pig	Liver	Septicemia	α, ß and γ-hemolytic streptococci, *Staphylococcus hyicus, Streptococcus porcinus, P. multocida, E. coli*
22	2089/07	Pig	Lung abscess	Pleuropneumonia	*P. multocida*, *K. pneumoniae, Pseudomonas* spp.
23*	2957/07	Pig	Lung	Bronchopneumonia and pleuropneumonia	*P. multocida, Pseudomonas* spp.
24*	2958/07	Pig	Lung	Bronchopneumonia	α-hemolytic streptococci, *Proteus* spp., *P. multocida*
25	3624/07	Pig	Ventricles	Opisthotonus	γ-hemolytic streptococci, *Klebsiella oxytoca, Aeromonas* spp., *E. coli*

No.	Strain/year of isolation	Animal	Specimen	Additional information	Additionally isolated microorganisms
26*	5939/08	Pig	Cervix	Abortion with vaginal discharge	*Staphylococcus epidermidis, E. coli*
27*	5940/08	Pig	Cervix	Abortion with vaginal discharge	α-hemolytic streptococci, *Staphylococcus chromogenes, Corynebacterium* spp.
28	6284/08	Pig	Lung	Pleuropneumonia	*Staphylococcus* spp.
29*	1070/09	Pig	Ear abscess	Perichondritis of ear cartilage	*S. hyicus, E. coli*
30*	1071/09	Pig	Liver	Septicemia	*S. epidermidis, E. coli*
31	1340/09	Pig	Lung	Septicemia	γ-hemolytic streptococci, *S. epidermidis, E. coli*
32	1342/09	Pig	Lung	n.d.	*P. multocida*
33	P2555/03	Sheep	n.d	n.d	n.d.
34	1870/07	Sheep	Kidney	Septicemia	*E. coli*
35	1971/07	Sheep	Skin	n.d	*S. aureus, Corynebacterium* spp., *Klebsiella* spp., *E. coli*
36	5144/08	Sheep	Skin	n.d	γ-hemolytic streptococci, *S. aureus, S. epidermidis*
37	2074/07	Goat	Lung	Septicemia	α-hemolytic streptococci, *E. coli*
38	36/03	Mountain Reedbuck	Tonsils	n.d.	n.d.
39	3303/07	Elk	Rumen	Septicemia	*E. coli*
40	3790/07	Yak	Lung	Thorax edema	*Mycoplasma pneumonia, P. multocida, E. coli*
41	4998/09	Deer	Lung	Pneumonia	γ-hemolytic streptococci, *E. coli*
42	5025/09	Deer	Brain	Septicemia	*A.* (*T.*) *pyogenes* in pure culture
43	3186/02	Horse	Wound	n.d.	n.d.
44	P2847/01	Dog	Ear	n.d.	n.d.
45	142/09	Dog	Vagina	Vaginitis and vaginal discharge	γ-hemolytic streptococci, *Staphylococcus* spp., Coliform bacteria, *E.coli*
46	6122/08	Cat	Anal gland	Perineal hernia	*Bacteroides* spp., *Prevotella* spp., *E. coli*
47	1514/09	Cat	Ear	Otitis and abscessation	*Corynebacterium* spp., *P. multocida*
48	852/09	Rabbit	Lung	Septicemia	*Acinetobacter* spp., Coliform bacteria, *E. coli*
49	1628/07	Rat	Liver	Septicemia	*Staphylococcus* spp., *K. pneumoniae, E. coli*
50	734/03	Bearded dragon	Lung	Septicemia	γ-hemolytic streptococci, *Pseudomonas* spp., Coliform bacteria
51	4984/03	Gecko	Intestine	Septicemia	*Acinetobacter* spp.

*The *A.* (*T.*) *pyogenes* strains numbered 23 and 24; 26 and 27; and 29 and 30, respectively, represent cultures isolated from different animals in the same farm domain; n.d. = no data available

Tab. 2: Origin of 23 *A.* (*T.*) *abortisuis* isolated from pigs and cows

No.	Strain/year of isolation	Animal	Specimen	Additional information	Additionally isolated microorganisms
1	P7403/99	Pig	Vagina	Vaginitis and vaginal discharge	γ-hemolytic streptococci, *E. coli*
2	P8609/00	Pig	Vagina	n.d.	n.d
3	P8612/00	Pig	Vagina	n.d.	n.d
4	1672/02	Pig	Vagina	Vaginitis and vaginal discharge	γ-hemolytic streptococci, *Proteus* spp., *S. aureus, E. coli*
5	P6572/02	Pig	Vagina	Vaginitis and vaginal discharge	*S. epidermidis, E. coli*
6	P7069/02	Pig	Vagina	Vaginal discharge and fertility problems	*Actinobacter* spp., *Corynebacterium* spp., *Flavobacterium* spp., *E. coli*
7	7115/03	Pig	Vagina	Vaginitis and vaginal discharge	*Acinetobacter spp., E. coli*
8	1142/04	Pig	Kidney	Post mortem: male, respiratory disorder	γ-hemolytic streptococci, *Acinetobacter* spp., *Branhamella* spp., *S. hyicus, E. coli*
9	4257/06	Pig	Urine	Vaginal discharge and urinary gravel	γ-hemolytic streptococci, *Acinetobacter* spp., *Bacillus* spp., *Corynebacterium* spp., *S. chromogenes, E. coli*
10	5041/06	Pig	Cervix	Vaginitis	*Enterococcus* spp., *E.coli*
11	815/07	Pig	Vagina	Blood discharge from vagina	γ-hemolytic streptococci, *Bacillus* spp., *E. coli.*
12	3064/07	Pig	Vagina	Vaginal discharge 3 days post partum	α-hemolytic streptococci, *Corynebacterium* spp., *S. epidermidis, E. coli*
13	5941/08	Pig	Cervix	Abortion, mummified fetuses and fertility problems in the farm domain	α, ß and γ-hemolytic streptococci, *Corynebacterium* spp.
14*	3369/09	Pig	CNS	Septicemia	α-hemolytic streptococci, *Micrococcus* spp., *E. coli*
15*	3370/09	Pig	Placenta	Post mortem: endometritis	*E. coli*
16	4022/09	Pig	Urine	Abortion, vaginal discharge and fertility problems in the farm domain	α-hemolytic streptococci, *Actinomyces* spp., *Staphylococcus* spp., *E. coli*
17	4044/09	Pig	Urine	Abortion, vaginal discharge, fertility problems and respiratory disorders in the farm domain	*Micrococcus* sp., *Enterococcus* spp., *Actinomyces* spp., *Corynebacterium* spp., *Staphylococcus* spp.
18*	341/10	Pig	Cervix	Dystocia, purulent vaginal discharge, fertility problems in the farm domain	α and ß-hemolytic streptococci, *Streptococcus dysgalactiae* subspecies e*quisimilis, S. hyicus, E. coli*
19*	342/10	Pig	Cervix	Dystocia, purulent vaginal discharge, fertility problems in the farm domain	α and γ-hemolytic streptococci, *Corynebacterium* spp., *S. hyicus, E. coli*
20*	343/10	Pig	Cervix	Dystocia, purulent vaginal discharge, fertility problems in the farm domain	α and γ-hemolytic streptococci, *Corynebacterium* spp., *S. hyicus, E. coli*
21	837/10	Pig	Vagina	Vaginal discharge, fertility problems in the farm domain	γ-hemolytic streptococci, *Corynebacterium* spp., *E. coli*
22*	6320/08	Cow	Vagina	Vaginitis and metritis in the farm domain	α and γ-hemolytic streptococci, *Pantoea* spp., *S. chromogenes*
23*	6322/08	Cow	Vagina	Vaginitis and metritis in the farm domain	α and γ-hemolytic streptococci, *Corynebacterium* spp.

*The *A.* (*T.*) *abortisuis* strains numbered 14 and 15; 18, 19 and 20; and 22, 23, respectively, represent cultures isolated from different animals in the same farm domain; n.d. = no data available.

Tab. 3: Origin of three *A. haemolyticum* isolated from three horses

No.	Strain/year of isolation	Animal	Specimen	Additional information	Additionally isolated microorganisms
1	2289/09	Horse	Castrational wound	Postcastrational complications	*Corynebacterium* spp., *Enterococcus* spp., *Branhamella* spp., *Prevotella* spp., *Bacteroides* spp., *Fusobacterium* spp.
2	P5648/10	Horse	Wound infection	n.d.	ß-hemolytic streptococci, *S. aureus, Erwinia* spp., Coliform bacteria
3	P579/11	Horse	Mallenders	n.d.	α-hemolytic streptococci, *S. aureus Actinomyces* spp., *Corynebacterium* spp.

n.d. = no data available

Tab. 4: Origin of three strains of the novel *Actinomyces* species

No.	Strain/year of isolation	Animal	Specimen	Additional information	Additionally isolated microorganisms
1	2298/06	Dog	Oral cavity	Wound infection caused by foreign body	α-hemolytic streptococci, aerobic spore forming bacilli, *Corynebacterium* spp., *Prevotella melaninogenica*
2	4321/06	Dog	Oral cavity	Gingivitis	ß and γ-hemolytic streptococci, *Staphylococcus intermedius* group, *S. epidermidis*, *Corynebacterium* spp., *Pasteurella canis*
3	1656/06	Dog	Oral cavity	Periodontitis and gingivitis	α-hemolytic streptococci, *Corynebacterium* spp., *P. canis*, *Neisseria* spp.

The reference strains of genera *Arcanobacterium*, *Trueperella* and *Actinomyces* were obtained from the strain collection of Institut für Pharmakologie und Toxikologie, Justus-Liebig-Universität Gießen and Institut für Hygiene und Infektionskrankheiten der Tiere, Justus-Liebig-Universität Gießen, Frankfurterstraße 85-89, 35392 Gießen. The *A.* (*T.*) *pyogenes, A.* (*T.*) *abortisuis* and *A. haemolyticum* strains and the three strains of the novel *Actinomyces* species were kindly obtained from Dr. R. Weiß and Dr. E. Prenger-Berninghoff (Institut für Hygiene und Infektionskrankheiten der Tiere) and from Dr. M. Zschöck (*A. pyogenes* of deer origin (n = 2)) from Landesbetrieb Hessisches Landeslabor (LHL), Schubertstraße 60, 35392 Gießen, Germany.

3.2 Cultivation on sheep and rabbit blood agar

The reference strains of genera *Arcanobacterium, Trueperella* and *Actinomyces* and the collected field isolates were cultivated on sheep blood agar and in parallel for determination of hemolytic properties on rabbit blood agar. The composition of the blood agar (base) (Merck, Darmstadt, Germany)* was as follows:

Nutrient substrate (heart extract and peptones)	20.0 g
Sodium chloride	5.0 g
Agar	15.0 g
Distilled water	up to 1000 ml

pH: 6.8 ± 0.2 at 25 °C.

The medium was prepared according to the manufacturers specifications. The components were added to distilled water, mixed thoroughly and heated under frequent agitation, finally boiled for 1 min to completely dissolve the medium and autoclaved for 15 min at 121 °C. The medium was cooled up to 50 °C and 5 % aseptically collected, defibrinated sheep or rabbit blood using ethylenediaminetetraacetic acid (EDTA, Sarstedt, Nümbrecht, Germany) was added and gently mixed. Sheep and rabbit blood were collected aseptically from donor animals of the Institut für Hygiene und Infektionskrankheiten der Tiere and from Zentrales Tierlabor, Justus-Liebig-Universität Gießen, Frankfurterstraße 105, 35392 Gießen, respectively. The medium was poured into agar plates which were kept at 4 °C for further use. The bacterial isolates were generally cultivated on sheep blood agar plates. The incubation of the inoculated culture media was carried out for 48 to 72 h at 37 °C under microaerobic conditions in a candle jar (Lenz Laborglas, Wertheim, Germany) and for some isolates under aerobic and under anaerobic conditions using the anaerobic system (AnaeroGen^{TM}, Oxoid, Wesel, Germany) in an anaerobic jar (Anaerocult, Merck) held in an incubator (Memmert GmbH + Co. KG, Schwabach, Germany). Subculturing of the bacteria was performed every four weeks.

* = If not otherwise stated, all chemicals were obtained from Merck, Darmstadt, Germany.

3.3 Preservation of the bacteria

For this purpose, the bacteria were cultivated on sheep blood agar (3.2). Using sterile cotton swabs (Böttger, Bodenmais, Germany) the freshly grown bacteria were harvested and transferred into a sterile micro tube with cap (Sarstedt) containing 1.5 ml of bovine serum (PAA Laboratories GmbH, Cölbe, Germany) with 6% glucose. The tubes were stored at -80 °C.

3.4 Identification and further characterization of the bacteria by conventional methods

3.4.1 Gram-staining and determination of bacterial cell morphology

Gram-staining was performed as described by Bisping and Amtsberg (1988). The captured images were viewed using a light microscope (Leica DM6000B, Wetzlar, Germany) at × 1000 equipped with the Leica Application Suite (LAS 2.8.1, Leica), kindly supported by Dr. K. Beuerlein, Rudolf-Buchheim-Institut für Pharmakologie, Frankfurterstraße 107, 35392 Gießen, Germany. The cell morphology was also investigated by phase contrast microscopy under an Axiphoto 2 Zeiss light microscope (Carl Zeiss, Jena, Germany) at × 1000, kindly supported by Mrs. G. Will and Prof. P. Kämpfer, Institut für Angewandte Mikrobiologie, Justus-Liebig-Universität Gießen, Heinrich-Buff-Ring 26-32, 35392 Gießen, Germany.

3.4.2 Detection of bacterial motility

Bacterial motility was investigated by inoculation of the bacteria into Hitchens semi-solid media (Hitchens, 1921).
The Hitchens semi-solid media was composed as follows:

Meat extract	5.0 g
Peptone	20.0 g
Potassium nitrate	2.0 g
Agar	1.3 g
Aqua dest.	up to 1000 ml

pH: 7.5 ± 0.2 at 25 °C.

The inoculation of the bacteria was performed by the stab method with a straight needle inserted to the bottom of the tube. The incubation of the inoculated tubes was carried out for 48 to 72 h at 37 °C under microaerobic conditions in a candle jar. Motility was indicated by a generalized turbidity of the growth medium or growth extending from the line of inoculation. Non-motile organisms grew only along the line of inoculation. *Proteus vulgaris* was used as motile control, while *Klebsiella oxytoca* served as non-motile control. Both control strains were kindly provided by Institut für Hygiene und Infektionskrankheiten der Tiere.

3.4.3 Hemolytic properties and CAMP-like hemolytic reactions

To investigate the colony morphology and hemolysis the bacterial cultures were cultivated on sheep or rabbit blood agar (3.2). The assessment was made after microaerobic incubation for 48-72 h at 37 °C. For some isolates the hemolytic properties were investigated by cultivation on sheep and rabbit blood agar and incubation under microaerobic conditions and/or in parallel under aerobic and under anaerobic conditions (3.2).

The reference strains and field isolates of the present study were additionally investigated for synergistic or CAMP-like and antagonistic or reverse hemolytic reactions. The well known CAMP-reaction, named after **C**hristie, **A**tkins and **M**unch **P**etersen (1944), is a characteristic synergistic hemolytic reaction of CAMP-factor of streptococci of serological group B (*Streptococcus agalactiae*) cultivated in the presence of incomplete staphylococcal β-hemolysin. The antagonistic or reverse CAMP reaction is a well known property of *A. haemolyticum* and *A. phocae* (Fraser, 1964; Johnson et al., 2003) showing an arc-shaped zone of inhibition of the exotoxins of *A. haemolyticum* and *A. phocae* in the area of staphylococcal β-hemolysin. The determination of CAMP-like and antagonistic hemolytic reactions was performed on sheep blood agar (3.2). For this ß-hemolytic *S. aureus, S. agalactiae*, *Rhodococcus equi*, *Psychrobacter phenylpyruvicus* and nine reference strains of genus *Arcanobacterium* and genus *Trueperella* used as indicator strains, respectively, were inoculated vertically and the strain to be tested horizontally up to 3-5 mm to the inoculation line of the indicator strain. The indicator strains were obtained from the strain collection of Institut für Pharmakologie und Toxikologie, Justus-Liebig-Universität Gießen. The incubation of the inoculated plates was subsequently carried out for 48 h at 37 °C under microaerobic conditions (3.2). A positive reaction could be observed as a half moon forming zone of complete hemolysis in the zone of incomplete staphylococcal-β-hemolysis or as a half moon forming zone of complete

hemolysis in close proximity of the other indicator strains. A positive antagonistic or reverse hemolytic reaction caused an arc-shaped inhibition zone of staphylococcal ß-hemolysin.

3.4.4 Biochemical characterization using the API Coryne test system

The API Coryne test system (Biomerieux, Nürtingen, Germany) was performed according to the manufacturers instructions. The API Coryne test system consists of 20 microtubes containing dehydrated substrates. These substrates enabled the detection of 11 enzymes and eight carbohydrate fermentation tests. The enzymatic tests are for nitrate reduction, pyrazinamidase, pyrrolidonyl arylamidase, alkaline phosphatase, β-glucuronidase, β-galactosidase, α-glucosidase, N-acetyl-β-glucosaminidase, esculin hydrolysis, Urease, gelatin hydrolysis and catalase, the eight carbohydrate fermentation tests for D-glucose, D-ribose, D-xylose, D-mannitol, D-maltose, D-lactose, D-saccharose and glycogen.

The incubation box (tray and lid) was prepared by distributing approximately 5 ml of distilled water into honey-combed wells of the tray to create a humid atmosphere. Following the manufacturer instructions the inoculum was prepared with freshly subcultured bacteria adjusted in a suspension medium (Biomerieux) with a turbidity greater than McFarland 6-standard (Biomerieux). In the first 11 microtubes of the API strip (NIT to GEL), 150 µl of the suspension were inoculated. For the fermentation tests 0.5 ml of the above mentioned suspension was added to 2 ml of the API GP medium (Biomerieux) containing phenol red as pH indicator, mixed and inoculated in a volume of 150 µl into the test tubes (0 to GLYC). The microtubes used for urea hydrolysis and carbohydrate fermentations were overlaid with mineral oil. The incubation was performed for 48 h at 37 °C. Before reading the results, one drop of Nit 1 and Nit 2 reagent (Biomerieux), respectively, were added to the nitrate microtube, one drop of PYZ reagent (Biomerieux) to pyrazinamidase and one drop of each Zym A and Zym B reagents (Biomerieux), respectively, to the pyrrolidonyl-arylamidase, alkaline phosphatase, β-glucuronidase, β-galactosidase, α-glucosidase and N-acetyl-β-glucosaminidase microtubes, respectively. Reading of the kit was performed through evaluation of the colours according to the reading table (Biomerieux) resulting in a seven-digit numerical code for identification of the bacteria. This code was compared with the API Coryne Analytical Profile Index (apiweb ^{TM}, version 1.2.1, 2003, Biomerieux). Catalase activity was determined by adding one drop of hydrogen peroxide (3 %) to the ESC or GEL test. The appearance of bubbles indicated a positive reaction.

3.4.5 Detection of the enzymes pyrrolidonyl arylamidase, alkaline phosphatase and α-mannosidase

These tests were performed according to the instructions provided by the manufacturer (Rosco Diagnostica A/S, Taastrup, Denmark). For this, bacterial suspensions of McFarland 4-standard were prepared from the isolates to be tested in 0.25 ml sterilized 0.9 % sodium chloride (Roth, Karlsruhe, Germany) in 1.5 ml Eppendorf tubes (Sarstedt). Subsequently a diagnostic test tablet for detection of pyrrolidonyl arylamidase, alkaline phosphatase and α-mannosidase (Rosco Diagnostica A/S), respectively, was added. The tube was tightly plugged and incubated for 4 h or up to 18-24 h at 37 °C. For the enzyme pyrrolidonyl arylamidase three drops of aminopeptidase reagent (Rosco Diagnostica A/S) was added and the colour reaction could be recorded after 5 min incubation at room temperature. The formation of a red colour indicated a positive reaction, a yellow or orange colour a negative reaction. *A. pluranimalium* DSM 13483^T was used as positive control and *A.* (*T.*) *abortisuis* DSM 19515^T served as negative control.

For alkaline phosphatase and α-mannosidase the formation of a yellow colour indicated a positive reaction, a colourless or very slight yellow was recorded as negative result. For the alkaline phosphatase test *A. phocae* DSM 10002^T was used as positive control and *A.* (*T.*) *pyogenes* DSM 20594 served as negative control, while for the α-mannosidase test *A. haemolyticum* DSM 20595^T was used as positive control and *A.* (*T.*) *bernardiae* DSM 9152^T as negative control.

3.4.6 Detection of the enzymes β-glucuronidase, β-galactosidase, α-glucosidase and N-acetyl-β-glucosaminidase with 4-methylumbelliferyl-conjugated substrates

The detection of these four bacterial enzymes was based on the generation of fluorescence when free 4-methylumbelliferone was released by enzymatic hydrolysis of the non-fluorescent 4-methylumbelliferyl-conjugated substrates. The substrates 4-methylumbelliferyl-β-D-glucuronide (for β-D-glucuronidase), 4-methylumbelliferyl-β-D-galactopyranoside (for β-D-galactosidase), 4-methylumbelliferyl-α-D-glucoside (for α-D-glucosidase) and 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide (for N-acetyl-β-D-glucosaminidase) (Sigma,

Steinheim, Germany) were used according to the information given by Maddocks and Greenan (1975), Slifkin and Gil (1983) and Bravo-Torres et al., (2003).

4-Methylumbelliferyl-β-D-glucuronide and 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide (15 µmol/l, respectively), and 4-methylumbelliferyl-β-D galactopyranoside and 4-methylumbelliferyl-α-D-glucoside (5 µmol/l, respectively) were dissolved according to Maddocks and Greenan (1975) and Slifkin and Gil (1983) in 0.2 ml of dimethyl sulfoxide (Roth). Then the volume of each solution was filled up to 10 ml with 0.2 mol/l sodium acetate buffer pH 5.2 (Maddocks and Greenan, 1975; Slifkin and Gil, 1983).

To test the presence of the bacterial enzymes, a loop of the freshly cultivated bacterial colonies was rubbed vigorously onto a filter paper (Macherey-Nagel, Düren, Germany). Then 20 µl of the 4-methylumbelliferyl-conjugated substrates were added to the bacterial smear and incubated at 37 °C for 1 h. After incubation, 20 µl of 0.1 mol/l NaOH was added to the inoculated substrate to increase the fluorescence intensity of methylumbelliferone. The fluorescence was investigated in a dark room under ultraviolet light (360 nm). For β-glucuronidase and β-galactosidase *A.* (*T.*) *pyogenes* DSM 20630^T was used as positive control and *A.* (*T.*) *bernardiae* DSM 9152^T served as negative control. For α-glucosidase and N-acetyl-β-glucosaminidase *A.* (*T.*) *pyogenes* DSM 20594 was used as positive control and *A.* (*T.*) *bonasi* DSM 17163^T as negative control.

3.4.7 Detection of the enzyme catalase

The detection of the enzyme catalase was additionally carried out by suspending some bacterial colonies in one to two drops of a 3 % H_2O_2 on a microscopic slide. A positive reaction through decomposition of hydrogen peroxide into water and gaseous oxygen was seen as bubble formation. A negative reaction appeared in a uniform turbidity of the solution without bubble formation (Brückler et al., 1994). *A. phocae* DSM 10002^T was used as positive control, while *A.* (*T.*) *bernardiae* DSM 9152^T served as negative control.

3.4.8 Growth on Loeffler medium

To investigate proteolytic enzymatic activities the bacterial isolates were cultivated on Loeffler medium (Hartwigk and Marcus, 1962). The basic ingredients of the medium were as follows:

Standard I nutrient broth contains:

Peptone	15.0 g
Yeast extract	3.0 g
Sodium chloride	6.0 g
D-Glucose	1.0 g
Aqua dest.	up to 1000 ml

pH: 7.2 ± 0.2 at 25 °C.

Loeffler medium was prepared in small glass Petri dishes by adding 7 parts of bovine serum (PAA Laboratories GmbH) to 3 parts standard I nutrient broth (containing additional 6 g glucose per liter). The Petri dishes were kept until solidification of the medium for about 50 min at 90 °C in a humid chamber and then stored at 4 °C before use. The incubation of the inoculated Loeffler medium was carried out for 48 h at 37 °C under microaerobic conditions. Formation of a groove in the solidified serum beneath the inoculation line was considered as positive reaction due to proteolytic activities (Hartwigk and Marcus, 1962). *A.* (*T.*) *pyogenes* DSM 20630^T was used as positive control, while *A.* (*T.*) *bernardiae* DSM 9152^T served as negative control.

3.4.9 Casein hydrolysis

Testing for casein hydrolysis was performed by cultivation of the bacteria on casein agar.
The casein agar was composed as follows:

Peptone	25.0 g
Sodium chloride	5.0 g
Casein (Serva, Heidelberg, Germany)	10.0 g
Agar	12.0 g
Aqua dest.	up to 1000 ml

pH: 7.4 ± 0.2 at 25 °C.

The incubation of the inoculated casein hydrolysis test agar was carried out for 48 h at 37 °C under microaerobic conditions (3.2). After incubation the medium was flooded with 10 % trichloroacetic acid (Sigma). The trichloroacetic acid precipitated the casein to form a turbid area. Any clear area around the growth of the culture indicated the breakdown of casein by the

organism due to the production of caseinase. *A.* (*T.*) *pyogenes* DSM 20630^T was used as positive control, while *A. phocae* DSM 10002^T served as negative control.

3.4.10 Detection of the enzyme DNase

In 1957, Jeffries et al. described a rapid agar method for demonstrating DNase activity of microorganisms. This procedure utilized a semi-synthetic medium with nucleic acid incorporated in the medium. The detection of enzyme DNase was carried out with DNase agar which was composed as follows:

Tryptose	20.0 g
Sodium chloride	5.0 g
DNA	2.0 g
Agar	15.0 g
Aqua dest.	up to 1000 ml

pH: 7.3 ± 0.2 at 25 °C.

The incubation of the inoculated DNase test agar plates was carried out for 48 h at 37 °C under microaerobic conditions (3.2). DNase is an extracellular enzyme that breaks down the DNA into subunits composed of nucleotides. The degradation of the DNA could be detected by flooding the surface of the medium with 1 N HCl and observing clear zones in the medium surrounding growth. The width of this clear zone is related to the amount of DNase produced. In the absence of DNase activity, the acid reacts with the intact nucleic acid, resulting in the formation of a cloudy precipitate. *A.* (*T.*) *pyogenes* DSM 20630^T was used as positive control, while *A.* (*T.*) *bonasi* DSM 17163^T served as negative control.

3.4.11 Detection of the enzyme hyaluronidase

The detection of the enzyme hyaluronidase was carried out on Brain-Heart Infusion agar (BHI, Oxoid). The composition of the BHI agar was a follows:

Calf brain infusion	12.5 g
Beef heart infusion	5.0 g
Proteose peptone	10.0 g
Glucose	2.0 g
Sodium chloride	5.0 g
Disodium hydrogen phosphate	2.5 g
Agar	10.0 g
Aqua dest.	up to 1000 ml

pH: 7.4 ± 0.2 at 25 °C.

The plate test for the detection of the enzyme hyaluronidase was performed according to Winkle (1979). For this the isolates to be investigated were cultivated in close proximity to a mucoid growing *Streptococcus equi* subsp. *equi* strain as indicator. A growth of the indicator strain in non mucoid colonies in close proximity of the isolate to be tested indicated a positive reaction. *S. aureus* DSM 346 was used as positive control, while *Staphylococcus delphini* DSM 20771^T served as negative control. The *S. equi* subsp. *equi* indicator strain and both staphylococcal strains were obtained from the strain collection of the Institut für Pharmakologie und Toxikologie, Justus-Liebig-Universität Gießen.

3.4.12 Detection of the enzyme amylase

Testing for starch hydrolysis due to the production of amylase was performed by cultivation of the bacteria on Mueller-Hinton-agar containing starch.
The Mueller-Hinton-agar containing soluble starch was composed as follows:

Beef infusion	5.0 g
Casein hydrolysate	17.5 g
Soluble starch	1.0 g
Agar	12.5 g
Aqua dest.	up to 1000 ml

pH: 7.2 ± 0.2 at 25 °C.

The incubation of the inoculated starch agar plates was carried out for 48 h at 37 °C under microaerobic conditions. After incubation the medium was flooded with Gram's iodine. The iodine reacts with starch to form a dark blue coloured complex. Any clear area around the growth of the culture after the addition of iodine indicated the breakdown of starch by the

organism due to its production of amylase. *A.* (*T.*) *pyogenes* DSM 20630^T was used as positive control, while *A.* (*T.*) *bonasi* DSM 17163^T served as negative control.

3.4.13 Detection of the enzyme oxidase

The oxidase test is based on bacterial production of the intracellular enzyme oxidase. This test was first described by Kovacs (1956) as a useful test for differentiation of *Pseudomonas* spp. and *Enterobacteriaceae*. The active substrate in the oxidase reagent is N,N,N',N'-Tetramethyl-1,4-phenylenediammonium dichloride which, in the oxidized form, builds a colored compound. The detection of the enzyme oxidase was carried out by the transfer of some bacterial colonies from the agar plate onto a filter paper moistened with 1 to 2 drops of oxidase reagent. The observation of a dark purple color within 5-10 s indicated a positive reaction (Kovacs, 1956). *Bordetella bronchiseptica* was used as positive control, while *E. coli* served as negative control.

3.4.14 Detection of indole production

The indole test was used to determine the ability of the bacteria to split indole from the amino acid tryptophan by the intracellular enzyme tryptophanase (Isenberg and Sundheim, 1958). For determination of indole production the bacteria were grown in peptone broth.
The peptone broth was composed as follows:

Peptones	10.0 g
Sodium chloride	3.0 g
Anhydrous trisodium phosphate	2.0 g
Aqua dest.	up to 1000 ml

pH: 7.4 ± 0.2 at 25 °C.

The incubation of the inoculated peptone broth was carried out for 48 h at 37 °C under microaerobic conditions (3.2). Following incubation 5 drops of Kovac's indole reagent (contains n-Butanol) were added to the culture broth. A positive result could be observed by the presence of a red or red-violet color in the alcohol layer (n-Butanol) surface of the broth. A negative result appeared yellow. *E. coli* was used as positive control, while *Klebsiella pneumoniae* served as negative control. The control strains used in 3.4.13 and 3.4.14 were kindly provided by Institut für Hygiene und Infektionskrankheiten der Tiere.

3.4.15 Cross reaction with streptococcal serogroup G specific antiserum

A cross reaction with streptococcal serogroup G specific antiserum could be used for tentative serological identification of *A.* (*T.*) *pyogenes* (Lämmler and Blobel, 1988). The determination of the cross reaction was performed with Streptococcal Grouping Kit (Oxoid) as described by Lämmler and Blobel (1988). For this test 2 to 5 colonies from freshly cultivated bacteria to be investigated were emulsified in 100 µl of the extraction enzyme provided by the kit (Oxoid) in an Eppendorf tube and subsequent incubation for 5 min at 37 ºC. Then the tube was shaken vigorously and the incubation was continued for another 5 min. The agglutination procedure was performed through mixing 20 µl of the enzymatic extract with 20 µl of the latex reagent coated with group G streptococcal antisera warmed to room temperature on a microscopic slide by gently rocking. The test was considered positive when agglutination occurred after 30 to 60 s. *A.* (*T.*) *pyogenes* DSM 20594 was used as positive control while *A. phocae* DSM 10003 served as negative control.

3.5 Identification of the bacteria by Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS)

3.5.1 Bacterial sample preparation

For protein extraction the Bruker ethanol formic acid preparation procedure (Bruker Daltonik, Bremen, Germany) was used. Briefly, few colonies of freshly cultivated bacteria were transferred to a 2.0 ml Eppendorf tube with a disposable loop and mixed thoroughly with 300 µl of HPLC grade water (Roth) to suspend the bacterial cells. This was followed by the addition of 900 µl of HPLC grade absolute ethanol (Roth). The samples were stored at -18 ^{o}C until processing. The samples were centrifuged for 2 min (Biofuge A, Heraeus, Rabenau, Germany) and the supernatant removed. Another centrifugation step was done for removing residual fluid by pipetting. The pellet was resuspended in 30 µl 70% formic acid. Subsequently, the same volume of pure acetonitrile was added and mixed. The suspension was centrifuged at 13.000 rpm for 2 min and 1 µl of the supernatant was transferred to a polished steel MALDI target plate (Bruker Daltonik) and allowed to dry at room temperature. Each sample was overlaid with 1 µl of matrix (10 mg/ml α-cyano-4-hydroxy-cinnamic acid in

50 % acetonitrile/2.5 % trifluoroacetic acid) and dried at room temperature. To increase the data reliability each bacterial sample was applied 2 times onto the MALDI target plate.

3.5.2 MALDI-TOF MS measurements and data analysis

MALDI-TOF MS analyses were performed with a microflex LT mass spectrometer (Bruker Daltonik). The data analyses were performed with the kind support of Dr. Markus Timke and Dr. Markus Kostrzewa (Bruker Daltonik). Instrument parameter settings were IS1 20 kV, IS2 18.45 kV, lens 8.5 kV, PIE 250 ns, no gating, detector gain 2,750 V. The peptide spectra were collected in the linear positive mode within the mass range 2 to 20 kDa. In advance to the sample measurements the mass spectrometer was calibrated using the Bacterial Test Standard (BTS, Bruker Daltonik) where the presence of eight specific proteins insured that the instrument calibration was set properly. The BTS contains a typical extract of *E. coli DH5alpha* spiked with the two additional proteins RNase A and myoglobin. Thus, the BTS covers an overall mass range from 4 to 17 kDa.

The database reference spectra (main spectra, MSP) for each sample were processed using the MALDI Biotyper version 2.0 software package (Bruker Daltonik). MALDI-TOF MS spectra were acquired using the automated functionality of flexControl 3.0 software (Bruker Daltonik). Raw spectra were quality-checked visually using flexAnalysis 3.0 software (Bruker Daltonik). At least six raw spectra were used to generate a main spectrum. A MSP contains the average mass and the average intensity of the selected peaks as well as the frequency of the peaks in multiple measurements.

The MSPs obtained in the present study were matched to the database included in the MALDI Biotyper 2.0 software package (version 3.1.1.0, 3740 entries) and to the new acquired MSPs from 12 reference strains representing ten species of genera *Arcanobacterium*, *Trueperella* and *Actinomyces* namely, *A.* (*T.*) *pyogenes* DSM 20630^T, *A.* (*T.*) *pyogenes* DSM 20594, *A.* (*T.*) *abortisuis* DSM 19515^T, *A.* (*T.*) *bernardiae* DSM 9152^T, *A.* (*T.*) *bialowiezense* DSM 17162^T, *A.* (*T.*) *bonasi* DSM 17163^T, *A. haemolyticum* DSM 20595^T, *A. hippocoleae* DSM 15539^T, *A. phocae* DSM 10002^T, *A. phocae* DSM 10003, *A. pluranimalium* DSM 13483^T and *Actinomyces bovis* DSM 43014^T. The software calculates a similarity score (log (score) value) by calculation of a value considering the proportion of matching peaks between the unknown spectrum and the main spectrum of the database or the known investigated reference strains of the present study, and the frequency of peaks in multiple measurements as

well as the consistency of the peak intensities between these spectra. The logarithmized score values range from 0 (no homology) to 3 (absolute identity). Log (score) values ≥ 2 are rated as identification of bacteria at species level. Log (score) values ≥ 1.7 and < 2.0 are considered as identification of microorganisms at least on genus level. Log (score) values < 1.7 indicated that a spectrum is not suitable for identification by the MALDI Biotyper **(Tab. 5)**. These thresholds have been shown to be widely applicable in previous studies (Mellmann et al., 2008; Barbuddhe et al., 2008; Lartigue et al., 2009; Ilina et al., 2009; Marklein et al., 2009; Nagy et al. 2009).

Tab. 5: MALDI Biotyper identification output (Bruker Daltonik)

Score values range*	Description	Symbol	Colour**
2.300 to 3.000	High probable species identification	(+++)	Green
2.000 to 2.299	Secure genus identification, probable species identification	(++)	Green
1.700 to 1.999	Probable genus identification	(+)	Yellow
0.000 to 1.699	No reliable identification	(-)	Red

* = Score value threshold for bacterial identification, ** = colours indicate different levels of bacterial identification.

3.6 Identification and molecular characterization of the bacteria by polymerase chain reaction (PCR)

3.6.1 DNA extraction and preparation using DNeasy Tissue-Kit

The extraction and preparation of DNA as a template for PCR reaction was performed with the DNeasy Tissue-Kit (Qiagen, Hilden, Germany). For this purpose a single colony of each isolate was cultivated and incubated for 48 h on sheep blood agar under microaerobic conditions (3.2). Five to 10 colonies of the freshly subcultured bacteria were subsequently suspended in 180 µl TE buffer (10 mmol/l Tris-HCl, 1 mmol/liter EDTA, pH 8.0), containing 5 µl mutanolysin (10U/µl, Sigma). After incubated for one hour at 37 °C, 25 µl proteinase K (Qiagen) and 200 µl of lysis buffer AL (Qiagen) were added and further incubated for 2 h at 56 °C. The DNA was subsequently isolated by using DNeasy silica membrane filters according to the manufacturers instructions (Qiagen). In short, approximately 200 µl ethanol (Roth) was added to the previously incubated mixture and mixed thoroughly. The reaction mixture was then transferred to the DNeasy silica membrane filter (Qiagen) and centrifuged

for 1 min at 6000 rpm. The eluant was discharged, the bound DNA washed from the silica filter by the addition of 500 µl buffer AW1 (Qiagen) followed by centrifugation for 1 min at 6000 rpm. The eluant was discharged again. This step was repeated using 500 µl buffer AW2 (Qiagen). After the second washing step a short centrifugation at 13000 rpm for 30 sec ensured the complete removal of the washing solution from the silica filter. Then the silica filter was transferred to another clean 2 ml Eppendorf tube. From the elution buffer AE (Qiagen) 200 µl were added to the filter and subsequently incubated for 5 min at room temperature. The DNA-containing eluant could be collected by centrifugation for 2 min at 6000 rpm. The resulting DNA eluate was cooled and used as template in the PCR reaction or stored at -20 °C.

3.6.2 Implementation of the PCR

The PCR reaction was initially carried out by the preparation of a master mix. The master mix was as follows:

Aqua bidest.	19.9 µl
PCR Gold buffer (10 x, Applied Biosystem, Darmstadt, Germany)[a]	3.0 µl
$MgCl_2$ (25 mmol/l, Applied Biosystem)	1.8 µl
dNTP (10 mmol/l, Fermentas, St. Leon-Rot, Germany)	0.6 µl
Primer 1 (10 pmol/l)	1.0 µl
Primer 2 (10 pmol/l)	1.0 µl
Taq-Polymerase (5 U/µl, Applied Biosystem)	0.2 µl

[a](150 mmol/l Tris-HCL, 500 mmol/l KCL, pH 8.0)

Alternatively the following master mix was used:

Aqua bidest.	21.7 µl
PCR buffer (10 x, contains 15 mmol/l $MgCl_2$, Sigma)[b]	3.0 µl
dNTP (10 mmol/l, Fermentas)	0.6 µl
Primer 1 (10 pmol/l)	1.0 µl
Primer 2 (10 pmol/l)	1.0 µl
Taq-Polymerase (5 U/µl, Applied Biosystem)	0.2 µl

[b](100 mmol/l Tris-HCL, 500 mmol/l KCL, pH 8.3)

Finally 27.5 µl of the master mix was dispensed in 0.2 ml sterile PCR reaction tubes and mixed with 2.5 µl of the prepared DNA (3.7.1). Subsequently, the samples were subjected to

specific temperature program cycles in a thermal cycler (Gene Amp PCR System 2400, (Perkin-Elmer, Rodgau, Germany) or MJ Mini™ Gradient Thermal Cycler, (Bio-Rad Laboratories, München, Germany)). The oligonucleotide primers used in the present study were synthesized by Eurofins MWG/Operon (Ebersberg, Germany) and TIB MOLBIOL Syntheselabor GmbH (Berlin, Germany).

3.6.3 Agarose gel electrophoresis

The amplicons were electrophoresed in an 1.5 % agarose gel by using 1 x Tris-acetate-EDTA buffer (TAE) (4.0 mmol/l Tris, 1 mmol/l EDTA, 1.14 mol/l glacial acetic acid, pH 8.0) as running buffer. To prepare the agarose gel 1.5 g agar (Biozym, Hess-Oldendorf, Germany) was dissolved in 100 ml of 1 x TAE buffer. For electrophoresis 8 µl of the PCR product was mixed with 2 µl of 6x loading dye solution (Fermentas) and loaded in the gel with a DNA ladder (GeneRuler™ DNA Ladder, size standard 100-1000 bp or 100-10000 bp-ladders, Fermentas) as molecular size standard. The electrophoresis was carried out at 100 mA for approximately 1 h.

3.6.4 Ethidium bromide staining and documentation

After successful completion of electrophoresis the gel was stained for approximately 30 min with an ethidium bromide solution (5 µg/ml, Sigma). Ethidium bromide staining material has the ability to interact and impregnate with the double stranded DNA. After staining the gel was carefully rinsed in aqua dest and the bands visualized by UV and photographed using Image Master® VDS (Pharmacia Biotech, Freiburg, Germany). The fragments could be seen as light bands in front of a dark background.

3.6.5 PCR amplicon purification

The purification of amplicons from PCR reactions was performed using the QIAquick PCR Purification Kit (Qiagen) according to the manufacturers instructions. Briefly, five volumes of binding buffer (PB buffer, Qiagen) were added to one volume of PCR sample in an Eppendorf tube, mixed well, transferred to a QIAquick spin column (Qiagen) and centrifuged for 1 min at 6000 rpm. The eluant was discharged and the bound DNA washed by addition of 750 µl

washing buffer (PE buffer, Qiagen) and centrifugation for 1 min at 6000 rpm. Again the eluant was discharged and the QIAquick column transferred to a new Eppendorf tube. Then 30 to 50 µl from the elution buffer EB (10 mmol/l Tris HCl, pH 8.5, Qiagen) was added to the center of the QIAquick membrane and subsequently incubated for 1 min at room temperature. The purified PCR amplicon could be collected by centrifugation of the QIAquick membrane for 1 min at 6000 rpm. The resulting eluant could be used for sequencing or it was stored at -20 °C.

3.6.6 DNA preparation from agarose gels and sequencing

The DNA preparation from agarose gels was performed with the QIAEX II Gel Extraction Kit according to the manufacturers instructions (Qiagen). The DNA preparation from the agarose gel was performed after electrophoresis and ethidium bromide staining (Sigma). For this approximately 250 mg material excised from the gel containing the gene amplicon of interest was transferred into an Eppendorf tube. After addition of 750 µl QX1 buffer (Qiagen) and 10 µl QIAEX II (Qiagen) the content was mixed and incubated for 10 min at 50 °C in a water bath until the gel slice had completely dissolved, and centrifuged at 14000 rpm for 1 min. The supernatant was discharged carefully and the sediment containing the DNA fragment was resuspended again in 500 µl QX1 followed by centrifugation at 14,000 rpm for 1 min and discharge of the supernatant. The sediment containing the DNA fragment was washed twice using 500 µl PE buffer containing ethanol (Qiagen) followed by centrifugation and discharging of the supernatant. Residual ethanol was completely removed through drying the DNA pellet at room temperature for 15 min with an open Eppendorf tube. The purified DNA was recovered by the addition of 20 µl of elution buffer EB (Qiagen), mixed and followed by centrifugation at 14,000 rpm for 1 min. The DNA-containing supernatant was then collected by pipetting and could be used as template for PCR reaction or for sequencing purposes.
The purified PCR or DNA products (3.6.5 and 3.6.6) were sequenced by SEQLAB Sequence Laboratories (Göttingen, Germany) or GATC Biotech AG (Konstanz, Germany). Sequence analysis and sequence comparison with the National Center for Biotechnology Information data base (NCBI: http://www.ncbi.nlm.nih.gov/) was performed with the aid of computer programs Finch TV version 1.4.0 and DNASTAR MegAlign version 8.0.2 (Clustal W method, Lasergene, Madison, USA) at the Institut für Pharmakologie und Toxikologie, Justus-Liebig-Universität Gießen.

3.6.7 Amplification and sequencing of the 16S rDNA

The 16S rDNA was amplified with an expected size of 1,403 bp by using the oligonucleotide primers 16SUNI-L (5`-AGA GTT TGA TCA TGG CTC AG-3') and 16SUNI-R (5`-GTG TGA CGG GCG GTG TGT AC-3') described by Kuhnert et al. (1996), which corresponded to bases 8 to 27 and to bases 1391 to 1410 of the 16S rDNA sequence of *E. coli* (NCBI accession number J01859), respectively. The PCR reaction was performed by preparation of the master mix mentioned in 3.6.2. The PCR thermal cycler program was carried out as follows:

1 cycle	94 °C	10	min	Initial denaturation
30 cycle	95 °C	30	sec	Denaturation
	58 °C	60	sec	Primer annealing
	72 °C	60	sec	Extension
1 cycle	72 °C	7	min	Final extension

For full length sequencing of the 16S rDNA two new oligonucleotide primers were designed by alignment studies using DNASTAR MegAlign version 8.0.2 (Clustal W method) and the program Oligonucleotide Properties Calculator (http://www.basic.northwestern.edu/biotools/oligocalc.html).

The oligonucleotide primer 1656-R with the sequence 5`-AGG TTC ACA ACC CGA AGG-3' was used together with the oligonucleotide primer 16SUNI-L mentioned before and produced an amplicon with an expected size of 426 bp representing the front part of the 16S rDNA. The second oligonucleotide primer 1656-F with the sequence 5`-GCT TCA CGC ATG CTA CAA TGG C-3' was used together with the oligonucleotide primer 16SUNI-R mentioned before and produced an amplicon with an expected size of 184 bp from the end part of the 16S rDNA. Both oligonucleotide primers 1656-R and 1656-F represented a slight modification of the *E. coli* 16S rDNA sequence (bases 417 to 434 and bases 1226 to 1247, respectively). The preparation of the master mix was described in 3.6.2. The PCR thermal cycler program for both PCR reactions was carried out as follows:

1 cycle	94 °C	10	min	Initial denaturation
30 cycle	95 °C	30	sec	Denaturation
	55 °C	30	sec	Primer annealing
	72 °C	30	sec	Extension
1 cycle	72 °C	7	min	Final extension

Sequencing of the amplicons and further sequence analyses were performed as described in 3.6.6.

3.6.8 Amplification and sequencing of the 16S-23S rDNA intergenic spacer region ISR

To amplify the ISR of the bacterial strains the oligonucleotide primers described by Kostman et al. (1995) and Chanter et al. (1997) were used. The oligonucleotide primers had for primer b the sequence 5`-GGT ACC TTA GAT GTT TCA GTT C-3' and for primer c the sequence 5`-TTG TAC ACA CCG CCC GTC A-3'. The preparation of the master mix was conducted as mentioned in 3.6.2. The PCR thermal cycler program was as follows:

1 cycle	94 °C	10	min	Initial denaturation
30 cycle	95 °C	70	sec	Denaturation
	45 °C	70	sec	Primer annealing
	72 °C	70	sec	Extension
1 cycle	72 °C	7	min	Final extension

Sequencing of the amplicons and further sequence analyses were performed as described in 3.6.6.

3.6.9 Amplification and sequencing of the 23S rDNA

The amplification of the 23S rDNA was performed by using the oligonucleotide primer 23S-1 with the sequence 5`-AGT TCC GAC CTG CAC GAA TGG C-3' described by Jost et al. (2004) and primer 23S-2a with the sequence 5`-CGT CCG TCC CGG TCC TCT-3' also described by Jost et al. (2004) but modified by Ülbegi-Mohyla et al. (2010). The preparation of the master mix was described in 3.6.2. The PCR thermal cycler program was carried out as follows:

1 cycle	94 °C	10	min	Initial denaturation
30 cycle	95 °C	30	sec	Denaturation
	60 °C	60	sec	Primer annealing
	72 °C	60	sec	Extension
1 cycle	72 °C	7	min	Final extension

Sequencing of the amplicons and further sequence analyses were performed as described in 3.6.6.

3.6.10 Amplification and sequencing of gene *cpn*60 encoding heat shock protein or chaperonin CPN60

The amplification of gene *cpn*60 was initially performed for the reference cultures *A.* (*T.*) *abortisuis* DSM 19515^T, *A.* (*T.*) *bernardiae* DSM 9152^T, *A.* (*T.*) *bialowiezense* DSM17162^T, *A.* (*T.*) *bonasi* DSM 17163^T and *A. haemolyticum* DSM 20595^T using the universal oligonucleotide primers HSP1-F with the sequence 5`-CGC TGC GTC GTG GCA TTG AC-3' and HSP1-R with the sequence 5`-TCC GCG ATG ATT GCG AGC GG-3', both designed with the help of the NCBI primer design tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) using the 60 kDa chaperonin gene *cpn*60 sequence of *A.* (*T.*) *pyogenes* (NCBI GenBank accession number AY691206). The preparation of the master mix was described in 3.6.2, the PCR thermal cycler program was carried out as follows:

1 cycle	94 °C	10	min	Initial denaturation
35 cycle	95 °C	60	sec	Denaturation
	50 °C	70	sec	Primer annealing
	72 °C	70	sec	Extension
1 cycle	72 °C	7	min	Final extension

The chaperonin encoding gene *cpn*60 from *A. phocae* DSM 10003 was amplified using the oligonucleotide primer HSP1-F described above and the new reverse primer HSP2-R with the sequence 5`-CCG GTC TGC ATG ACC TTC TC-3'. The HSP2-R primer sequence was designed using the before obtained *cpn*60 sequences of *A.* (*T.*) *abortisuis* DSM 19515^T, *A.* (*T.*) *bernardiae* DSM 9152^T, *A.* (*T.*) *bialowiezense* DSM17162^T, *A.* (*T.*) *bonasi* DSM 17163^T and *A. haemolyticum* DSM 20595^T (GenBank accession number FR681838, FR681839, FR681841, FR681840 and FR681842), respectively and *A.* (*T.*) *pyogenes cpn*60, as described

in 3.6.6. Preparation of the master mix was mentioned in 3.6.2. The PCR thermal cycler program was carried out as follows:

1 cycle	94 °C	10	min	Initial denaturation
35 cycle	95 °C	60	sec	Denaturation
	45 °C	70	sec	Primer annealing
	72 °C	70	sec	Extension
1 cycle	72 °C	7	min	Final extension

Chaperonin encoding gene *cpn*60 of *A. hippocoleae* DSM 15539^T, *A. pluranimalium* DSM 13483^T and of the three cultures isolated from dogs, representing a novel species of genus *Actinomyces* was finally amplified using the primer HSP2-F with the sequence 5`-AAG GTC GGC AAG GAA GGC G-3' and HSP2-R described above. The HSP2-F sequence was designed as described in 3.6.6, by using the *cpn*60 genes of species of both genera *Arcanobacterium* and *Trueperella* mentioned above. The preparation of master mix was mentioned in 3.6.2, the PCR thermal cycler program was carried out as follows:

1 cycle	94 °C	10	min	Initial denaturation
35 cycle	95 °C	60	sec	Denaturation
	40 °C	70	sec	Primer annealing
	72 °C	70	sec	Extension
1 cycle	72 °C	7	min	Final extension

Sequencing of the amplicons and further sequence analyses were performed as described in 3.6.6.

3.6.11 PCR-mediated identification by amplification of species specific parts

3.6.11.1 Amplification of *A.* (*T.*) *pyogenes* ISR

The amplification of *A.* (*T.*) *pyogenes* specific regions of the ISR was done with the oligonucleotide primer sequences given by Ülbegi (2010). These sequences were obtained from the ISR sequence of *A.* (*T.*) *pyogenes* DSM 20630^T (GenBank accession number EU194563). The oligonucleotide primer Apy-ISR-F had the sequence 5`-GTT TTG CTT GTG ATC GTG GTG GTT ATG A-3' and Apy ISR-R the sequence 5'-AAG CAG GCC CAC

GCG CAG G-3'. The PCR reaction mixture was described in 3.6.2, the PCR thermal cycler program was carried out as follows:

1 cycle	94 °C	10	min	Initial denaturation
30 cycle	95 °C	30	sec	Denaturation
	64 °C	15	sec	Primer annealing
	72 °C	30	sec	Extension
1 cycle	72 °C	7	min	Final extension

3.6.11.2 Amplification of *A.* (*T.*) *pyogenes* superoxide dismutase A encoding gene *sod*A

For amplification of *A.* (*T.*) *pyogenes* specific regions of gene *sod*A the oligonucleotide primer Apy-*sod*A-F with the sequence 5´-CGA GCT CGC CGA CGC TAT TGC T-3´ and Apy-*sod*A-R with the sequence 5´ GAG CAT GAG AAT CGG GTA AGT GCC A-3´ were used. These sequences were obtained from the *sod*A gene sequence of *A.* (*T.*) *pyogenes* DSM 20630^T (GenBank accession number AM949566) and designed as mentioned in 3.6.6. The PCR reaction mixture was described in 3.6.2 and the PCR thermal cycler program was carried out as follows:

1 cycle	94 °C	10	min	Initial denaturation
25 cycle	94 °C	20	sec	Denaturation
	62 °C	20	sec	Primer annealing
	72 °C	30	sec	Extension
1 cycle	72 °C	10	min	Final extension

3.6.11.3 Amplification of *A.* (*T.*) *abortisuis* ISR

The amplification of *A.* (*T.*) *abortisuis* specific regions of the ISR was done with the oligonucleotide primer Aab-ISR-F with the sequence 5`-AAG GAG CCT CAT GTG TGC CTG T-3' and Aab-ISR-R with the sequence 5'-GCT ACA ACG TCC TTC ATC GGC TC-3'. These sequences were obtained from the ISR sequence of *A.* (*T.*) *abortisuis* DSM 19515^T (GenBank accession number FN667627) and designed as mentioned in 3.6.6. The PCR reaction mixture was described in 3.6.2 and the PCR thermal cycler program was carried out as follows:

1 cycle	94 °C	10	min	Initial denaturation
30 cycle	95 °C	30	sec	Denaturation
	58 °C	30	sec	Primer annealing
	72 °C	60	sec	Extension
1 cycle	72 °C	7	min	Final extension

3.6.11.4 Amplification of *A. haemolyticum* ISR and 23S rDNA

The amplification of *A. haemolyticum* specific regions of the ISR-23S rDNA was done with the oligonucleotide primer sequence data given by Ülbegi (2010). The oligonucleotide primer Aha-ISR-F had the sequence 5`-CCT AGC CTG GTG GTT GGG TAG-3` and Aha-23S rDNA-R the sequence 5`-GTG CGG GTA ACC AGA AAT AAC TCT G-3`. The PCR reaction mixture was described in 3.6.2 and the PCR thermal cycler program was carried out as follows:

1 cycle	95 °C	10	min	Initial denaturation
30 cycle	95 °C	8	sec	Denaturation
	66 °C	10	sec	Primer annealing
	72 °C	10	sec	Extension
1 cycle	72 °C	7	min	Final extension

3.6.11.5 Amplification of *A. haemolyticum* phospholipase D encoding gene *pld*

The amplification of *A. haemolyticum*-specific regions of phospholipase D encoding gene *pld* was done with the oligonucleotide primer sequence data given by Hassan et al. (2009). The oligonucleotide primer Ah-F had the sequence 5`-ATG TAC GAC GAT GAA GAC GCG-3` and Ah-R the sequence 5`-GCT TCC TTG TCG TTG AGA TTA TTA GC-3`. The PCR reaction mixture was described in 3.6.2 and the PCR thermal cycler program was carried out as follows:

1 cycle	95 °C	10	min	Initial denaturation
30 cycle	95 °C	30	sec	Denaturation
	60 °C	60	sec	Primer annealing
	72 °C	60	sec	Extension
1 cycle	72 °C	7	min	Final extension

3.6.11.6 Amplification of *A. haemolyticum* CAMP factor family protein encoding gene *cfa*

The amplification of *A. haemolyticum* specific regions of CAMP factor family protein encoding gene *cfa* was done with the oligonucleotide primer camp-F with the sequence 5´-ACT TGC TTG ACC GTG GAG CGT G-3´ and camp-R with the sequence 5´-TCT CAG TAG TGT CCG CGC ACG-3´. These sequences were designed using the *cfa* gene sequence given by the *A. haemolyticum* DSM 20595^{T} complete genome project (NC_014218, Gene ID 9260882) with the help of the NCBI primer design tool as mentioned in 3.6.10. The PCR reaction mixture was described in 3.6.2 and the PCR thermal cycler program was carried out as follows:

1 cycle	95 °C	10	min	Initial denaturation
30 cycle	95 °C	45	sec	Denaturation
	55 °C	60	sec	Primer annealing
	72 °C	60	sec	Extension
1 cycle	72 °C	7	min	Final extension

3.6.12 PCR-mediated amplification of *A.* (*T.*) *pyogenes* putative virulence factor encoding genes

3.6.12.1 Amplification of *A.* (*T.*) *pyogenes* pyolysin encoding gene *plo*

The amplification of *A.* (*T.*) *pyogenes* pyolysin encoding gene *plo* was performed with the oligonucleotide primer sequence data given by Ülbegi (2010). The oligonucleotide primer *plo*-F had the sequence 5`-CGA TCC CTC TGG TGT ACT TGC-3` and *plo*-R the sequence 5`-GCT TGA CAA AAA TCT GGC GTC C-3`. The PCR reaction mixture was described in 3.6.2, the PCR thermal cycler program was carried out as follows:

1 cycle	95 °C	10	min	Initial denaturation
30 cycle	95 °C	60	sec	Denaturation
	62 °C	60	sec	Primer annealing
	72 °C	60	sec	Extension
1 cycle	72 °C	7	min	Final extension

3.6.12.2 Amplification of *A.* (*T.*) *pyogenes* collagen-binding protein encoding gene *cbp*A

The amplification of *A.* (*T.*) *pyogenes* collagen-binding protein encoding gene *cbp*A was done with the oligonucleotide primer sequence data given by Ülbegi (2010). The oligonucleotide primer *cbp*-F had the sequence 5′-CTT GAA ATC GAA CTT AAG GCT GG-3′ and *cbp*-R the sequence 5′-ATC GCC AGT CAC CTT AGA CG-3′. The PCR reaction mixture was described in 3.6.2, the thermal cycler program was carried out as follows:

1 cycle	94 °C	10	min	Initial denaturation
30 cycle	95 °C	30	sec	Denaturation
	58 °C	30	sec	Primer annealing
	72 °C	60	sec	Extension
1 cycle	72 °C	7	min	Final extension

3.6.12.3 Amplification *A.* (*T.*) *pyogenes* neuraminidase encoding genes *nan*H and *nan*P

The amplification of the *A.* (*T.*) *pyogenes* neuraminidase encoding genes *nan*H and *nan*P was performed with the oligonucleotide primer sequence data given by Silva et al. (2008). For *nan*H the oligonucleotide primer *nan*H-F had the sequence 5′-CGC TAG TGC TGT AGC GTT GTT AAG T-3′ and *nan*H-R the sequence 5′-CCG AGG AGT TTT GAC TGA CTT TGT-3′, while for *nan*P the oligonucleotide primer *nan*P-F had the sequence 5′TTG AGC GTA CGC AGC TCT TC-3′ and *nan*P-R the sequence 5′-CCA CGA AAT CGG CCT TAT TG-3′. The PCR reaction mixture was described in 3.6.2, the thermal cycler program was carried out as follows:

1 cycle	94 °C	10	min	Initial denaturation
35 cycle	94 °C	60	sec	Denaturation
	60 °C	60	sec	Primer annealing
	72 °C	180	sec	Extension
1 cycle	72 °C	7	min	Final extension

3.6.12.4 Amplification of *A.* (*T.*) *pyogenes* fimbriae encoding genes *fim*A, *fim*C and *fim*E

The amplification of *A.* (*T.*) *pyogenes* fimbriae encoding genes *fim*A, *fim*C and *fim*E was performed with oligonucleotide primer sequence data given by Silva et al. (2008). For amplification of fimbriae encoding gene *fim*A the oligonucleotide primer *fim*A-F had the sequence 5′-CAC TAC GCT CAC CAT TCA CAA G-3′ and *fim*A-R the sequence 5′-GCT GTA ATC CGC TTT GTC TGT G-3′. For fimbriae encoding gene *fim*C the oligonucleotide primer *fim*C-F had the sequence 5′-TGT CGA AGG TGA CGT TCT TCG-3′ and *fim*C-R the sequence 5′-CAA GGT CAC CGA GAC TGC TGG-3′ and for fimbriae encoding gene *fim*E the oligonucleotide primer *fim*E-F had the sequence 5′-GCC CAG GAC CGA GAG CGA GGG C-3′ and *fim*E-R the sequence 5′-GCC TTC CAC AAA TAA CAG CAA CC -3′.
The PCR reaction mixture was described in 3.6.2, the PCR thermal cycler program was carried out as follows:

1 cycle	94 °C	10	min	Initial denaturation
35 cycle	94 °C	60	sec	Denaturation
	× °C	60	sec	Primer annealing
	72 °C	180	sec	Extension
1 cycle	72 °C	7	min	Final extension

× = Primer annealing temperatures (*fim*A =57 °C, *fim*C = 60 °C and *fim*E = 55 °C)

3.7 Statistical analysis

Statistical analyses were kindly performed by Dr. K. Failing, Unit for Biomathematics and Data processing, Justus-Liebig-Universität Gießen, Frankfurterstraße 95, 35392 Gießen, using the statistical program package BMDP (Dixon, W. J. (chief editor), 1993. BMDP Statistical Software Manual, Volume 1 and 2. University of California Press, Berkeley, Los Angeles, London).
First, the statistical analysis was performed by forming fourfold tables for the comparison of the frequency of gene expression between different animal groups followed by the two-sided exact test of Fisher (program BMDP4F). For the comparison of the log (score) values obtained by MALDI-TOF MS analysis the two way analysis of variance with repeated measures on the same element known as ANOVA test was used (program BMDP2V).

4 Results

4.1 Identification and further characterization of the bacteria using conventional and molecular techniques

In the present study a total of 80 bacterial cultures could be identified and classified to genus *Arcanobacterium* and *Trueperella* or to genus *Actinomyces*. The characterization was performed by cultural methods, by various phenotypical tests, by MALDI-TOF MS and by PCR-mediated amplification of various molecular targets. The use of reference strains of genera *Arcanobacterium* and *Trueperella* (n = 11) and genus *Actinomyces* (n = 2) for comparative purposes allowed a final identification of the bacteria as *A.* (*T.*) *pyogenes* (n = 51), *A.* (*T.*) *abortisuis* (n = 23) and *A. haemolyticum* (n = 3) and as *Actinomyces weissii* (n = 3), representing a novel species of genus *Actinomyces*.
The bacteria of genus *Arcanobacterium* and *Trueperella* were further characterized by investigating various potential virulence genes.

4.1.1 Phenotypic and genotypic properties of *A.* (*T.*) *pyogenes*

4.1.1.1 Phenotypic results

4.1.1.1.1 Hemolytic properties and CAMP-like hemolytic reactions

The determination of hemolytic properties on sheep and rabbit blood agar (3.2) and the investigation of CAMP-like hemolytic reactions (3.4.3) revealed that all 51 *A.* (*T.*) *pyogenes* strains investigated and the *A.* (*T.*) *pyogenes* reference strains *A.* (*T.*) *pyogenes* DSM 20630^T and *A.* (*T.*) *pyogenes* DSM 20594 displayed, after cultivation on sheep blood agar under microaerobic conditions in a candle jar (3.2), a relatively wide zone of hemolysis. No enhanced hemolysis of the *A.* (*T.*) *pyogenes* strains could be observed after cultivation on rabbit blood agar compared to sheep blood agar **(Tab. 6)**. In addition, all 51 *A.* (*T.*) *pyogenes* and the two *A.* (*T.*) *pyogenes* reference strains displayed CAMP-like hemolytic reactions with β-hemolytic *S. aureus, R. equi, A. haemolyticum* and *A. phocae* as indicator strains (**Fig. 4a, b; Tab. 6)**.

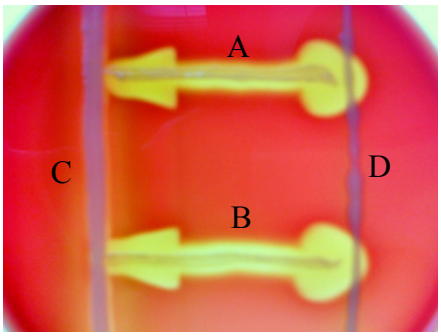


Fig. 4a: CAMP-like hemolytic reaction of *A.* (*T.*) *pyogenes* 3201/07 (A, horizontal) and *A.* (*T.*) *pyogenes* 6248/08 (B, horizontal) of the present study with β-hemolysin of *S. aureus* (C, vertical) and *R. equi* (D, vertical) as indicator strains.

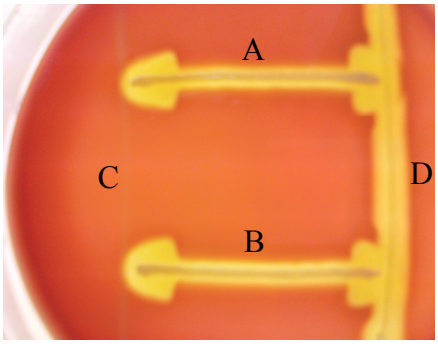


Fig. 4b: CAMP-like hemolytic reaction of *A.* (*T.*) *pyogenes* 2958/07 (A, horizontal) and *A.* (*T.*) *pyogenes* 1886/08 (B, horizontal) of the present study with *A. haemolyticum* DSM 20595^T (C, vertical) and *A. phocae* DSM 10002^T (D, vertical) as indicator strains.

Tab. 6: Hemolytic properties and CAMP-like hemolytic reactions of 51 *A.* (*T.*) *pyogenes* and both *A.* (*T.*) *pyogenes* reference strains.

		A. (*T.*) *pyogenes* n = 51	*A.* (*T.*) *pyogenes* DSM 20630^T	*A.* (*T.*) *pyogenes* DSM 20594
Hemolysis on	Sheep blood agar	+* (51)	+	+
	Rabbit blood agar	+ (51)	+	+
CAMP-like hemolysis with	*S. aureus* β-hemolysin zone	+** (51)	+	+
	S. agalactiae	-	-	-
	R. equi	+ (51)	+	+
	P. phenylpyruvicus	-	-	-
	A. haemolyticum	+ (51)	+	+
	A. phocae	+ (51)	+	+
Reverse CAMP reaction		-	-	-

n = Number of strains; * = (+), +, +(+), ++ degree of hemolysis; ** = CAMP-like hemolytic reaction or reverse CAMP reaction. The number of positive strains is shown in parentheses.

4.1.1.1.2 Additional properties

Phenotypical properties of the 51 *A.* (*T.*) *pyogenes* strains of different animal origin as well as the two reference strains *A.* (*T.*) *pyogenes* DSM 20630^T and *A.* (*T.*) *pyogenes* DSM 20594 are summarized in **Tab. 7**.

All of the investigated *A.* (*T.*) *pyogenes* strains showed a positive reaction for the enzymes β-D-glucuronidase, β-D-galactosidase, α-D-glucosidase and N-acetyl-β-D-glucosaminidase (3.4.6) and a negative reaction for the enzymes catalase (3.4.7) and hyaluronidase (3.4.11). Positive reactions were also observed investigating serolysis on Loeffler medium (3.4.8), casein hydrolysis (3.4.9) and for the enzyme DNase (3.4.10). Variable results could be observed for the enzymes pyrrolidonyl arylamidase, alkaline phosphatase, α-mannosidase (3.4.5) and amylase (3.4.12). Amylase enzyme activities could be detected in all *A.* (*T.*) *pyogenes* isolated from wild ruminants, horse and rabbit, less pronounced in *A.* (*T.*) *pyogenes* from pigs (62 %), small ruminants (60 %) and from dogs and cats (50 %). *A.* (*T.*) *pyogenes* from bovines, rat and reptiles did not demonstrate amylase activities. The differences in the production of the enzyme amylase of the *A.* (*T.*) *pyogenes* strains isolated from ruminants and pigs appeared to be significant ($P = 0.047$). Amylase enzyme activity could also be found for reference strain *A.* (*T.*) *pyogenes* DSM 20630^T originally isolated from a pig, but not for *A.* (*T.*) *pyogenes* DSM 20594 of bovine origin **(Tab. 7)**.

Enzymatic extracts of all 51 *A.* (*T.*) *pyogenes* and the two *A.* (*T.*) *pyogenes* reference strains displayed cross reactions with streptococcal serogroup G specific antiserum (3.4.15) **(Tab. 7).**

Tab. 7: Phenotypic properties of 51 *A.* (*T.*) *pyogenes* strains and the two *A.* (*T.*) *pyogenes* reference strains.

Phenotypic properties	*A.* (*T.*) *pyogenes* (n = 51)	*A.* (*T.*) *pyogenes* DSM 20630^T	*A.* (*T.*) *pyogenes* DSM 20594
Pyrrolidonyl arylamidase	+(28); (+)(2)[1]	+[1]	-[1]
Alkaline phosphatase	+(42)[1]	-[1]	-[1]
α-Mannosidase	+(9); (+)(1)[1]	-[1]	-[1]
β-Glucuronidase	+(51)[2]	+[2]	+[2]
β-Galactosidase	+(51)[2]	+[2]	+[2]
α-Glucosidase	+(51)[2]	+[2]	+[2]
N-Acetyl-β-Glucosaminidase	+(51)[2]	+[2]	+[2]
Catalase	-	-	-
Serolysis on Loeffler medium	+(51)	+	+
Casein hydrolysis	+(51)	+	+
DNase	+(51)	+	+
Hyaluronidase	-	-	-
Amylase*	+(18); (+)(2)	+	-
Cross reaction with streptococcal serogroup G specific antiserum	+(51)	+	+

n = Number of strains; + = positive reaction; (+) = weak reaction; - = negative reaction. The number of positive strains is shown in parentheses. 1 = Tablets containing substrates (3.4.5); 2 = 4-methylumbelliferyl-conjugated substrates (3.4.6), * = significant difference between *A.* (*T.*) *pyogenes* isolated from ruminants and pigs with $P = 0.047$.

4.1.1.2 Identification of *A.* (*T.*) *pyogenes* and eight other species of genera *Arcanobacterium* and *Trueperella* by MALDI-TOF MS

MALDI-TOF MS (3.5) could be used to identify 11 reference strains of the present study representing nine species of genera *Arcanobacterium* and *Trueperella* to the species level by matching against reference library entries using the MALDI Biotyper 2.0 software package (3.5.2) and the newly acquired MSPs from the 11 reference strains. All nine species were reliably differentiated with no false positive cluster formation. There were major differences in the peak pattern of the raw spectra of the nine species, respectively **(Fig. 5)**, also reflected by low log (score) values obtained by comparing the log (score) values of the nine species among each other. **(Tab. 8)**. A dendrogram analysis of MALDI-TOF main spectra of the eleven reference strains is shown in **Fig. 6**.

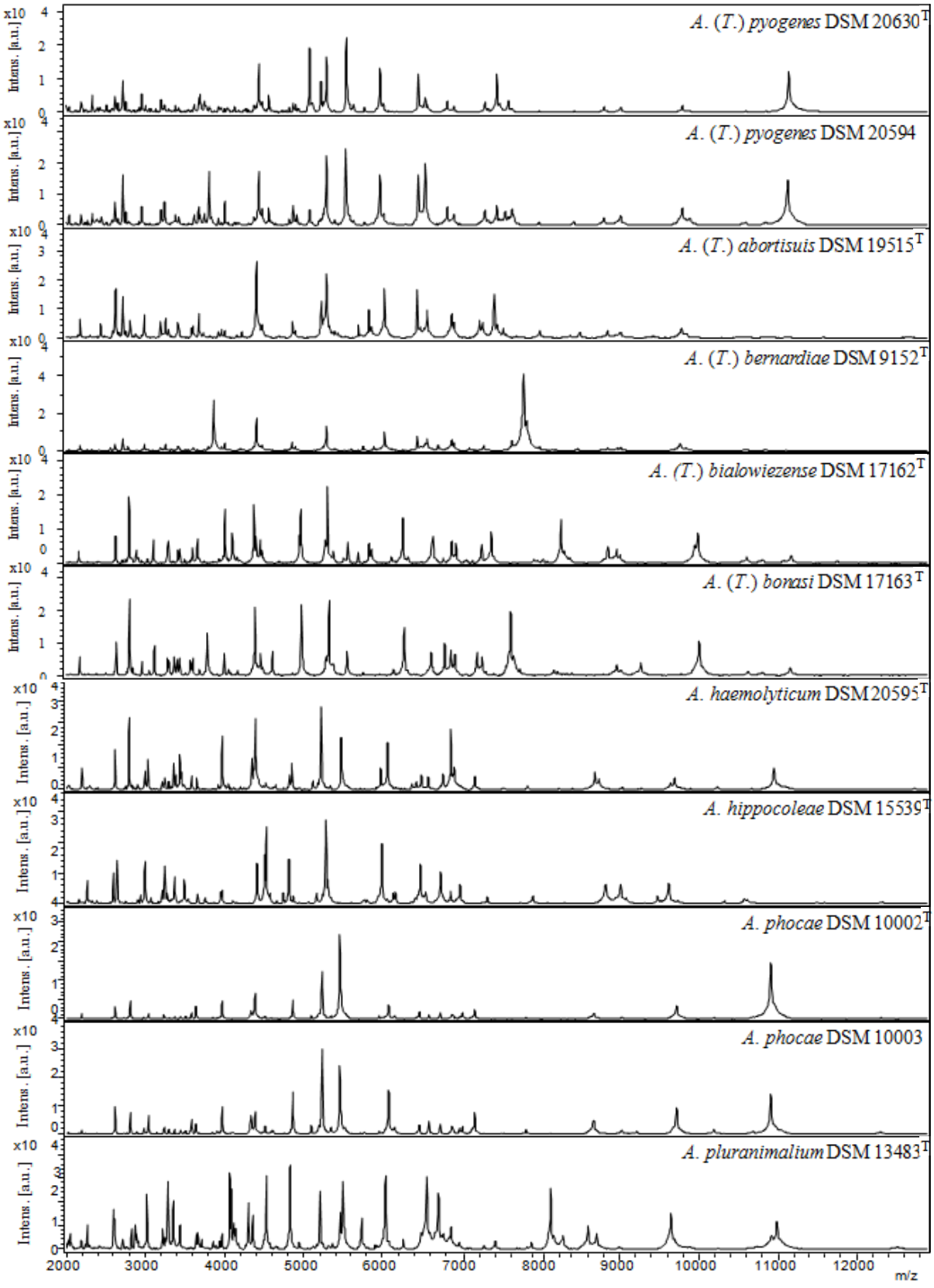


Fig. 5: Typical MALDI-TOF raw spectra of whole-cell extracts of 11 reference strains of genera *Arcanobacterium* and *Trueperella* in the range from 2,000 to 12,600 Da. The relative intensities of the ions are shown on the *y* axis, the mass to charge ratios are shown on the *x* axis.

Tab. 8: Log (score) value matrix of 11 reference strains of genera *Arcanobacterium* and *Trueperella* among each other.

Reference strains	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)
(1) *A.* (*T.*) *pyogenes* DSM 20630^T	3*	2.15	1.22	1.12	0	0.45	0.32	0.39	0	0	0.88
(2) *A.* (*T.*) *pyogenes* DSM 20594		3	1.11	1.13	0	0	0.66	0.70	0	0	0.59
(3) *A.* (*T.*) *abortisuis* DSM 19515^T			3	1.77	1.18	0	0.76	0	0	0.37	0.05
(4) *A.* (*T.*) *bernardiae* DSM 9152^T				3	0.68	0.02	0.83	0.42	0	0.74	0.08
(5) *A.* (*T.*) *bialowiezense* DSM 17162^T					3	0.85	0.83	0.16	0.53	0.07	0.5
(6) *A.* (*T.*) *bonasi* DSM 17163^T						3	0	0.28	0	0	0
(7) *A. haemolyticum* DSM 20595^T							3	0.65	0.83	0.72	0.16
(8) *A. hippocoleae* DSM 15539^T								3	0.73	0	0
(9) *A. phocae* DSM 10002^T									3	2.69	0.83
(10) *A. phocae* DSM 10003										3	0
(11) *A. pluranimalium* DSM 13483^T											3

* = Maximum log (score) value is 3, indicating that all peak list parameters match completely to the compared known MSP of the present study.

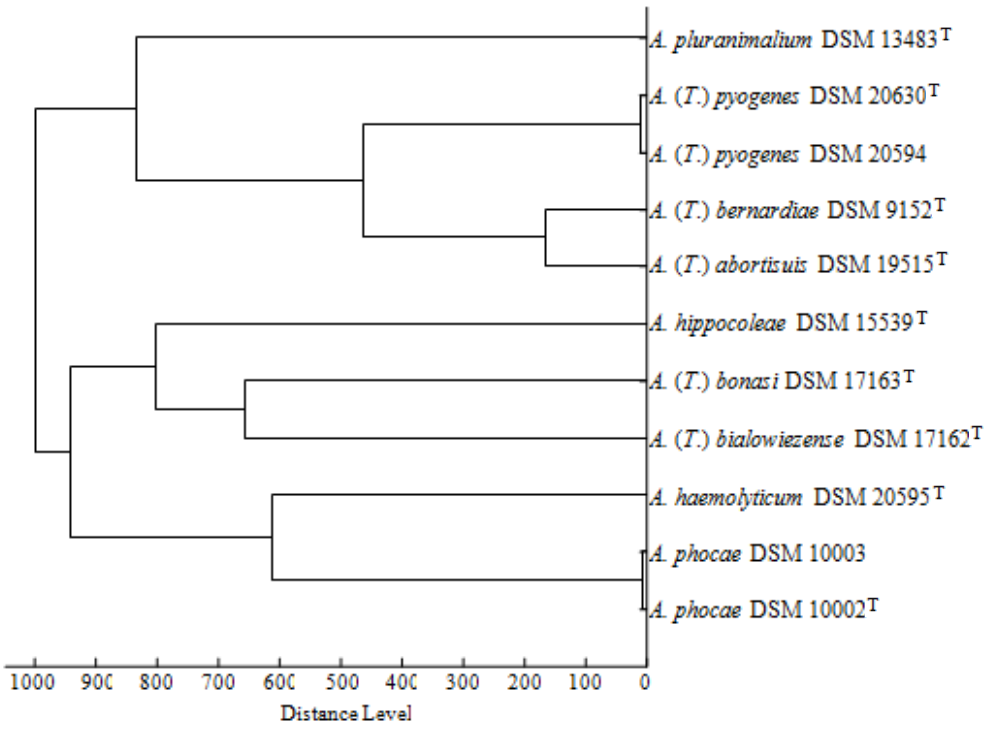


Fig. 6: A score oriented dendrogram of MALDI-TOF main spectra profiles of eleven reference strains of genera *Arcanobacterium* and *Trueperella*. The dendrogram was generated using the MALDI Biotyper 2.0 software with the following settings for peak picking: lower bound 3,000, upper bound 15,000, maximum peaks 100 and threshold 0.001, the distance measure was set at correlation and linkage set at average.

In addition, MALDI-TOF MS fingerprint analysis allowed the identification of all 51 *A.* (*T.*) *pyogenes* strains. A comparison of raw spectra of four selected *A.* (*T.*) *pyogenes* from different animal origins and the two *A.* (*T.*) *pyogenes* reference strains is shown in **Fig. 7**.

MALDI-TOF MS correctly identified all *A.* (*T.*) *pyogenes* strains to species level. The 51 strains matched against reference library entries (3.5.2) and MSPs of the two reference strains *A.* (*T.*) *pyogenes* DSM 20630^T and *A.* (*T.*) *pyogenes* DSM 20594 of the present study with log (score) values ranging from 2.11 to 2.63 **(Fig. 8)**. A dendrogram analysis of MALDI-TOF main spectra of 20 selected *A.* (*T.*) *pyogenes* strains from different animal origins and 11 reference strains of genera *Arcanobacterium* and *Trueperella* is shown in **Fig. 9**.

A comparison of the log (score) values of the 51 *A.* (*T.*) *pyogenes* strains among each other yielded the lowest log (score) value of 1.89 of *A.* (*T.*) *pyogenes* 2089/07 of pig origin to *A.* (*T.*) *pyogenes* 142/09 of dog origin to a maximal score of 2.63 of *A.* (*T.*) *pyogenes* 734/03 obtained from a bearded dragon to *A.* (*T.*) *pyogenes* 36/03 obtained from a mountain reedbuck. *A.* (*T.*) *pyogenes* strains 2957/07 and 2958/07, 5939/08 and 5940/08, and 1070/09 and 1071/09, representing strains isolated from two pigs of three farm domains, respectively matched in MALDI Biotyper analysis to each other with log (score) values of 2.30, 2.37 and 2.14, respectively.

The MALDI-TOF MS software package (version 3.1.1.0) furthermore allowed the matching of the MSPs of the 51 *A.* (*T.*) *pyogenes* strains isolated from different animal origins against the two reference strains *A.* (*T.*) *pyogenes* DSM 20594 of bovine origin and *A.* (*T.*) *pyogenes* DSM 20630^T of pig origin. Based on two-way ANOVA, the mean of the log (score) values of the strains isolated from bovines, pigs and from other animal origin appeared to be significantly higher (P = 0.0001) using reference strain *A.* (*T.*) *pyogenes* DSM 20594 as matching partner than using *A.* (*T.*) *pyogenes* DSM 20630^T. In addition, using both reference strains as matching partner the mean log (score) values of the *A.* (*T.*) *pyogenes* strains isolated from bovines were significantly higher (P = 0.0087) than the mean log (score) values of *A.* (*T.*) *pyogenes* isolated from pigs. In addition, the mean values of strains isolated from ruminants were also significantly higher (P = 0.0059) than of strains isolated from non-ruminants. A typical similarity matrix of the MSPs of the two *A.* (*T.*) *pyogenes* reference strains and nine selected *A.* (*T.*) *pyogenes* strains of the present study among each other is shown in **Tab. 9**.

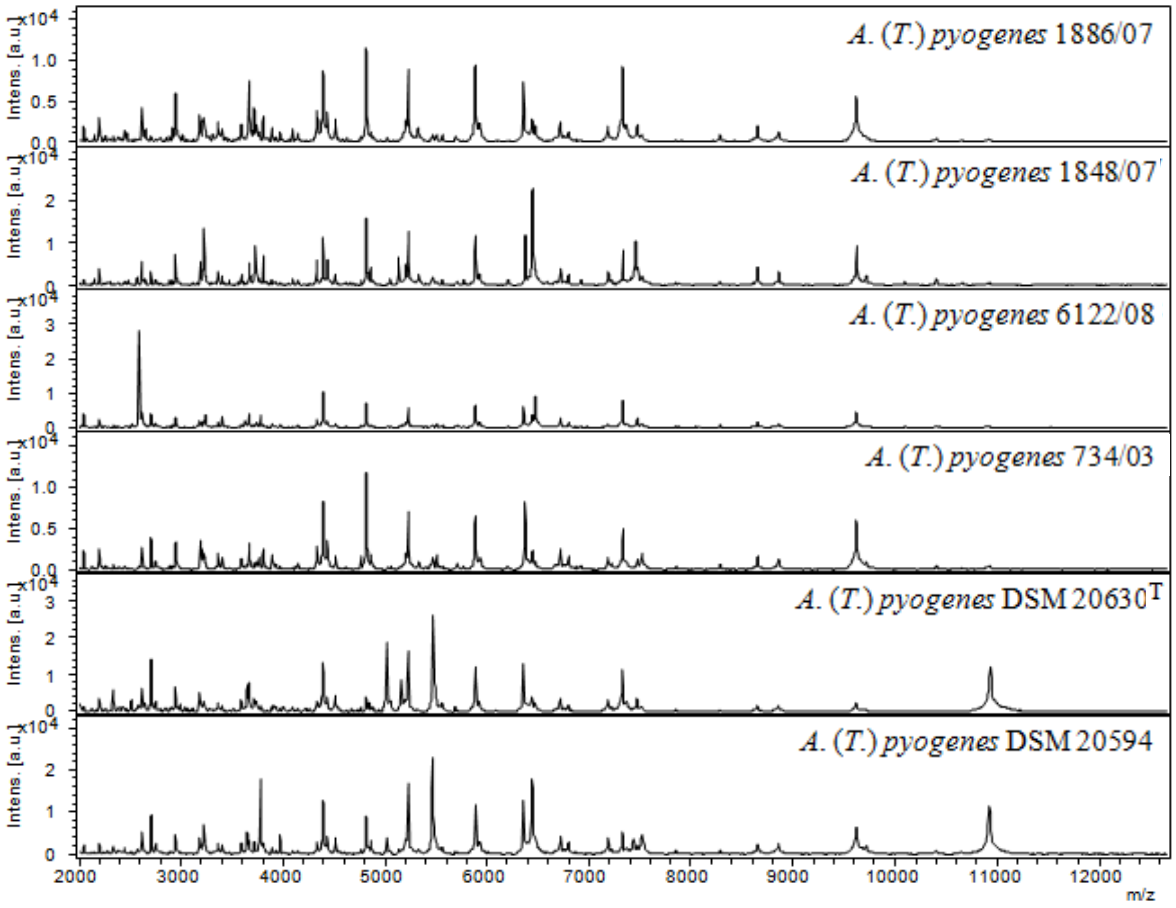


Fig. 7: MALDI-TOF raw spectra of whole-cell extract of six *A*. (*T*.) *pyogenes* strains in the range from 2,000 to 12,600 Da. The relative intensities of the ions are shown on the *y* axis, the mass to charge ratios are shown on the *x* axis. The patterns display the overall similarities among the *A*. (*T*.) *pyogenes* strains.

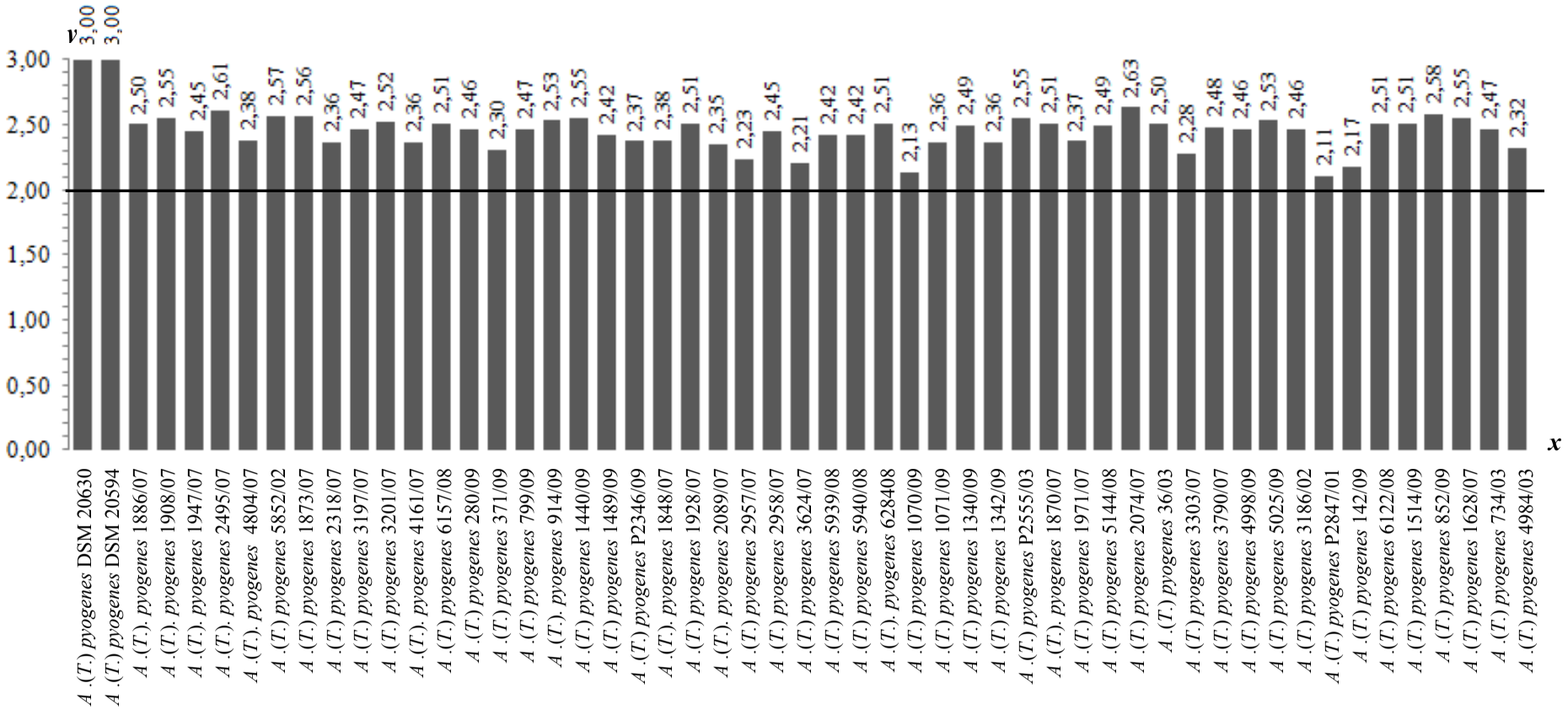


Fig. 8: Log (score) values (*y* axis) of 51 *A*. (*T*.) *pyogenes* and the two *A*. (*T*.) *pyogenes* reference strains (*x* axis) matched against reference library entries and the MSP of the two reference strains *A*. (*T*.) *pyogenes* DSM 20630^{T} and *A*. (*T*.) *pyogenes* DSM 20594 of the present study. Log (score) values ≥ 2.00 are rated as identification of the bacteria at species level.

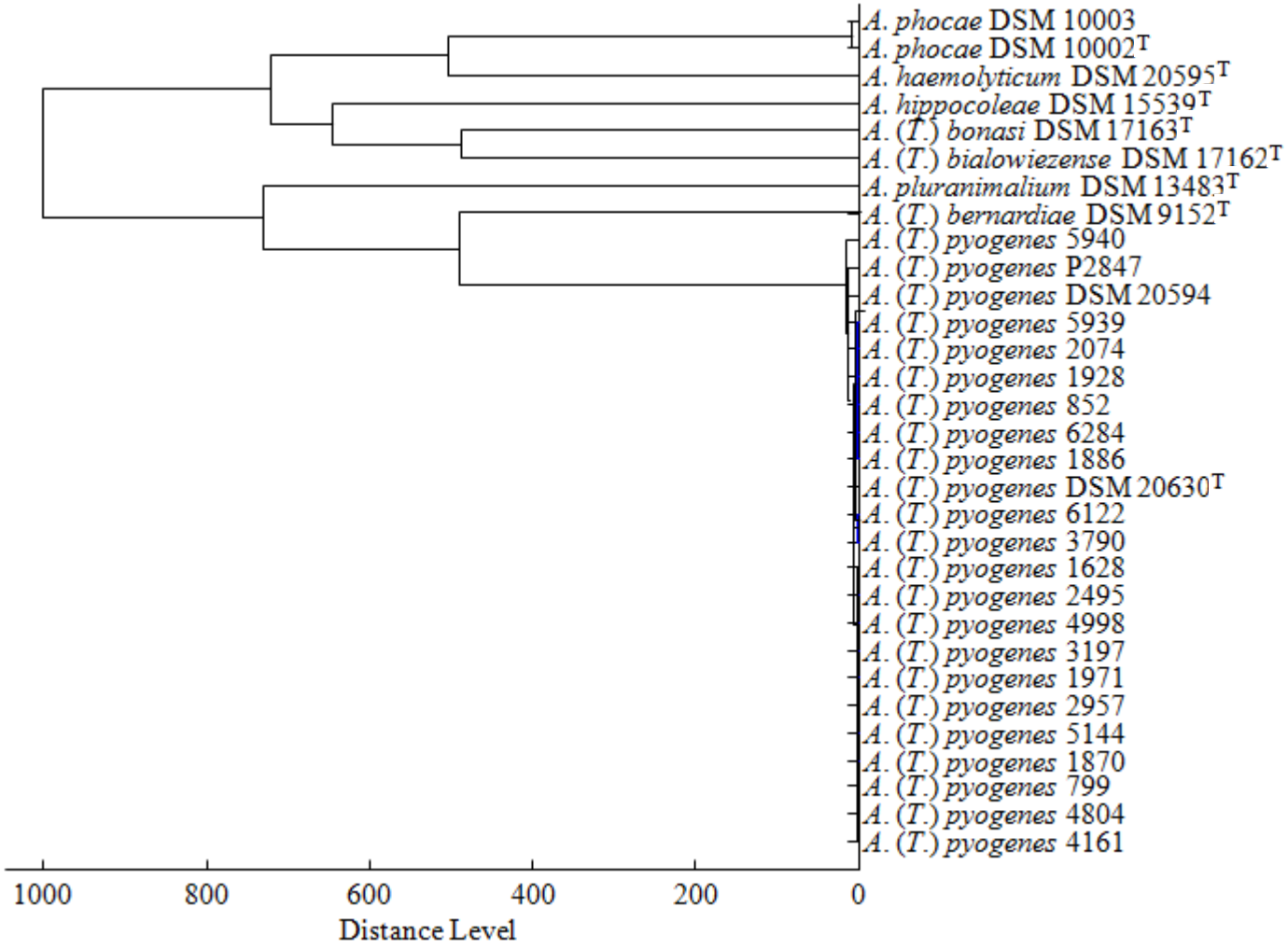


Fig. 9: A score oriented dendrogram of MALDI-TOF main spectra profiles of 20 selected *A*. (*T*.) *pyogenes* strains of the present study with eleven reference strains of genera *Arcanobacterium* and *Trueperella*, also including the two *A*. (*T*.) *pyogenes* reference strains. For generating of the dendrogram see **Fig. 6**.

Tab. 9: Typical log (score) value matrix of the two *A*. (*T*.) *pyogenes* reference strains and nine *A*. (*T*.) *pyogenes* strains of the present study among each other.

A. (*T*.) *pyogenes* strains	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)
(1) *A*. (*T*.) *pyogenes* DSM 20630^T	3	2.16	2.50	2.55	1.97	2.34	2.26	2.35	2.35	1.84	2.32
(2) *A*. (*T*.) *pyogenes* DSM 20594		3	2.48	2.44	2.13	2.51	2.55	2.50	2.46	2.17	2.47
(3) *A*. (*T*.) *pyogenes* 1886/07			3	2.61	2.13	2.55	2.54	2.59	2.60	2.29	2.56
(4) *A*. (*T*.) *pyogenes* 1908/07				3	2.06	2.26	2.31	2.42	2.29	2.15	2.37
(5) *A*. (*T*.) *pyogenes* 1070/09					3	2.05	2.26	2.14	2.13	1.94	2.08
(6) *A*. (*T*.) *pyogenes* 1928/07						3	2.56	2.60	2.31	2.35	2.61
(7) *A*. (*T*.) *pyogenes* P2555/03							3	2.56	2.29	2.39	2.55
(8) *A*. (*T*.) *pyogenes* 36/03								3	2.46	2.51	2.63
(9) *A*. (*T*.) *pyogenes* 3186/02									3	2.23	2.47
(10) *A*. (*T*.) *pyogenes* 142/09										3	2.56
(11) *A*. (*T*.) *pyogenes* 734/03											3

4.1.1.3 PCR-mediated identification and characterization of *A.* (*T.*) *pyogenes*

4.1.1.3.1 PCR-mediated identification by amplification of species specific parts

4.1.1.3.1.1 Amplification of *A.* (*T.*) *pyogenes* ISR

The amplification of ISR specific parts of *A.* (*T.*) *pyogenes* was performed using the oligonucleotide primers Apy-F and Apy-R described in 3.6.11.2. With these oligonucleotide primers an amplicon with an approximate size of 120 bp (122 bp expected size) could be detected for all 51 investigated *A*. (*T*.) *pyogenes* strains and for the two reference strains *A*. (*T*.) *pyogenes* DSM 20630^T and *A.* (*T*.) *pyogenes* DSM 20594 **(Fig. 10)**. No cross reactivity could be observed with any of the control strains representing eight other species of genera *Arcanobacterium* and *Trueperella*.

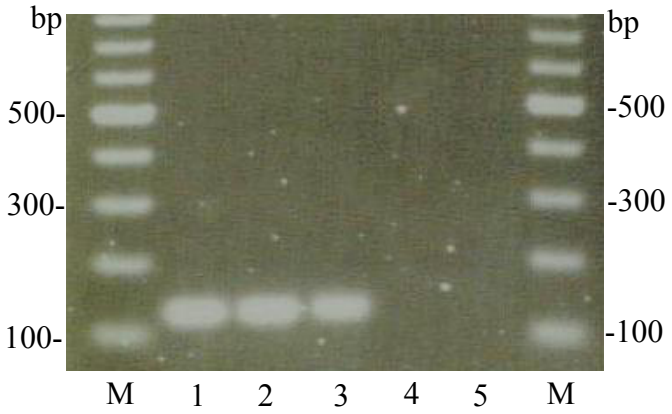


Fig. 10: Typical amplicons of *A.* (*T.*) *pyogenes* (1, 2 and 3) with a size of approximately 120 bp using *A.* (*T.*) *pyogenes* ISR specific oligonucleotide primers Apy-F and Apy-R. *A.* (*T.*) *abortisuis* DSM 19515^T (4) and *A.* (*T.*) *bernardiae* DSM 9152^T (5) served as negative control. M = GeneRuler 100-1000 bp DNA ladder (3.6.3).

4.1.1.3.1.2 Amplification of *A.* (*T.*) *pyogenes sod*A gene

The amplification of *A.* (*T.*) *pyogenes sod*A gene specific parts was performed using the oligonucleotide primers Apy-*sod*A-F and Apy-*sod*A-R described in 3.6.11.3. With these oligonucleotide primers an amplicon with an approximate size of 200 bp (199 bp expected size) could be detected for all 51 investigated *A.* (*T.*) *pyogenes* strains and for both *A.* (*T.*) *pyogenes* reference strains **(Fig. 11)**. No cross reactivity could be observed with any of the control strains representing eight other species of genera *Arcanobacterium* and *Trueperella*.

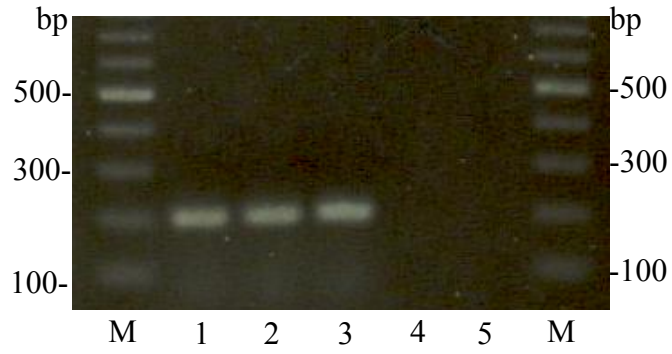


Fig. 11: Typical amplicons of *A.* (*T.*) *pyogenes* (1, 2 and 3) with a size of approximately 200 bp using *A.* (*T.*) *pyogenes sod*A specific oligonucleotide primers Apy-*sod*A-F and Apy-*sod*A-R. *A.* (*T.*) *bialowiezense* DSM 17162^T (4) and *A.* (*T.*) *bonasi* DSM 17163^T (5) served as negative control. M = see **Fig. 10**.

4.1.1.3.2 PCR-mediated amplification of putative virulence factor encoding genes of *A.* (*T.*) *pyogenes*

4.1.1.3.2.1 Amplification of pyolysin encoding gene *plo*

The amplification of gene *plo* was performed using the oligonucleotide primers *plo*-F and *plo*-R described in 3.6.12.1. With these oligonucleotide primers an amplicon with an approximate size of 700 bp (704 bp expected size) could be detected for all 51 investigated *A.* (*T.*) *pyogenes* strains and for both *A.* (*T.*) *pyogenes* reference strains **(Fig. 12; Tab. 10)**. No cross reactivity could be observed with any of the control strains representing eight other species of genera *Arcanobacterium* and *Trueperella*.

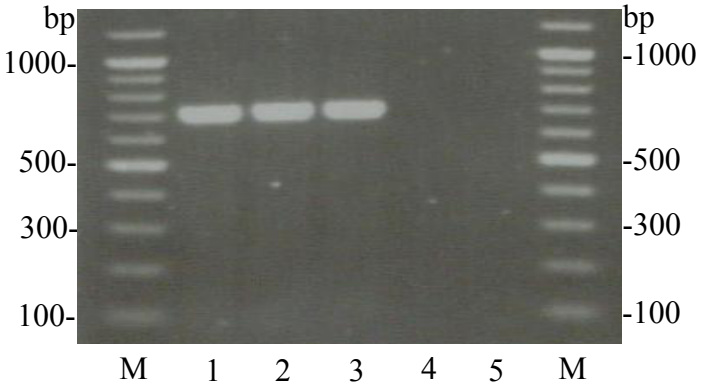


Fig. 12: Amplicons of gene *plo* of *A.* (*T.*) *pyogenes* DSM 20630^T (1) and of two *A.* (*T.*) *pyogenes* strains of the present study (2 and 3) with a size of approximately 700 bp using oligonucleotide primers *plo*-F and *plo*-R. Negative reaction of *A.* (*T.*) *abortisuis* DSM 19515^T (4) and *A.* (*T.*) *bernardiae* DSM 9152^T (5). M = see **Fig. 10**.

4.1.1.3.2.2 Amplification of collagen-binding protein encoding gene *cbp*A

The amplification of gene *cbp*A was carried out as described (3.6.12.2). Using the oligonucleotide primers *cbp*-F and *cbp*-R an amplicon with an approximate size of 330 bp (327 bp expected size) was successfully amplified for 11 (22 %) of the 51 *A.* (*T.*) *pyogenes* strains investigated **(Fig. 13; Tab. 10)**. Comparing the presence of CbpA encoding gene *cbp*A and the origin of the strains revealed that *cbp*A could be detected rarely in *A.* (*T.*) *pyogenes* of bovine origin (5 %), more frequently in *A.* (*T.*) *pyogenes* isolated from pigs (54 %), dogs and cats (25 %), (1 of 4 strains) and both reptiles (100 %). *A.* (*T.*) *pyogenes* from small ruminants

and wild ruminants, horse, rabbit and rat did not carry gene *cbp*A. As shown in **Tab. 10** the differences in the presence of gene *cbp*A between isolates from ruminants and pigs appeared to be significant (P = 0.0004). Gene *cbp*A could also be found for reference strain *A.* (*T.*) *pyogenes* DSM 20630^T, but not for *A.* (*T.*) *pyogenes* DSM 20594 **(Tab. 10)**. No cross reactivity could be observed with any of the control strains representing eight other species of genera *Arcanobacterium* and *Trueperella*.

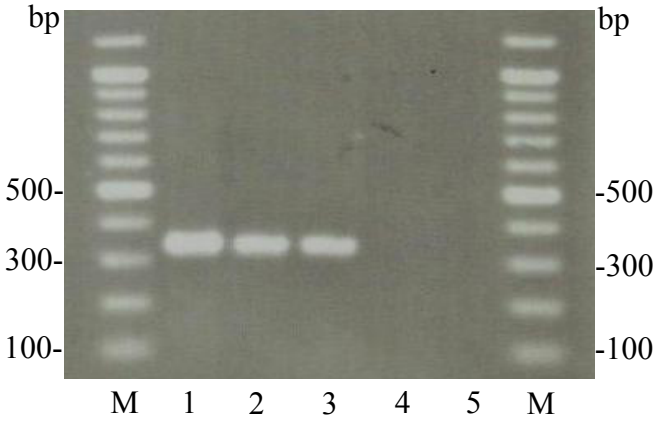


Fig. 13: Typical amplicons of gene *cpb*A of *A.* (*T.*) *pyogenes* DSM 20630^T (1) and the two *A.* (*T.*) *pyogenes* strains 1928/07 and 2089/07 (2 and 3, respectively) with a size of approximately 330 bp using oligonucleotide primers *cbp*-F and *cbp*-R. Negative reaction of *A.* (*T.*) *pyogenes* DSM 20594 (4) and *A. haemolyticum* DSM 20595^T (5). M = see **Fig. 10**.

4.1.1.3.2.3 Amplification of neuraminidase encoding genes *nan*H and *nan*P

The amplification of the genes *nan*H and *nan*P was carried out as described (3.6.12.3). Using the oligonucleotide primers *nan*H-F and *nan*H-R a *nan*H amplicon with an approximate size of 780 bp (781 bp expected size) could successfully be amplified for 47 (92 %) of the 51 *A.* (*T.*) *pyogenes* strains investigated **(Fig. 14; Tab. 10)**. Using the oligonucleotide primers *nan*P-F and *nan*P-R gene *nan*P with an approximate amplicon size of 150 bp (150 bp expected size) could be amplified for 40 (78 %) of the 51 *A.* (*T.*) *pyogenes* strains investigated **(Fig. 15; Tab. 10)**.

Neuraminidase encoding gene *nan*H and *nan*P negative strains occurred singly (n = 1, negative for *nan*H; n = 8, negative for *nan*P) or in combination (n = 3, negative for both *nan*H and *nan*P), with no significant differences between the *A.* (*T.*) *pyogenes* strains of various origins (P □ 0.05). The two *A.* (*T.*) *pyogenes* reference strains carried both genes *nan*H and

*nan*P **(Tab. 10)**. No cross reactivities for both genes could be observed with any of the control strains representing eight other species of genera *Arcanobacterium* and *Trueperella*.

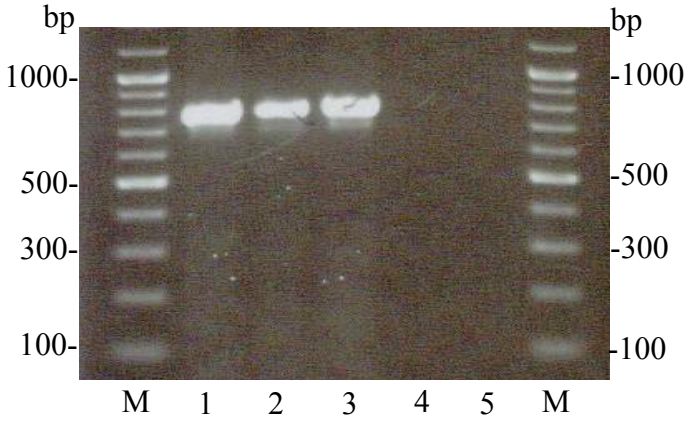


Fig. 14: Typical *nan*H amplicons of *A.* (*T.*) *pyogenes* DSM 20630^T (1) and the two *A.* (*T.*) *pyogenes* strains 5852/02 and 1873/07 (2 and 3, respectively) with a size of approximately 780 bp using oligonucleotide primers *nan*H-F and *nan*H-R. Negative reaction of *A. hippocoleae* DSM 15539^T (4) and *A. pluranimalium* DSM 13483^T (5). M = see **Fig. 10**.

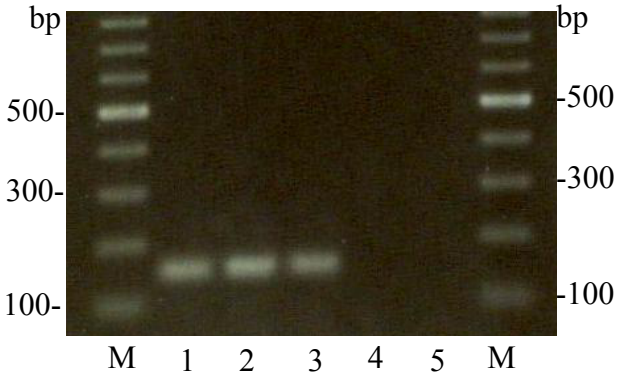


Fig. 15: Typical *nan*P amplicons of *A.* (*T.*) *pyogenes* DSM 20630^T (1) and the two *A.* (*T.*) *pyogenes* strains 1873/07 and 1928/07 (2 and 3, respectively) with a size of approximately 150 bp using oligonucleotide primers *nan*P-F and *nan*P-R. Negative reaction of *A.* (*T.*) *abortisuis* DSM 19515^T (4) and *A.* (*T.*) *bernardiae* DSM 9152^T (5). M = see **Fig. 10**.

4.1.1.3.2.4 Amplification of the fimbriae encoding genes *fim*A, *fim*C and *fim*E

The amplification of the genes *fim*A, *fim*C and *fim*E was carried out as described in 3.6.14.4 using the oligonucleotide primers *fim*A-F and *fim*A-R for gene *fim*A, the oligonucleotide primers *fim*C-F and *fim*C-R for gene *fim*C and the oligonucleotide primers *fim*E-F and *fim*E-R for gene *fim*E, respectively. Using the oligonucleotide primers *fim*A-F and *fim*A-R an amplicon with an approximate size of 610 bp (605 bp expected size) could successfully be

amplified for 50 (98 %) of the 51 *A.* (*T.*) *pyogenes* strains investigated **(Fig. 16; Tab. 10)**. Gene *fim*C could be amplified for 37 (73 %) of the 51 *A.* (*T.*) *pyogenes* strains investigated with an amplicon size of 850 bp (843 bp expected size) **(Fig. 17; Tab. 10)** and gene *fim*E for 50 (98 %) of the 51 *A.* (*T.*) *pyogenes* strains with an approximate amplicon size of 780 bp (775 bp expected size) **(Fig. 18; Tab. 10)**.

Comparing the origin of the strains and the presence of fimbrial encoding genes revealed that gene *fim*C appeared to be present in *A.* (*T.*) *pyogenes* of bovine origin (95 %), in *A.* (*T.*) *pyogenes* strains from wild ruminants, horse, rat and reptiles (100 %), but less pronounced in *A.* (*T.*) *pyogenes* from pigs (46 %), small ruminants (40 %) and from dogs and cats (50 %). The *A.* (*T.*) *pyogenes* strain isolated from a rabbit did not carry gene *fim*C. The differences in the presence of *fim*C between *A.* (*T.*) *pyogenes* strains from ruminants and pigs appeared to be significant ($P = 0.011$).

Both *A.* (*T.*) *pyogenes* reference strains were positive for *fim*C and *fim*E, *A.* (*T.*) *pyogenes* was DSM 20594 positive for *fim*A **(Tab. 10)**. No cross reactivity for *fim*A, *fim*C or *fim*E could be observed with any of the control strains representing eight other species of genera *Arcanobacterium* and *Trueperella*.

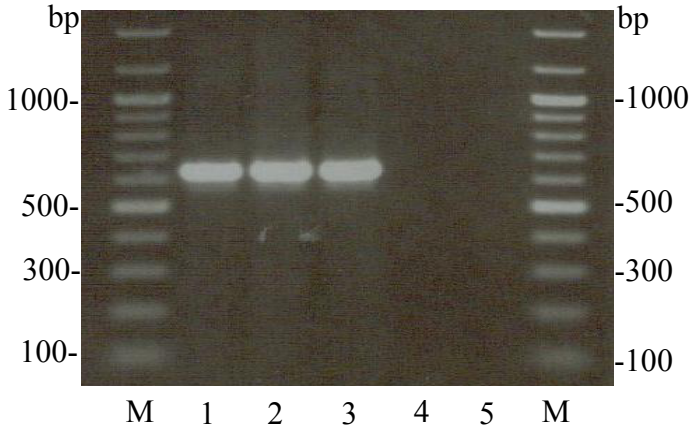


Fig. 16: Amplicons of *fim*A of *A.* (*T.*) *pyogenes* DSM 20594 (1) and the two *A.* (*T.*) *pyogenes* strains 1848/07 and P2346/09 (2 and 3, respectively) with a size of approximately 610 bp using oligonucleotide primers *fim*A-F and *fim*A-R. Negative reaction of *A.* (*T.*) *pyogenes* DSM 20630^T (4) and *A. haemolyticum* DSM 20595^T (5). M = see **Fig. 10**.

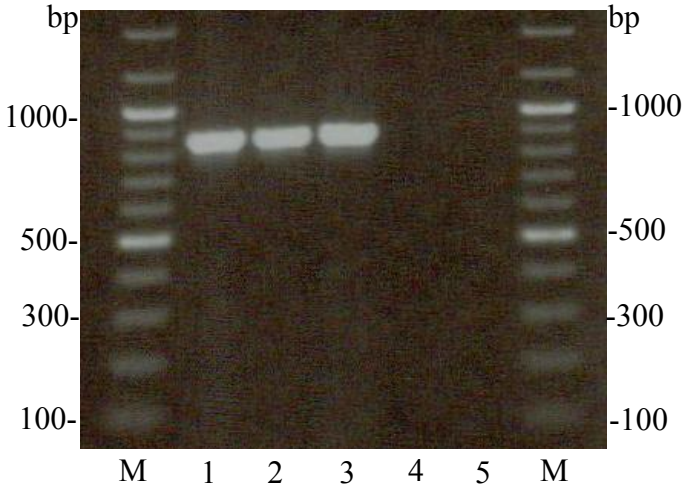


Fig. 17: Amplicons of *fim*C of *A.* (*T.*) *pyogenes* DSM 20630^T (1) and the two *A.* (*T.*) *pyogenes* strains 5939/08 and 5940/08 (2 and 3, respectively) with a size of approximately 850 bp using oligonucleotide primers *fim*C-F and *fim*C-R. Negative reaction of *A. phocae* DSM 10002^T (4) and *A. pluranimalium* DSM 13483^T (5). M = see **Fig. 10**.

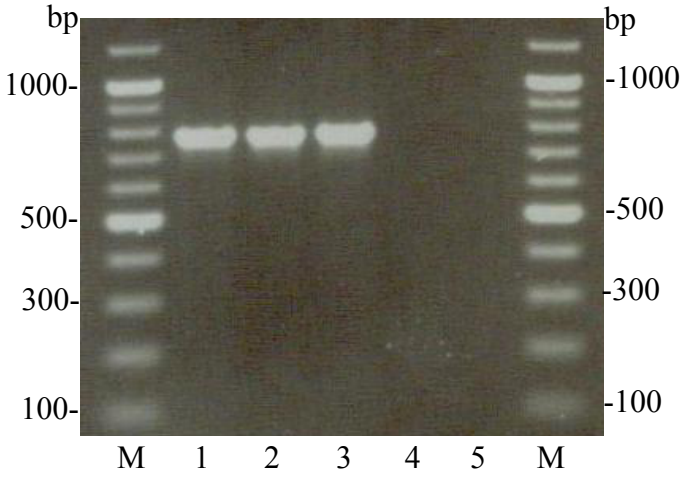


Fig. 18: Amplicons of *fim*E of *A.* (*T.*) *pyogenes* DSM 20594 (1) and the two *A.* (*T.*) *pyogenes* strains 4984/03 and 734/03 (2 and 3, respectively) with a size of approximately 780 bp using oligonucleotide primers *fim*E-F and *fim*E-R. Negative reaction of *A. hippocoleae* DSM 15539^T (4) and *A. phocae* DSM 10003 (5). M = see **Fig. 10**.

Tab. 10: PCR mediated amplification of various putative virulence factor encoding genes of *A.* (*T.*) *pyogenes*.

Origin of the *A.* (*T.*) *pyogenes* strains	Virulence factor encoding genes								Number of putative virulence genes present in all strains
	n	*plo* (704 bp)*	*cbp*A*** (327 bp)	*nan*H (781 bp)	*nan*P (150 bp)	*fim*A (605 bp)	*fim*C*** (843 bp)	*fim*E (775 bp)	
Bovine	19	19**	1	17	13	19	18	19	3
Pig	13	13	7	13	12	13	6	13	4
Small ruminants	5	5	0	4	4	5	2	5	3
Wild ruminants	5	5	0	5	4	5	5	5	5
Horse	1	1	0	1	1	1	1	1	6
Dog and cat	4	4	1	4	3	3	2	4	3
Rabbit	1	1	0	1	1	1	0	0	4
Rat	1	1	0	0	0	1	1	1	4
Reptiles	2	2	2	2	2	2	2	2	7
∑	51	51 (100 %)	11 (22 %)	47 (92 %)	44 (86 %)	50 (98 %)	37 (73 %)	50 (98 %)	
A. (*T.*) *pyogenes* DSM 20630^{T}		Positive	Positive	Positive	Positive	Negative	Positive	Positive	
A. (*T.*) *pyogenes* DSM 20594		Positive	Negative	Positive	Positive	Positive	Positive	Positive	

n = Number of strains: * = expected amplicon size; ** = number of strains with the respective property; *** = significant difference between *A.* (*T.*) *pyogenes* isolated from ruminants and pigs with $P = 0.0004$ and $P = 0.011$ for genes *cbp*A and *fim*C, respectively.

4.1.2 Phenotypic and genotypic properties of *A.* (*T.*) *abortisuis*

4.1.2.1 Phenotypic results

4.1.2.1.1 Hemolytic properties and CAMP-like hemolytic reactions

The 23 *A.* (*T.*) *abortisuis* strains and reference strain *A.* (*T.*) *abortisuis* DSM 19515^T could be cultivated under aerobic conditions, microaerobic conditions in a candle jar and under anaerobic conditions (3.2) and produced a narrow zone of complete hemolysis on sheep and rabbit blood agar **(Tab. 11)**. Cultivation of *A.* (*T.*) *abortisuis* under anaerobic conditions and under microaerobic conditions, compared to aerobic conditions, resulted in a slightly enhanced growth (data not shown).

In addition, all 23 *A.* (*T.*) *abortisuis* and *A.* (*T.*) *abortisuis* DSM 19515^T displayed CAMP-like hemolytic reactions with β-hemolytic *S. aureus, R. equi, A. haemolyticum* and *A. phocae* as indicator strains (**Fig. 19a, b; Tab. 11**).

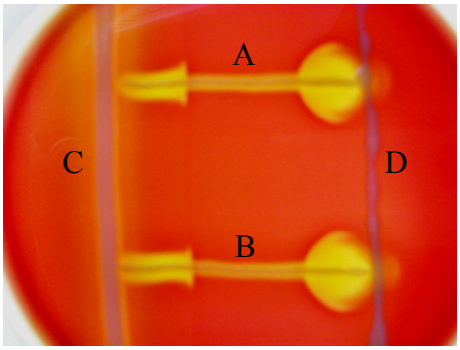


Fig. 19a: CAMP-like hemolytic reaction of *A.* (*T.*) *abortisuis* DSM 19515^T (A, horizontal) and *A.* (*T.*) *abortisuis* 1142/04 (B, horizontal) of the present study with β-hemolysin of *S. aureus* (C, vertical) and *R. equi* (D, vertical) as indicator strains.

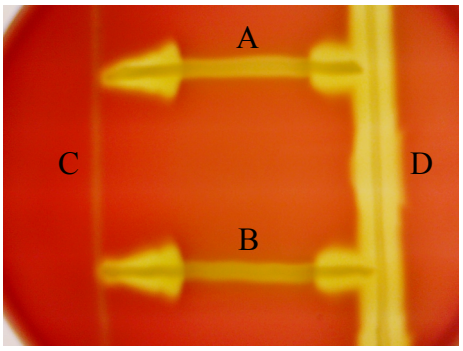


Fig. 19b: CAMP-like hemolytic reaction of *A.* (*T.*) *abortisuis* DSM 19515^T (A, horizontal) and *A.* (*T.*) *abortisuis* 837/10 (B, horizontal) of the present study with *A. haemolyticum* DSM 20595^T (C, vertical) and *A. phocae* DSM 10002^T (D, vertical) as indicator strains.

Tab. 11: Hemolytic properties after cultivation under microaerobic conditions and CAMP-like hemolytic reactions of 23 *A.* (*T.*) *abortisuis* and the *A.* (*T.*) *abortisuis* reference strain.

		***A.* (*T.*) *abortisuis* n = 23**	***A.* (*T.*) *abortisuis* DSM 19515^T**
Hemolysis on	**Sheep blood agar**	(+)* (23)	(+)
	Rabbit blood agar	(+) (23)	(+)
CAMP-like hemolysis with	***S. aureus* β-hemolysin zone**	+** (23)	+
	S. agalactiae	-	-
	R. equi	+ (23)	+
	P. phenylpyruvicus	-	-
	A. haemolyticum	+ (23)	+
	A. phocae	+ (23)	+
Reverse CAMP reaction		-	-

n = Number of strains; * = (+), +, +(+), ++ degree of hemolysis; ** = CAMP-like hemolytic reaction or reverse CAMP reaction. The number of positive strains is shown in parenthesis.

4.1.2.1.2 Characterization of *A.* (*T.*) *abortisuis* using API-Coryne-test system and other phenotypical tests

The results of the API-Coryne-test system (3.4.4), tablets containing various substrates (3.4.5), 4-methylumbelliferyl conjugated substrates (3.4.6) and from various other phenotypic tests (3.4.7 to 3.4.12 and 3.4.15) of the 23 *A.* (*T.*) *abortisuis* and *A.* (*T.*) *abortisuis* DSM 19515^T are summarized in **Tab. 12**. Using the API-Coryne-test system a seven-digit code was obtained for each strain yielding 15 numerical profiles for the 24 *A.* (*T.*) *abortisuis* strains

investigated *A.* (*T.*) (2650122 (n = 2); 2750120 (n = 1), 3650132 (n = 2); 3650133 (n = 3); 3650162 (n = 1); 3650322 (n = 1); 3650323 (n = 1); 3650361 (n = 1); 3650372 (n = 4); 3750323 (n = 1); 3750363 (n = 2); 3750373 (n = 1); 3750773 (n = 2); 7750322 (n = 1) and 2650363 for *A.* (*T.*) *abortisuis* DSM 19515^T). These numerical profiles could not be identified by API-Coryne database (version 1.2.1, 2003) (3.4.4).

All 23 *A.* (*T.*) *abortisuis* strains and *A.* (*T.*) *abortisuis* DSM 19515^T showed a positive reaction for the enzymes pyrazinamidase, β-D-glucuronidase, β-D-galactosidase, α-D-glucosidase and esculin hydrolysis (3.4.4; 3.4.6), DNase (3.4.10), amylase (3.4.12) and for degradation of D-glucose and D-maltose (3.4.4). Negative reactions were observed for the enzymes N-acetyl-β-D-glucosaminidase (3.4.4; 3.4.6), urease and gelatinase (3.4.4), α-mannosidase (3.4.5), catalase (3.4.7) and hyaluronidase (3.4.11), for serolysis of Loeffler medium (3.4.8) and for casein hydrolysis (3.4.9). Variable results could be detected for nitrate reduction (3.4.4), the enzymes pyrrolidonyl arylamidase and alkaline phosphatase (3.4.4; 3.4.5) and for degradation of D-ribose, D-xylose, D-mannitol, D-lactose, D-saccharose and glycogen (3.4.4). Enzymatic extracts of the 23 *A.* (*T.*) *abortisuis* strains and *A.* (*T.*) *abortisuis* DSM 19515^T displayed no cross reaction with streptococcal serogroup G specific antiserum (3.4.15) **(Tab. 12)**.

Tab. 12: Phenotypic properties of 23 *A.* (*T.*) *abortisuis* strains and *A.* (*T.*) *abortisuis* reference strain.

Phenotypic properties	*A.* (*T.*) *abortisuis* (n=23)	*A.* (*T.*) *abortisuis* DSM 19515^T
Nitrate reduction	+(21)[1]	-[1]
Pyrazinamidase	+(19), (+)(4)[1]	+[1]
Pyrrolidonyl arylamidase	+(1)[1]; +(2)[2]	-[1,2]
Alkaline phosphatase	+(2), (+)(6)[1]; -[2]	-[1.2]
β-Glucuronidase	+(23)[1,3]	+[1,3]
β-Galactosidase	+(23)[1,3]	+[1,3]
α-Glucosidase	+(23)[1,3]	+[1,3]
N-Acetyl-β-Glucosaminidase	-[1,3]	-[1,3]
Esculin (β-Glucosidase)	+(22), (+)(1)[1]	+[1]
Urease	-[1]	-[1]
Gelatine	-[1]	-[1]
D-Glucose	+(22), (+)(1)[1]	+[1]
D-Ribose	+(12), (+)(2)[1]	+[1]
D-Xylose	(+)(2)[1]	-[1]
D-Mannitol	+(10), (+)(1)[1]	-[1]
D-Maltose	+(21), (+)(2)[1]	+[1]
D-Lactose	+(6), (+)(4)[1]	+[1]
D-Saccharose	+(7), (+)(4)[1]	+[1]
Glycogen	+(18), (+)(3)[1]	+[1]
α-Mannosidase	-[2]	-[2]
Catalase	-	-
Serolysis on Loeffler medium	-	-
Caseinase	-	-
DNase	+(23)	+
Hyaluronidase	-	-
Amylase	+(23)	+
Cross reaction with streptococcal serogroup G specific antiserum	-	-

n = Number of strains; + = positive reaction; (+) = weak reaction; - = negative reaction. The number of positive strains is shown in parentheses. 1 = Api-Coryne-test system (3.4.4); 2 = tablets containing substrates (3.4.5); 3 = 4-methylumbelliferyl conjugated substrates (3.4.6).

4.1.2.2 Identification of *A.* (*T.*) *abortisuis* by MALDI-TOF MS

MALDI-TOF MS fingerprint analysis could also be used to successfully identify reference strain *A.* (*T.*) *abortisuis* DSM 19515^T and 22 (96 %) of the 23 *A.* (*T.*) *abortisuis* strains investigated (3.5.2). A comparison of raw spectra of *A.* (*T.*) *abortisuis* 6320/08 and *A.* (*T.*) *abortisuis* 6322/08 of bovine origin, *A.* (*T.*) *abortisuis* 1142/04 of pig origin and *A.* (*T.*) *abortisuis* DSM 19515^T is shown in **Fig. 19**. MALDI-TOF MS correctly identified 22 *A.* (*T.*) *abortisuis* strains to the

species level matching against the reference strain *A.* (*T.*) *abortisuis* DSM 19515^T of the present study with log (score) values ranging from 2.36 to 2.70. This also included both *A.* (*T.*) *abortisuis* isolates of bovine origin which matched with log (score) values of 2.53 and 2.40, respectively **(Fig. 20)**. A comparison of the log (score) values of the 22 strains among each other revealed the lowest log (score) value of 2.14 of *A.* (*T.*) *abortisuis* strain 6320/08 of bovine origin to *A.* (*T.*) *abortisuis* strain 1672/02 of pig origin to a maximal score of 2.83 of *A.* (*T.*) *abortisuis* strain 341/10 to *A.* (*T.*) *abortisuis* strain 343/10, both isolated from two different pigs of the same farm. Moreover, both strains of bovine origin (*A.* (*T.*) *abortisuis* 6320/08 and *A.* (*T.*) *abortisuis* 6322/08) isolated from two different animals of the same farm matched in MALDI Biotyper analysis to each other with a log (score) value of 2.83. A similarity matrix of the MSPs of *A.* (*T.*) *abortisuis* DSM 19515^T and ten selected *A.* (*T.*) *abortisuis* strains of the present study among each other is shown in **Tab. 13**. *A.* (*T.*) *abortisuis* strain P8609/00 resulted in a log (score) value of 1.77 **(Fig. 20, Tab. 13)**. This strain could be identified to genus level. A dendrogram analysis of MALDI-TOF main spectra of all 23 *A.* (*T.*) *abortisuis* strains investigated in the present study and eleven reference strains of genera *Arcanobacterium* and *Trueperella* is shown in **Fig. 21**.

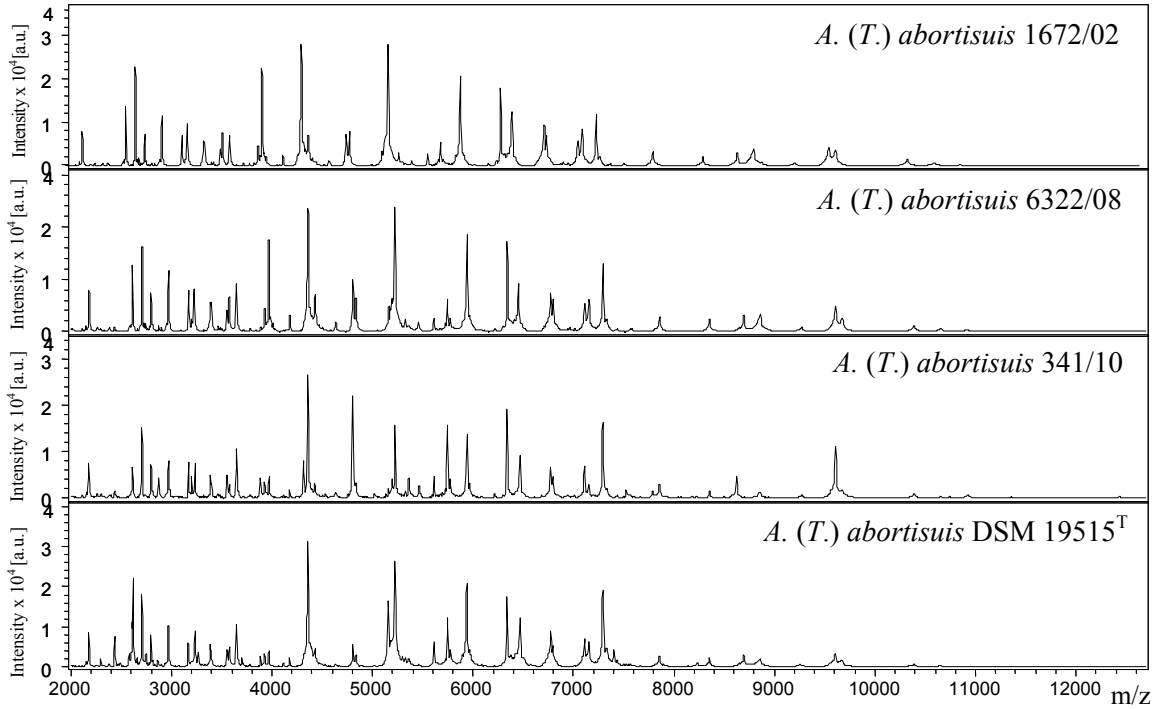


Fig. 19: Typical MALDI-TOF raw spectra of whole-cell extracts of four *A.* (*T.*) *abortisuis* strains in the range from 2,000 to 12,600 Da. The relative intensities of the ions are shown on the *y* axis, the mass to charge ratios are shown on the *x* axis. The patterns display the overall similarities among these *A. abortisuis* strains.

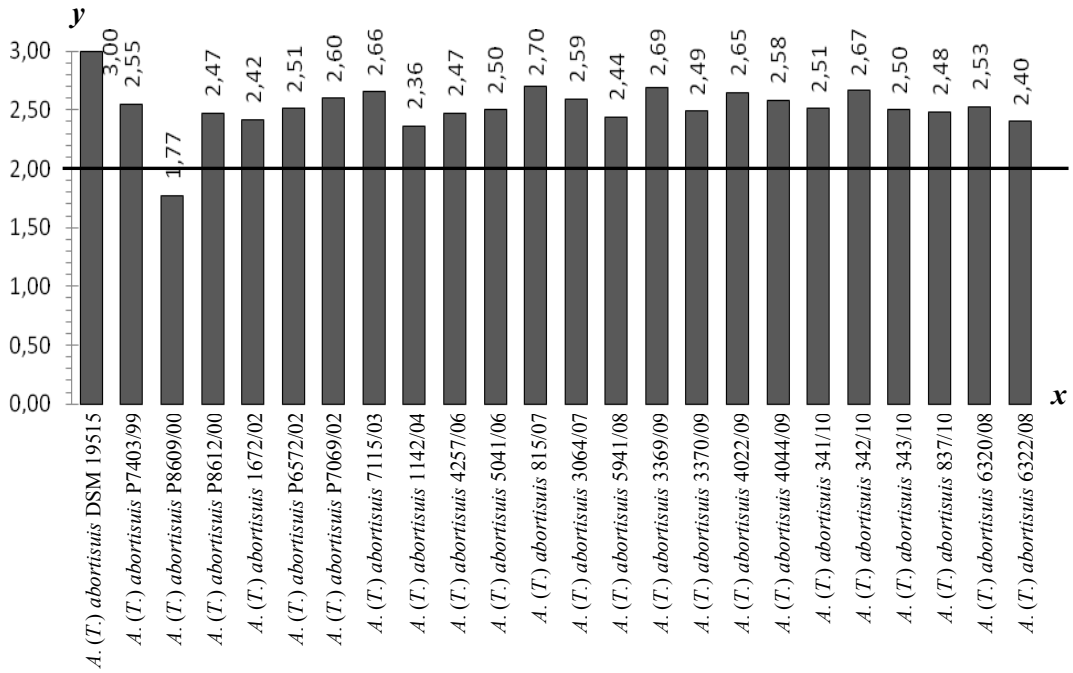


Fig. 20: Log (score) values (*y* axis) of 23 *A.* (*T.*) *abortisuis* (*x* axis) matching against the reference strain *A.* (*T.*) *abortisuis* DSM 19515^{T} of the present study. Log (score) values ≥ 2.00 are rated as identification of bacteria at species level.

Tab. 13: Typical log (score) value matrix of *A.* (*T.*) *abortisuis* DSM 19515^{T} and ten *A.* (*T.*) *abortisuis* of the present study among each other.

A. abortisuis strains	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)
(1) *A.* (*T.*) *abortisuis* DSM 19515^{T}	3	1.77*	2.42	2.60	2.50	2.44	2.51	2.67	2.50	2.53	2.40
(2) *A.* (*T.*) *abortisuis* P8609/00*		3	1.65	1.86	1.82	1.37	1.65	1.85	1.53	1.50	1.66
(3) *A.* (*T.*) *abortisuis* 1672/02			3	2.40	2.60	2.61	2.60	2.43	2.52	2.14	2.32
(4) *A.* (*T.*) *abortisuis* P7069/02				3	2.55	2.44	2.50	2.70	2.50	2.50	2.50
(5) *A.* (*T.*) *abortisuis* 5041/06					3	2.52	2.75	2.57	2.73	2.39	2.50
(6) *A.* (*T.*) *abortisuis* 5941/08						3	2.48	2.45	2.50	2.36	2.47
(7) *A.* (*T.*) *abortisuis* 341/10							3	2.66	2.83	2.45	2.50
(8) *A.* (*T.*) *abortisuis* 342/10								3	2.62	2.53	2.55
(9) *A.* (*T.*) *abortisuis* 343/10									3	2.37	2.46
(10) *A.* (*T.*) *abortisuis* 6320/08										3	2.83
(11) *A.* (*T.*) *abortisuis* 6322/08											3

* = identified to genus level.

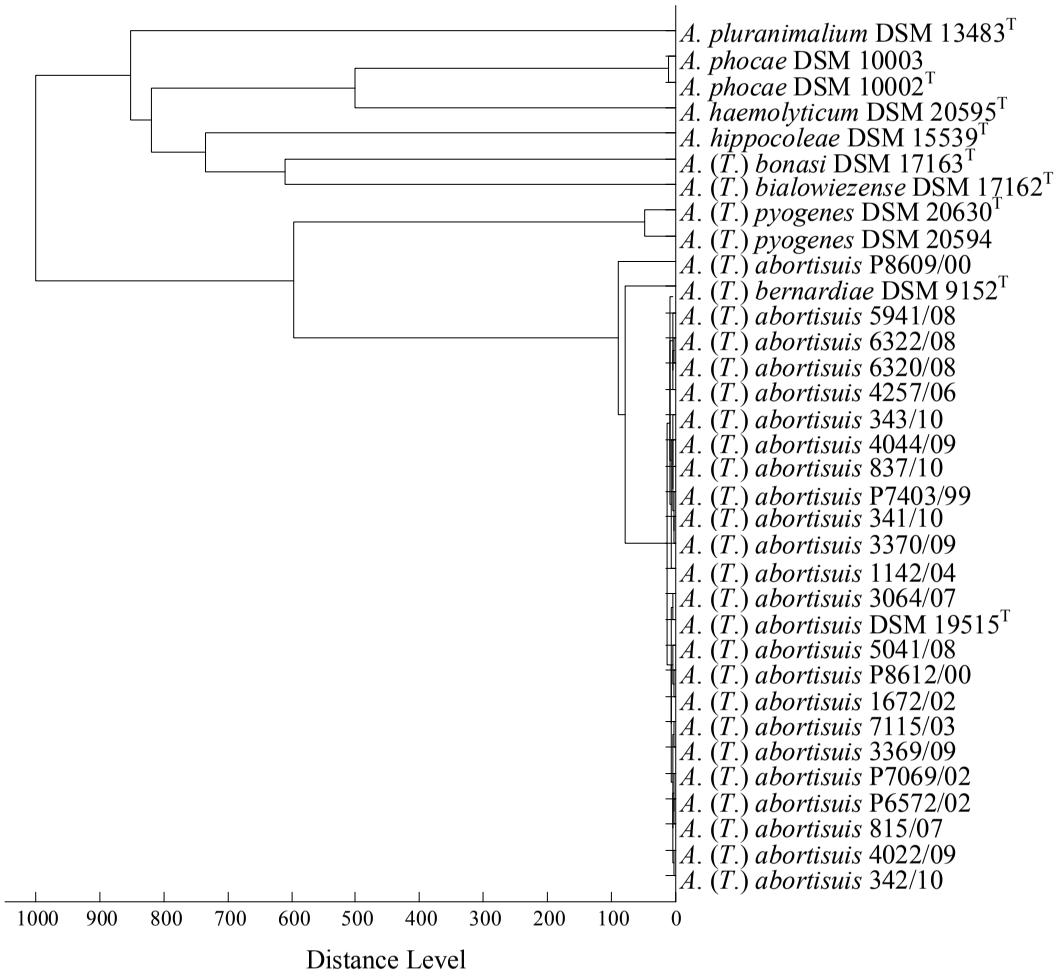


Fig. 21: A score oriented dendrogram of MALDI-TOF main spectra profiles of 23 *A.* (*T.*) *abortisuis* strains and eleven reference strains of genera *Arcanobacterium* and *Trueperella* also including *A.* (*T.*) *abortisuis* DSM 19515^T. For generating of the dendrogram see **Fig. 6**.

4.1.2.3 PCR-mediated identification of *A.* (*T.*) *abortisuis*

4.1.2.3.1 Amplification and sequencing of 16S rDNA

As mentioned in 4.1.2.2 MALDI-TOF MS analysis of *A.* (*T.*) *abortisuis* P8609/00 yielded a log (score) value of 1.77. This strain could not be identified to species level but to genus level. To verify that this strain belongs to the species *A.* (*T.*) *abortisuis* the 16S rDNA was amplified and sequenced. Using the oligonucleotide primers 16SUNI-L and 16SUNI-R described in 3.7.7 an amplicon with approximate size of 1400 bp (1403 bp expected size) could be detected for this strain (data not shown). Sequencing the 16S rRNA gene of *A.* (*T.*) *abortisuis* P8609/00 (3.6.7) revealed a continuous stretch of 1146 bp (GenBank accession number FR837910) with a sequence homology of 99.7 % to 16S rDNA of *A.* (*T.*) *abortisuis* reference strain DSM 19515^{T} (accession number AB305159); (**Fig. 22**). A dendrogram analysis of the 16S rDNA sequences of *A.* (*T.*) *abortisuis* P8609/00 and *A.* (*T.*) *abortisuis* DSM 19515^{T} together with eight reference strains of genera *Arcanobacterium* and *Trueperella* is shown in **Fig. 23**.

```
                                  121                                                 180
A. (T.) abortisuis P8609/00       CCTGCCCTTGTCTTTGGGATAAGCCTGGGAAACTGGGTCTAATACTGGATATTCTGCTTC
A. (T.) abortisuis DSM 19515T     CCTGCCCTTGTCTTTGGGATAAGCCTGGGAAACTGGGTCTAATACCGGATATTCTGCTTC
                                  181                                                 240
A. (T.) abortisuis P8609/00       TGCCGCATGGTGGGGGTTGGAAAGATTTTTTGGATGGGGATGGGCTCACGGCCTATCAGC
A. (T.) abortisuis DSM 19515T     TGCCGCATGGTGGGGGTTGGAAAGATTTTTTGGATGGGGATGGGCTCACGGCCTATCAGC
                                  241                                                 300
A. (T.) abortisuis P8609/00       TTGTTGGTGGGGTGATGGCCTACCAAGGCGTCGACGGGTAGCCGGCCTGAGAGGGTGACC
A. (T.) abortisuis DSM 19515T     TTGTTGGTGGGGTGATGGCCTACCAAGGCGTCGACGGGTAGCCGGCCTGAGAGGGTGACC
                                  301                                                 360
A. (T.) abortisuis P8609/00       GGCCACATTGGGACTGAGATACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATT
A. (T.) abortisuis DSM 19515T     GGCCACATTGGGACTGAGATACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATT
                                  361                                                 420
A. (T.) abortisuis P8609/00       GCACAATGGACGCAAGTCTGATGCAGCGACGCCGCGTGGGGGATGAAGGCTTTCGGGTTG
A. (T.) abortisuis DSM 19515T     GCACAATGGACGCAAGTCTGATGCAGCGACGCCGCGTGGGGGATGAAGGCTTTCGGGTTG
                                  421                                                 480
A. (T.) abortisuis P8609/00       TAAACTCCTTTCAGTACAGAACAAGGCCTTTTTGGTTGAGGGTATGTGCAGAAGAAGCGC
A. (T.) abortisuis DSM 19515T     TAAACTCCTTTCAGTACAGAACAAGGCCTTTGTGGTTGAGGGTATGTGCAGAAGAAGCGC
                                  481                                                 540
A. (T.) abortisuis P8609/00       CGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCGAGCGTTGTCCGGAATTA
A. (T.) abortisuis DSM 19515T     CGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCGAGCGTTGTCCGGAATTA
                                  541                                                 600
A. (T.) abortisuis P8609/00       TTGGGCGTAAAGAGCTCGTAGGCGGTTTGTTGCGCCTGCTGTGAAAGACCGGGGCTTAAC
A. (T.) abortisuis DSM 19515T     TTGGGCGTAAAGAGCTCGTAGGCGGTTTGTTGCGCCTGCTGTGAAAGACCGGGGCTTAAC
                                  601                                                 660
A. (T.) abortisuis P8609/00       TTCGGGGTTGCAGTGGGTACGGGCAGACTAGAGTGTGGTAGGGGTAATTGGAATTCCTGG
A. (T.) abortisuis DSM 19515T     TTCGGGGTTGCAGTGGGTACGGGCAGACTAGAGTGTGGTAGGGGTAATTGGAATTCCTGG
                                  661                                                 720
A. (T.) abortisuis P8609/00       TGTAGCGGTGGAATGCGCAGATATCAGGAGGAACACCGATGGC-GAAGGCAGGTTACTGG
A. (T.) abortisuis DSM 19515T     TGTAGCGGTGGAATGCGCAGATATCAGGAGGAACACCGATGGCCGAAGGCAGGTTACTGG
                                  721                                                 780
A. (T.) abortisuis P8609/00       GCCATTACTGACGCTGAGGAGCGAAAGCGTGGGTAGCGAACAGGATTAGATACCCTGGTA
A. (T.) abortisuis DSM 19515T     GCCATTACTGACGCTGAGGAGCGAAAGCGTGGGTAGCGAACAGGATTAGATACCCTGGTA
```

```
                                781                                                 840
A. (T.) abortisuis P8609/00     GTCCACGCCGTAAACGTTGGGCACTAGGTGTGGGGCCTTTTCCATGGGTTCTGCGCCGTA
A. (T.) abortisuis DSM 19515T   GTCCACGCCGTAAACGTTGGGCACTAGGTGTGGGGCCTTTTCCATGGGTTCTGCGCCGTA
                                841                                                 900
A. (T.) abortisuis P8609/00     GCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAAT
A. (T.) abortisuis DSM 19515T   GCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAAT
                                901                                                 960
A. (T.) abortisuis P8609/00     TGACGGGGGCCCGCACAAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACC
A. (T.) abortisuis DSM 19515T   TGACGGGGGCCCGCACAAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACC
                                961                                                 1020
A. (T.) abortisuis P8609/00     TTACCAAGGCTTGACATACACTGCGATGTGCCAGAGATGGTGCAGCCTTCGGGGTGGTGT
A. (T.) abortisuis DSM 19515T   TTACCAAGGCTTGACATACACTGCGATGTGCCAGAGATGGTGCAGCCTTTGGGGTGGTGT
                                1021                                                1080
A. (T.) abortisuis P8609/00     ACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAAC
A. (T.) abortisuis DSM 19515T   ACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAAC
                                1081                                                1140
A. (T.) abortisuis P8609/00     GAGCGCAACCCTTGTCCTGTGTTGCCAGCAAGTTGTGTTGGGGACTCACGGGAGACTGCC
A. (T.) abortisuis DSM 19515T   GAGCGCAACCCTTGTCCTGTGTTGCCAGCAAGTTGTGTTGGGGACTCACGGGAGACTGCC
                                1141                                                1200
A. (T.) abortisuis P8609/00     GGGGTTAACTCGGAGGAAGGTGGGGAACGTCAAATCATCATGCCCCTTATGTCTTGGG
A. (T.) abortisuis DSM 19515T   GGGGTTAACTCGGAGGAAGGTGGGGAACGTCAAATCATCATGCCCCTTATGTCTTGGG
```

Fig. 22: Sequence alignment of 16S rDNA (partial sequence) of *A.* (*T.*) *abortisuis* P6809/00 and *A.* (*T.*) *abortisuis* reference strain DSM 19515^T using Clustal W method (3.6.6). The differences are marked.

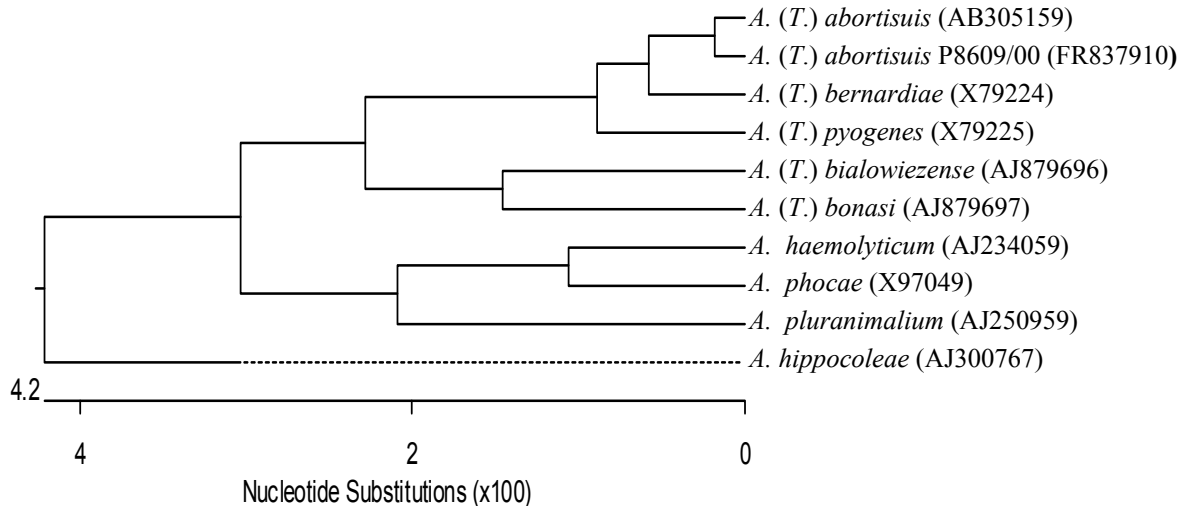


Fig. 23: Dendrogram analysis of 16S rDNA sequences of *A.* (*T.*) *abortisuis* P6809/00 of the present study and nine reference strains of genera *Arcanobacterium* and *Trueperella* obtained from NCBI GenBank using Clustal W method (3.6.6).

4.1.2.3.2 Amplification of *A.* (*T.*) *abortisuis* ISR specific parts

The amplification of *A.* (*T.*) *abortisuis* ISR specific parts was performed using the oligonucleotide primers Aab-F and Aab-R described in 3.6.11.4. With these oligonucleotide primers an amplicon with an approximate size of 270 bp (271 bp expected size) could be

detected for all 23 investigated *A.* (*T.*) *abortisuis* strains and for *A.* (*T.*) *abortisuis* reference strain DSM 19515^T **(Fig. 24)**. No cross reactivity could be observed with any of the control strains representing eight other species of genera *Arcanobacterium* and *Trueperella*.

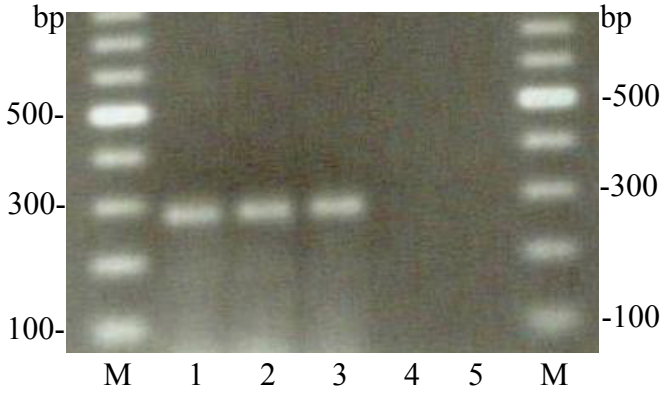


Fig. 24: Typical amplicons of *A.* (*T.*) *abortisuis* (1, 2 and 3) with a size of approximately 270 bp using the *A.* (*T.*) *abortisuis* ISR specific oligonucleotide primers Aab-F and Aab-R. *A.* (*T.*) *pyogenes* DSM 20630^T (4) and *A.* (*T.*) *bernardiae* DSM 9152^T (5) served as negative control. M = see **Fig. 10**.

4.1.3 Phenotypic and genotypic properties of *A. haemolyticum*

4.1.3.1 Phenotypic results

4.1.3.1.1 Hemolytic properties and CAMP-like hemolytic reactions

The three *A. haemolyticum* strains and *A. haemolyticum* reference strain DSM 20595^T produced after cultivation under microaerobic conditions in a candle jar (3.2) a narrow zone of complete hemolysis on sheep blood agar. After cultivation on rabbit blood agar all three *A. haemolyticum* and the *A. haemolyticum* DSM 20595^T displayed an enhanced hemolysis compared to sheep blood agar **(Fig. 25; Tab. 14)**.

Moreover, all three *A. haemolyticum* strains and *A. haemolyticum* DSM 20595^T displayed CAMP-like hemolytic reactions with *S. agalactiae, R. equi, P. phenylpyruvicus, A.* (*T.*) *pyogenes*, *A.* (*T.*) *abortisuis*, *A. hippocoleae* and *A. pluranimalium* as indicator strains, and a reverse CAMP reaction in the zone of *S. aureus* β-hemolysin (**Fig. 26a, b; Tab. 14**).

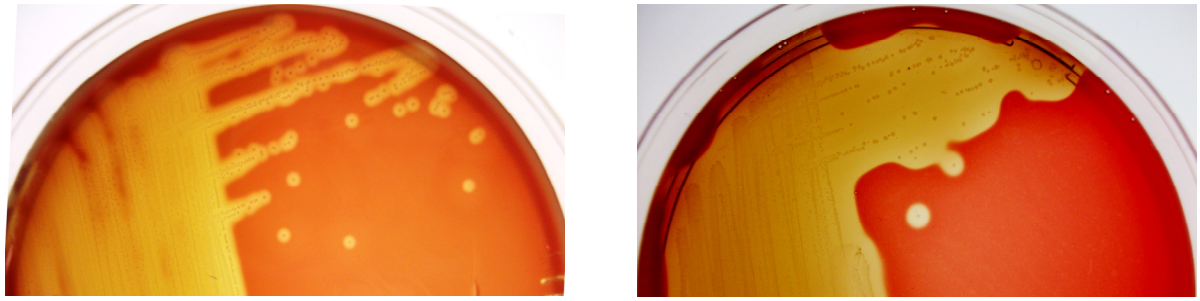


Fig. 25: Hemolysis of *A. haemolyticum* 2289/09 after cultivation on sheep blood agar (A); enhanced hemolysis after cultivation on rabbit blood agar (B).

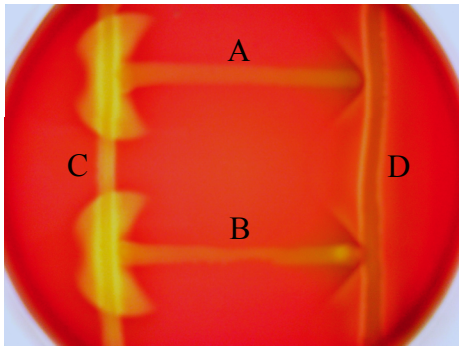


Fig. 26a: CAMP-like hemolytic reaction of *A. haemolyticum* 2289/09 (A, horizontal) and P5648/10 (B, horizontal) of the present study with *S. agalactiae* (C, vertical) as indicator strain and reverse CAMP-like reaction with β-hemolysin of *S. aureus* (D, vertical).

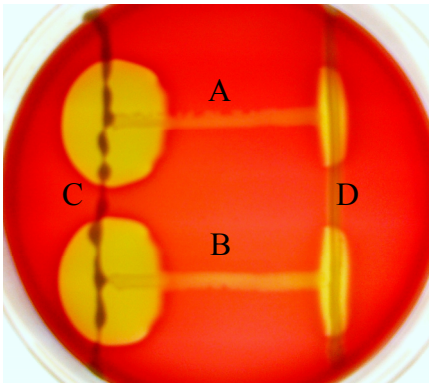


Fig. 26b: CAMP-like hemolytic reaction of *A. haemolyticum* 2289/09 (A, horizontal) and P564810 (B, horizontal) of the present study with *R. equi* (C, vertical) and *P. phenylpyruvicus* (D, vertical) as indicator strains.

Tab. 14: Hemolytic properties, CAMP-like hemolytic reactions and reverse CAMP reaction of three *A. haemolyticum* strains and *A. haemolyticum* reference strain.

		A. haemolyticum n = 3	*A. haemolyticum* DSM 20595^T
Hemolysis on	Sheep blood agar	+* (3)	(+)
	Rabbit blood agar	++ (3)	++
CAMP-like hemolysis with	*S. aureus* β-hemolysin zone	-**	-
	S. agalactiae	+ (3)	+
	R. equi	+ (3)	+
	P. phenylpyruvicus	+ (3)	+
	A. (*T.*) *pyogenes*	+ (3)	+
	A. (*T.*) *abortisuis*	+ (3)	+
	A. hippocoleae	+ (3)	+
	A. pluranimalium	+ (3)	+
Reverse CAMP reaction		+ (3)	+

n= Number of strains; * = (+), +, +(+), ++ degree of hemolysis; ** = CAMP-like hemolytic reaction or reverse CAMP reactions. The number of positive strains is shown in parenthesis.

4.1.3.1.2 Characterization of *A. haemolyticum* using API-Coryne-test system and other phenotypical tests

The results of the API-Coryne-test system (3.4.4), tablets containing various substrates (3.4.5), 4-methylumbelliferyl conjugated substrates (3.4.6) and from various other phenotypic tests (3.4.7 to 3.4.12 and 3.4.15) of the three *A. haemolyticum* and *A. haemolyticum* DSM 20595^T are summarized in **Tab. 15**. Using the API-Coryne-test system a seven-digit code was obtained for each strain yielding 3 numerical profiles (6530360 (n = 1); 6730360 (n = 2) and 2530360 for *A. haemolyticum* DSM 20595^T). Using API-Coryne database (3.4.4) these numerical profiles allowed identification (99.9 %) of all four strains as *A. haemolyticum.*

All three investigated *A. haemolyticum* strains and *A. haemolyticum* DSM 20595^T showed a positive reaction for the enzymes pyrazinamidase, β-D-galactosidase, α-D-glucosidase, N-acetyl-β-D-glucosaminidase (3.4.4; 3.4.6), α-mannosidase (3.4.5) and DNase (3.4.10), and for degradation of D-glucose, D-ribose, D-maltose and D-lactose (3.4.4). Negative reactions were observed for nitrate reduction, esculin hydrolysis, urease, gelatinase (3.4.4), catalase (3.4.7) and for hyaluronidase (3.4.11), and for degradation of D-xylose, D-

mannitol, D-saccharose and glycogen (3.4.4). Variable results could be detected for the enzymes pyrrolidonyl arylamidase, alkaline phosphatase and β-D-glucuronidase (3.4.4; 3.4.5; 3.4.6), and for serolysis of Loeffler medium (3.4.8), casein hydrolysis (3.4.9) and amylase (3.4.12). Enzymatic extracts of the three *A. haemolyticum* strains and *A. haemolyticum* reference strain displayed no cross reaction with streptococcal serogroup G specific antiserum (3.4.15) **(Tab. 15)**.

Tab. 15: Phenotypic properties of three *A. haemolyticum* strains and *A. haemolyticum* reference strain.

Phenotypic properties	*A. haemolyticum* 2289/09	*A. haemolyticum* P5648/10	*A. haemolyticum* P579/11	*A. haemolyticum* DSM 20595^T
Nitrate reduction	-[1]	-[1]	-[1]	-[1]
Pyrazinamidase	(+)[1]	+[1]	+[1]	+[1]
Pyrrolidonyl arylamidase	(+)[1],+[2]	+[1,2]	+[1,2]	-[1, 2]
Alkaline phosphatase	+[1,2]	+[1,2]	+[1,2]	+[1],- [2]
β-Glucuronidase	-[1],+[3]	(+)[1],+[3]	+[1],+[3]	-[1, 3]
β-Galactosidase	+[1,3]	+[1,3]	+[1,3]	+[1, 3]
α-Glucosidase	+[1,3]	+[1,3]	+[1,3]	+ [1,3]
N-Acetyl-β-glucosaminidase	+[1,3]	+[1,3]	+[1,3]	+[1, 3]
Esculin (β-Glucosidase)	-[1]	-[1]	-[1]	-[1]
Urease	-[1]	-[1]	-[1]	-[1]
Gelatine	-[1]	-[1]	-[1]	-[1]
D-Glucose	+[1]	+[1]	+[1]	+[1]
D-Ribose	+[1]	+[1]	+[1]	+[1]
D-Xylose	-[1]	-[1]	-[1]	-[1]
D-Mannitol	-[1]	-[1]	-[1]	-[1]
D-Maltose	+[1]	+[1]	+[1]	+[1]
D-Lactose	+[1]	+[1]	+[1]	+[1]
D-Saccharose	-[1]	-[1]	-[1]	-[1]
Glycogen	-[1]	-[1]	-[1]	-[1]
Catalase	-	-	-	-
α-Mannosidase	+[2]	+[2]	+[2]	+[2]
Serolysis on Loeffler medium	(+)	(+)	-	-
Casein hydrolysis	(+)	(+)	-	-
DNase	+	+	+	+
Hyaluronidase	-	-	-	-
Amylase	+	+	+	-
Cross reaction with streptococcal serogroup G specific antiserum	-	-	-	-

+ = Positive reaction; (+) = weak reaction; - = negative reaction. The number of positive strains is shown in parenthesis. 1 = Api-Coryne test system (3.4.4); 2 = tablets containing substrates (3.4.5); 3 = 4-methylumbelliferyl conjugated substrates (3.4.6).

4.1.3.2 Identification of *A. haemolyticum* using MALDI-TOF MS

MALDI-TOF MS analysis also allowed an identification of all three *A. haemolyticum* strains of the present study and *A. haemolyticum* reference strain DSM 20595^T. A comparison of raw spectra of the three *A. haemolyticum* strains and *A. haemolyticum* DSM 20595^T revealed minor differences in the peak pattern of the raw spectra **(Fig. 27)** also reflected by high log (score) values of the strains among each other.

MALDI-TOF MS correctly identified the three *A. haemolyticum* strains to species level matching against reference library entries (3.5.2) and reference strain *A. haemolyticum* DSM 20595^T of the present study with log (score) values of 2.44, 2.43 and 2.41 for *A. haemolyticum* 2289/09, *A. haemolyticum* P5648/10 and *A. haemolyticum* P579/11, respectively. A comparison of the log (score) values of the three *A. haemolyticum* strains to each other revealed score values between 2.60 to 2.70. A dendrogram analysis of MALDI-TOF main spectra of the three investigated strains is shown in **Fig. 28.**

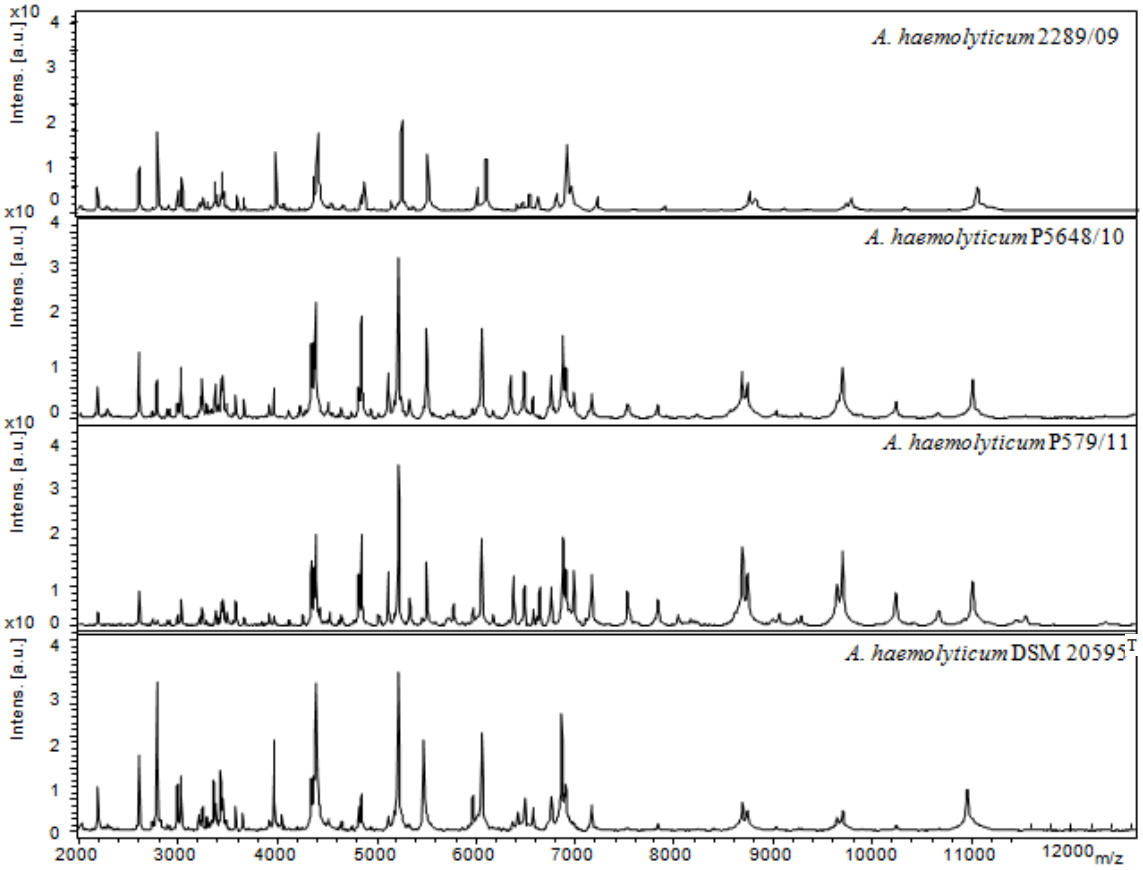


Fig. 27: Typical MALDI-TOF raw spectra of whole-cell extracts of four *A. haemolyticum* strains in the range from 2,000 to 12,600 Da. The relative intensities of the ions are shown on the *y* axis, the mass to charge ratios are shown on the *x* axis. The patterns display the overall similarities among the *A. haemolyticum* strains.

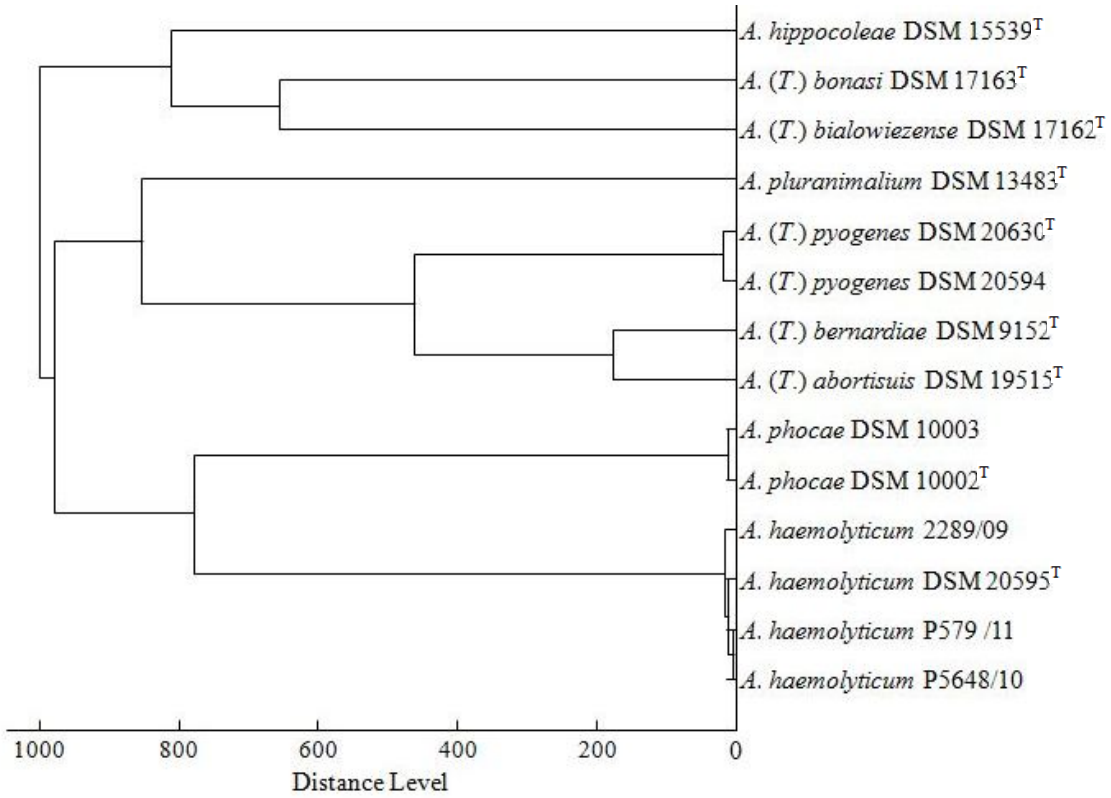


Fig. 28: A score oriented dendrogram of MALDI-TOF main spectra profiles of the three *A. haemolyticum* strains with eleven reference strains of genera *Arcanobacterium* and *Trueperella*, also including *A. haemolyticum* DSM 20595^T. For generating of the dendrogram see **Fig. 6**.

4.1.3.3 PCR-mediated identification of *A. haemolyticum*

4.1.3.3.1 Amplification and sequencing of the ISR

The amplification of the ISR of three *A. haemolyticum* strains 2289/09, P5648/10 and P579/11 was carried out using the universal oligonucleotide primers b and c described in 3.6.8 with a PCR product of approximate 600 bp amplicon size **(Fig. 29)**. The ISR sequences of the three *A. haemolyticum* strains (accession numbers FN551181, FR745891 and FR822748, respectively) yielded an almost complete identity ranging from 99.0 % to 99.8 % to the corresponding sequence of the *A. haemolyticum* DSM 20595^T obtained from GenBank (accession number EU194564), and a sequence homology ranging from 99.1 % to 100 % among each other. A dendrogram analysis of the ISR sequences of the three *A. haemolyticum* strains together with nine reference strains of genera *Arcanobacterium* and *Trueperella*, also including *A. haemolyticum* DSM 20595^T is shown in **Fig. 30**.

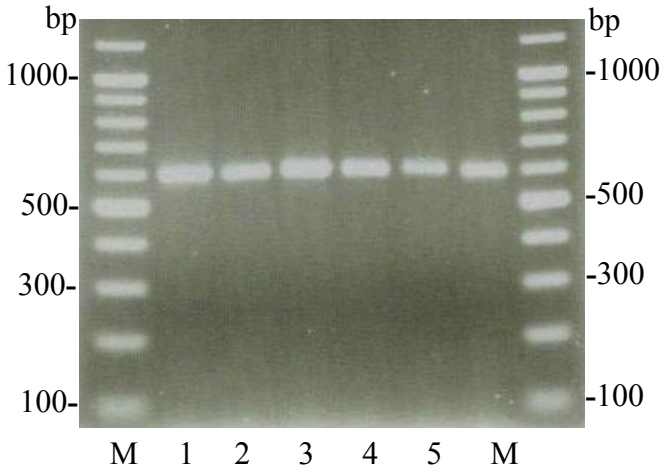


Fig. 29: Typical ISR amplicons of the three *A. haemolyticum* strains 2289/09, P5648/10 and P579/1119515 of the present study (1, 2 and 3, respectively), *A. haemolyticum* DSM 20595^T (4), *A. bonasi* DSM 17163^T (5) and *A. hippocoleae* DSM 15539^T (6) with a size of approximate 600 bp using the universal oligonucleotide primers b and c. M = see **Fig. 10**.

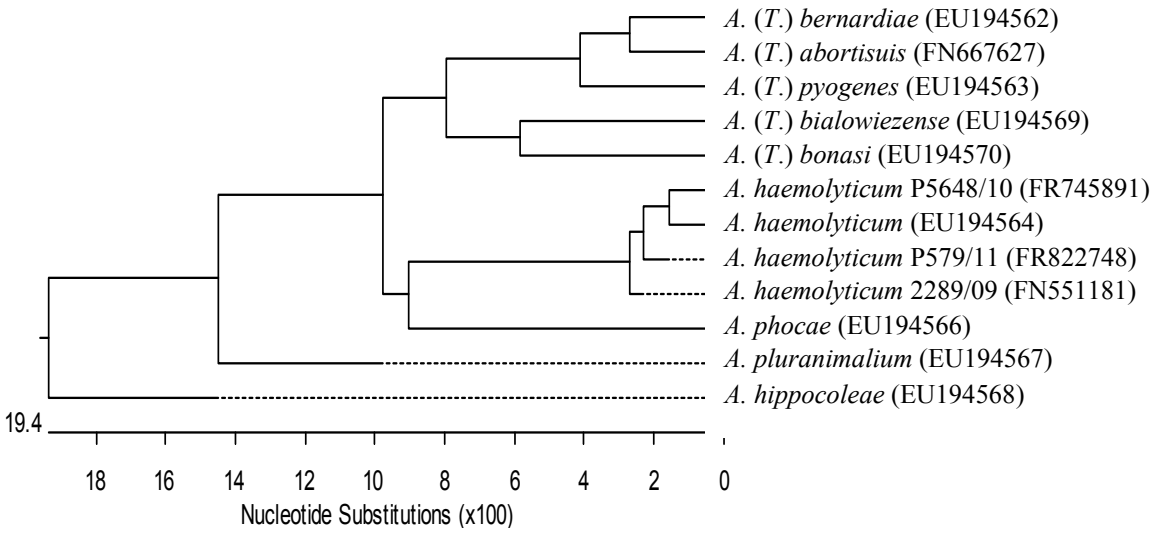


Fig. 30: Dendrogram analysis of ISR of the three *A. haemolyticum* strains 2289/09, P5648/10 and P579/11 of the present study and nine reference strains of genera *Arcanobacterium* and *Trueperella* obtained from NCBI GenBank using Clustal W method (3.6.6).

4.1.3.3.2 PCR-mediated identification of *A. haemolyticum* by amplification of various species specific parts

4.1.3.3.2.1 Amplification of *A. haemolyticum* ISR-23S rDNA

The amplification of *A. haemolyticum* ISR-23S rDNA specific parts was performed using the oligonucleotide primers Aha-ISR-F and Aha-23S-R described in 3.6.11.5. Using these oligonucleotide primers an amplicon with an approximate size of 350 bp (345 bp expected size) could be detected for all three investigated *A. haemolyticum* strains and for *A. haemolyticum* DSM 20595^T **(Fig. 31).** No cross reactivity could be observed with any of the control strains representing eight other species of genera *Arcanobacterium* and *Trueperella*.

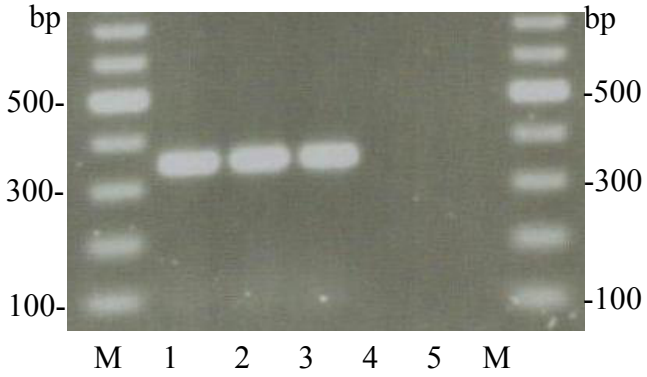


Fig. 31: Typical amplicons of *A. haemolyticum* (1, 2 and 3) with a size of approximately 350 bp using *A. haemolyticum* ISR-23S rDNA specific oligonucleotide primers Aha-ISR-F and Aha-23S-R. *A. hippocoleae* DSM 15539^T (4) and *A. phocae* DSM 10002^T (5) served as negative control. M = see **Fig. 10**.

4.1.3.3.2.2 Amplification of *A. haemolyticum* gene *pld*

The amplification of *A. haemolyticum* gene *pld* could be performed by using the oligonucleotide primers Ah-F and Ah-R described in 3.6.11.6. With these oligonucleotide primers an amplicon with an approximate size of 530 bp (528 bp expected size) was detected for all three investigated *A. haemolyticum* strains and *A. haemolyticum* DSM 20595^T **(Fig. 32)**. No cross reactivity could be observed with any of the control strains representing eight other species of genera *Arcanobacterium* and *Trueperella*.

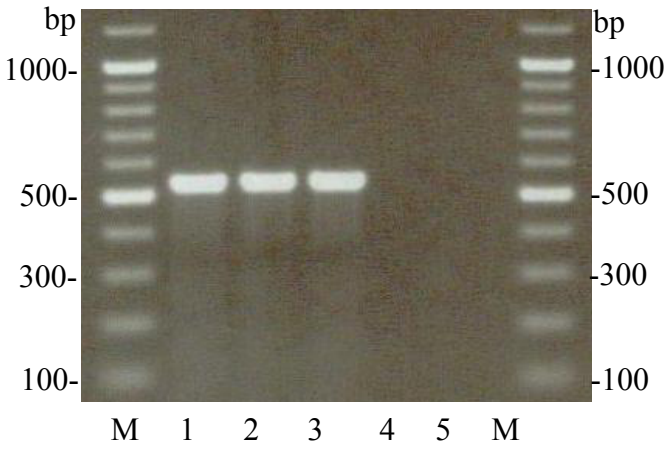


Fig. 32: Typical amplicons of *A. haemolyticum* (1, 2 and 3) with a size of approximately 530 bp using *A. haemolyticum pld* specific oligonucleotide primers Ah-F and Ah-R. *A. phocae* DSM 10003 (4) and *A. pluranimalium* DSM 13483^T (5) served as negative control. M = see **Fig. 10**.

4.1.3.3.2.3 Amplification of *A. haemolyticum* gene *cfa*

The amplification of *A. haemolyticum* gene *cfa* was performed using the oligonucleotide primers camp-F and camp-R described in 3.6.11.7. Using these oligonucleotide primers an amplicon with an approximate size of 650 bp (650 bp expected size) could be detected for all three investigated *A. haemolyticum* strains and *A. haemolyticum* DSM 20595^T **(Fig. 33).** No cross reactivity could be observed with any of the control strains representing eight other species of genera *Arcanobacterium* and *Trueperella*.

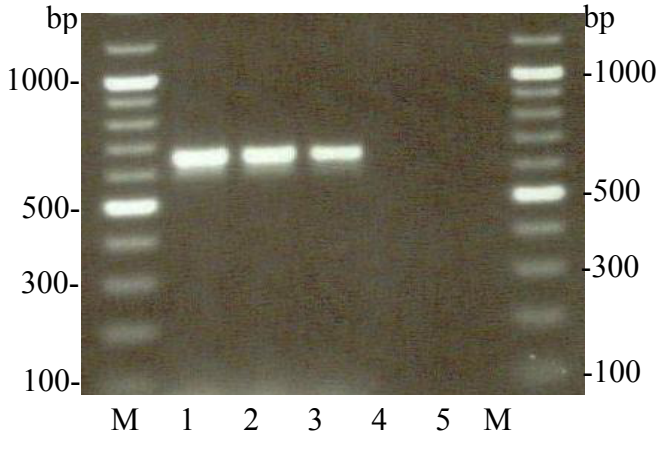
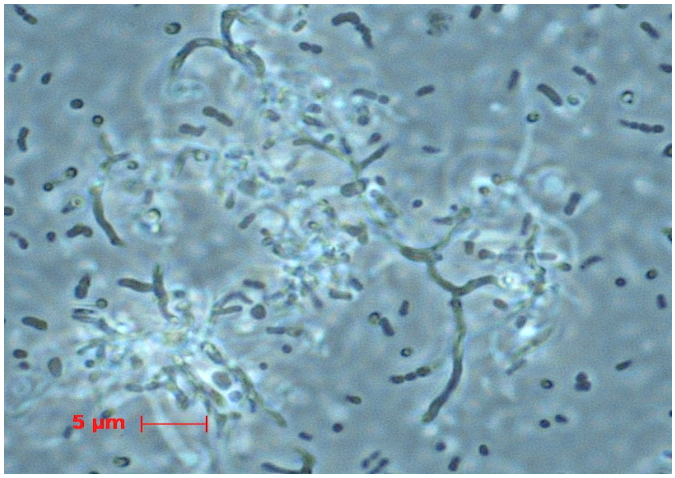


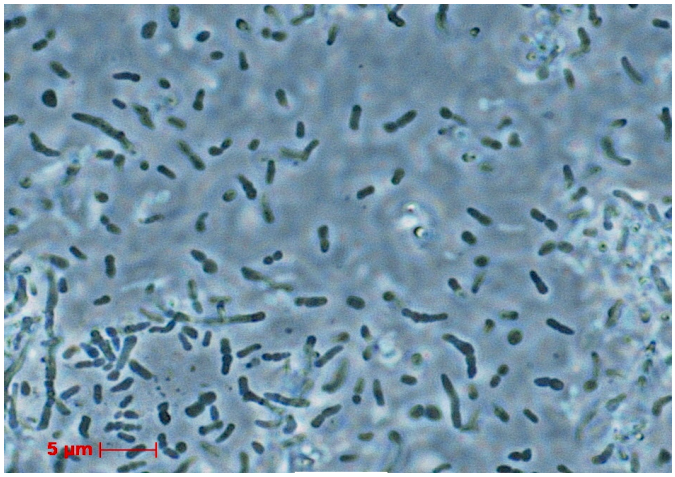
Fig. 33: Typical amplicons of *A. haemolyticum* (1, 2 and 3) with a size of approximately 650 bp using the *A. haemolyticum cfa* gene specific oligonucleotide primers camp-F and camp-R. *A.* (*T.*) *bialowiezense* DSM 17162^T (4) and *A.* (*T.*) *bonasi* DSM 17163^T (5) served as negative control. M = see **Fig. 10**.

4.1.4 Identification and further characterization of *Actinomyces weissii* sp. nov.

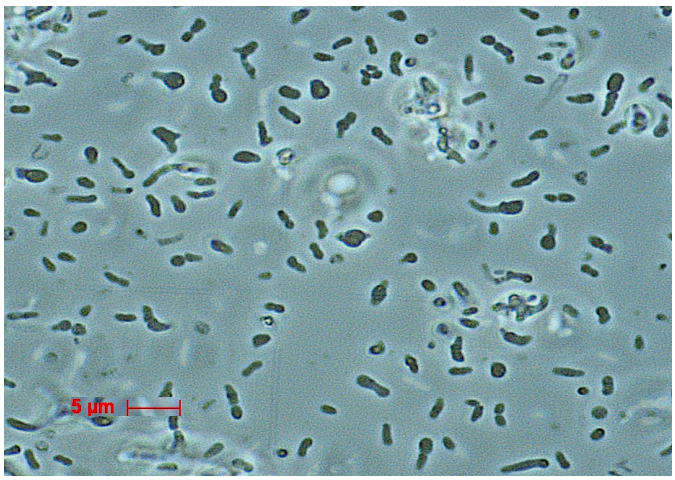
The three *A. weissii* strains 2298, 4321 and 1656 investigated in the present study were isolated from the oral cavity of three dogs. These strains represent a new species of genus *Actinomyces*, for which the name *A. weissii* (N.L. gen. n. weissii, of Weiss, to honour Reinhard Weiss, a contemporary German microbiologist, for his contributions to veterinary microbiology with the type strain *A. weissii* 2298^T (= CIP 110333^T = LMG 26472^T = CCM 7951^T) was proposed. The *A. weissii* strains were Gram-positive, rod shaped, non-motile and non spore-forming bacteria. The non spore-forming feature was investigated using phase contrast microscopy. According to phase contrast microscopy *A. weissii* 2298^T and *A. weissii* 4321 additionally appeared as irregular rod shaped bacterium forming long branching filamentous structured hyphae, *A. weissii* 1656 appeared rod shaped without hyphae **(Fig. 34a)**. Gram-staining also confirmed that the long branching filamentous structured hyphae were only formed by *A. weissii* 2298^T and *A. weissii* 4321, but not by *A. weissii* 1656 **(Fig. 34b)**.



(A)

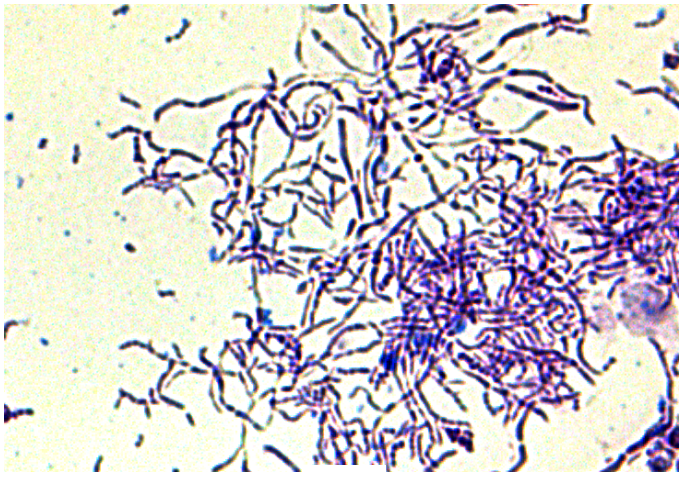


(B)

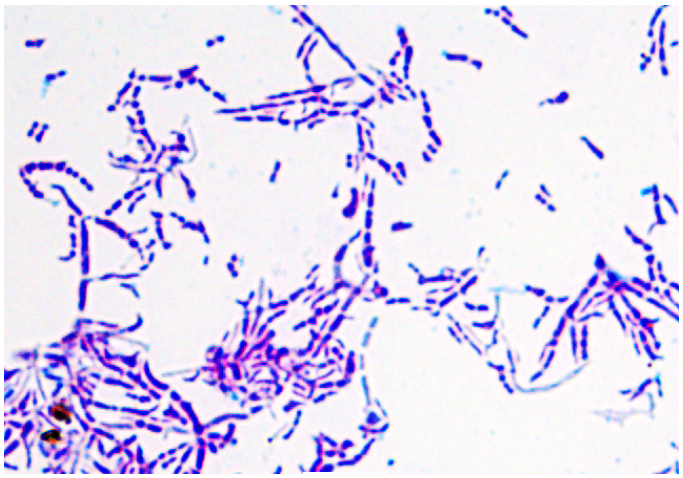


(C)

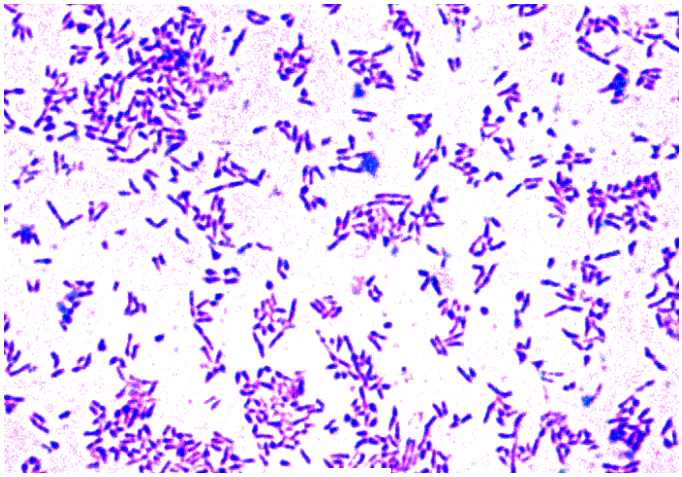
Fig. 34a: *A. weissii* type strain 2298^{T} (= CIP 110333^{T}) (A), *A. weissii* 4321 (B) and *A. weissii* 1656 (C) showing irregular rods with 2-10 µm in length and 1-1.5 µm in width using phase contrast microscopy.



(A)



(B)



(C)

Fig. 34b: Typical Gram-positive *A. weissii* 2298^{T} (= CIP 110333^{T}) (A) and *A. weissii* 4321 (B) showing irregular rod shaped bacteria forming long branching filamentous structured hyphae and rod shaped *A. weissii* 1656 without hyphae (C).

4.1.4.1 Phenotypic results

4.1.4.1.1 Hemolytic properties and CAMP-like hemolytic reactions

A. weissii 2298^T (= CIP 110333^T), *A. weissii* 4321 and *A. weissii* 1656 could be cultivated with a weak zone of hemolysis on sheep blood agar under microaerobic conditions in a candle jar, less pronounced under aerobic and anaerobic conditions **(Fig. 35)**. No enhanced hemolysis could be observed after cultivation on rabbit blood agar compared to sheep blood agar **(Tab. 16)**.

In addition, all three *A. weissii* strains investigated in the present study displayed CAMP-like hemolytic reactions with *S. agalactiae, R. equi, P. phenylpyruvicus, A.* (*T.*) *pyogenes, A.* (*T.*) *abortisuis, A. hippocoleae* and *A. pluranimalium* as indicator strain and a reverse CAMP reaction in the zone of *S. aureus* β-hemolysin. The two reference strains *A. bovis* DSM 43014^T and *A. canis* DSM 15536^T investigated in parallel were found to be negative for CAMP-like hemolytic reactions and for the reverse CAMP reaction (**Fig. 36a, b; Tab. 10**).

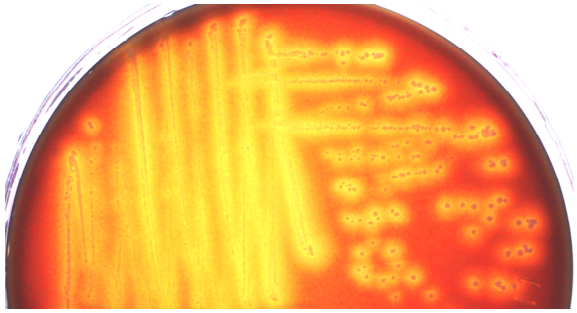


Fig. 35: Hemolytic properties of *A. weissii* 2298^T (= CIP 110333^T) on sheep blood agar after microaerobic incubation for 96 h at 37 °C.

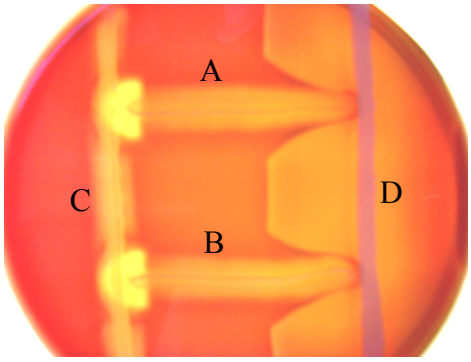


Fig. 36a: Typical CAMP-like hemolytic reactions of *A. weissii* 2298^T (= CIP 110333^T) and *A. weissii* 4321 (A and B, horizontal, respectively) with *S. agalactiae* (C, vertical) as indicator strain and reverse CAMP reaction with β-hemolysin of *S. aureus* (D, vertical).

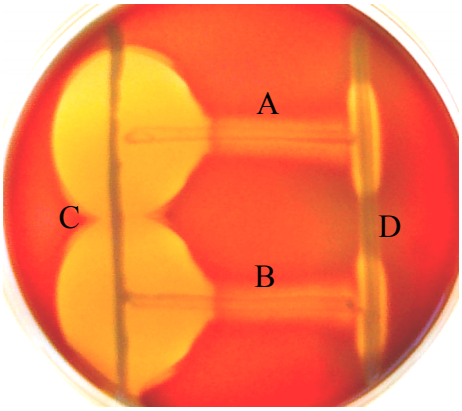


Fig. 36b: Typical CAMP-like hemolytic reactions of *A. weissii* 2298^T (= CIP 110333^T) and *A. weissii* 4321 (A and B, horizontal, respectively) with *R. equi* (C, vertical), and *P. phenylpyruvicus* (D, vertical) as indicator strains.

4.1.4.1.2 Characterization of *A. weissii*, *A. bovis* DSM 43014^T and *A. canis* DSM 15536^T using the API-Coryne-test system and other phenotypical tests

The results of the API-Coryne-test system (3.4.4), tablets containing various substrates (3.4.5), 4-methylumbelliferyl conjugated substrates (3.4.6) and from various other phenotypic tests (3.4.7 to 3.4.15) for the three *A. weissii* strains, *A. bovis* DSM 43014^T and *A. canis* DSM 15536^T are summarized in **Tab. 17**. A seven-digit code was obtained for the three *A. weissii* strains investigated yielding two numerical profiles (0570167 for *A. weissii* 2298^T CIP 110333^T and *A. weissii* 4321; 0530167 for *A. weissii* 1656). These numerical profiles could not be identified by API-Coryne database (3.4.4). *A. bovis* DSM 43014^T and *A. canis* DSM 15536^T yielded the two numerical profiles 2470163 and 0430767, respectively. Both reference strains also could not be identified by API-Coryne database.

All three investigated *A. weissii* strains showed a positive reaction for the enzymes alkaline phosphatase, β-D-galactosidase, α-D-glucosidase and N-acetyl-β-D-glucosaminidase, catalase and amylase, for degradation of D-glucose, D-maltose, D-lactose, D-saccharose and glycogen. Negative reactions were observed for nitrate reduction, pyrazinamidase, pyrrolidonyl arylamidase, β-D-glucuronidase, urease, gelatinase, for serolysis on Loeffler medium, for casein hydrolysis, hyaluronidase, oxidase and indole production and for degradation of D-ribose, D-xylose, D-mannitol. Variable results could be detected for esculin hydrolysis and for the enzymes α-mannosidase and DNase **(Tab. 17)**.

Tab. 16: Hemolytic properties and CAMP-like hemolytic reactions of the three *A. weissii* strains, *A. bovis* DSM 43014^T and *A. canis* DSM 15536^T.

		A. weissii **n = 3**	***A. bovis*** **DSM 43014^T**	***A. canis*** **DSM 15536^T**
Hemolysis on	**Sheep blood agar**	(+)* (3)	(+)	(+)
	Rabbit blood agar	(+) (3)	(+)	(+)
CAMP-like hemolysis with	***S. aureus* β-hemolysin zone**	-**	-	-
	S. agalactiae	+ (3)	-	-
	R. equi	+ (3)	-	-
	P. phenylpyruvicus	+ (3)	-	-
	A. (T.) pyogenes	+ (3)	-	-
	A. (T.) abortisuis	+ (3)	-	-
	A. hippocoleae	+ (3)	-	-
	A. pluranimalium	+ (3)	-	-
Reverse CAMP reaction		+ (3)	-	-

n= Number of strains; * = (+), +, +(+), ++ degree of hemolysis; ** = negative or positive CAMP-like hemolytic reaction or reverse CAMP reaction. The number of positive strains is shown in parenthesis.

Tab. 17: Phenotypic properties of the three *A. weissii* strains, *A. bovis* DSM 43014^T and *A. canis* DSM 15536^T.

Phenotypic properties	*A. weissii* 2298^T (CIP 110333^T)	*A. weissii* 4321	*A. weissii* 1656	*A. bovis* DSM 43014^T	*A. canis* DSM 15536^T
Nitrate reduction	-[1]	-[1]	-[1]	-[1]	-[1]
Pyrazinamidase	-[1]	-[1]	-[1]	(+)[1]	-[1]
Pyrrolidonyl arylamidase	-[1,2]	-[1,2]	-[1,2]	-[1,2]	-[1,2]
Alkaline phosphatase	+[1,2]	+[1,2]	+[1,2]	-[1,2]	-[1,2]
β-Glucuronidase	-[1,3]	-[1,3]	-[1,3]	-[1,3]	-[1,3]
β-Galactosidase	+[1, 3]	+[1, 3]	+[1,3]	+[1,3]	+[1,3]
α-Glucosidase	+[1,3]	+[1,3]	+[1,3]	(+)[1], -[3]	+[1,3]
N-Acetyl-β-Glucosaminidase	+[1, 3]	+[1, 3]	+[1,3]	+[1,3]	+[1,3]
Esculin (β-Glucosidase)	+[1]	+[1]	-[1]	+[1]	-[1]
Urease	-[1]	-[1]	-[1]	-[1]	-[1]
Gelatine	-[1]	-[1]	-[1]	-[1]	-[1]
D-Glucose	+[1]	+[1]	+[1]	+[1]	+[1]
D-Ribose	-[1]	-[1]	-[1]	-[1]	+[1]
D-Xylose	-[1]	-[1]	-[1]	-[1]	+[1]
D-Mannitol	-[1]	-[1]	-[1]	-[1]	-[1]
D-Maltose	+[1]	+[1]	+[1]	+[1]	(+)[1]
D-Lactose	+[1]	+[1]	+[1]	+[1]	+[1]
D-Saccharose	+[1]	+[1]	(+)[1]	+[1]	+[1]
Glycogen	+[1]	+[1]	+[1]	+[1]	+[1]
α-Mannosidase	+[2]	+[2]	-[2]	-[2]	-[2]
Catalase	+	+	+	-	+
Serolysis on Loeffler medium	-	-	-	-	-
Caseinase	-	-	-	-	-
DNase	+	+	-	+	+
Hyaluronidase	-	-	-	-	-
Amylase	+	+	+	+	+

+ = positive reaction; (+) = weak reaction; - = negative reaction. 1 = Api-Coryne-test system (3.4.4); 2 = tablets containing substrates (3.4.5); 3 = 4-methylumbelliferyl conjugated substrates (3.4.6).

4.1.4.2 Identification of *A. weissii* by MALDI-TOF MS

With the available MALDI Biotyper 2.0 software package (3.5.2) all three *A. weissii* strains could not be identified to the species level. A comparison of raw spectra of the three *A. weissii* strains revealed minor differences in the peak pattern of the raw spectra also reflected by high log (score) values of the strains among each other. *A. bovis* DSM 43014^T and *A. canis* DSM 15536^T showed differences in the peak pattern **(Fig. 37)**.

The MALDI Biotyper 2.0 software package (3.6.2) allowed a comparison of the log (score) values of the three *A. weissii* strains, *A. bovis* DSM 43014^T and *A. canis* DSM 15536^T among each other. The *A. weissii* strains showed log (score) values between 2.55 to 2.70 among each other and log (score) values between 0.38 and 0.72 and between 0.43 and 0.84 to *A. bovis* DSM 43014^T and *A. canis* DSM 15536^T, respectively **(Tab. 18)**. A dendrogram analysis of the MSPs of the three *A. weissii* strains investigated in the present study and available reference strains of genus *Actinomyces* included in MALDI Biotyper 2.0 software package is shown in **Fig. 38**. This hierarchical cluster analysis showed that *A. weissii* is representing a novel species of genus *Actinomyces* and formed a distinct cluster separate from other species of genus *Actinomyces*.

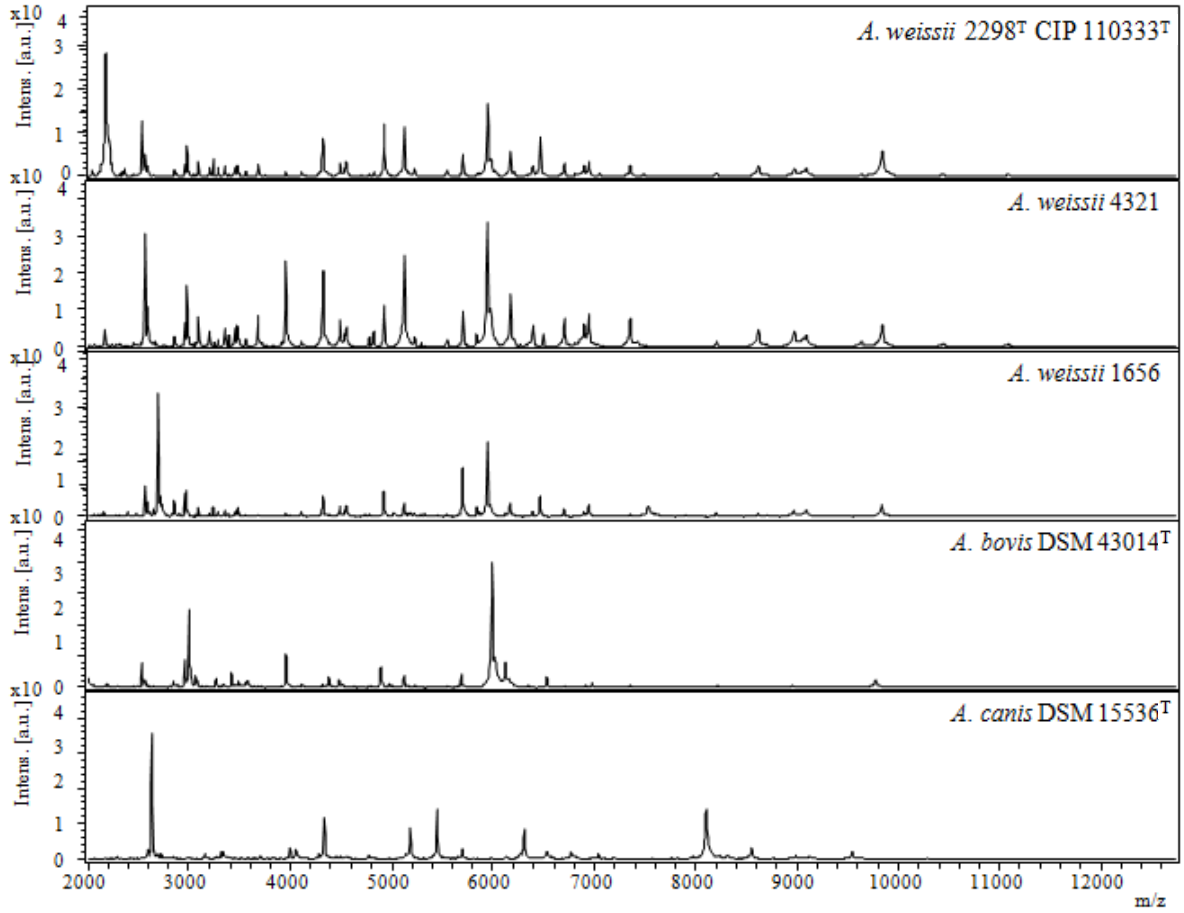


Fig. 37: Typical MALDI-TOF raw spectra of whole-cell extracts of the three *A. weissii* strains, *A. bovis* DSM 43014^T and *A. canis* DSM 15536^T in the range from 2,000 to 12,600 Da. The relative intensities of the ions are shown on the *y* axis, the mass to charge ratios are shown on the *x* axis. The patterns display the overall similarities among the three *A. weissii* strains.

Tab. 18: Log (score) value matrix of the three *A. weissii* strains of the present study and the two reference strains *A. bovis* DSM 43014^T and *A. canis* DSM 15536^T.

	(1)	(2)	(3)	(4)	(5)
(1) *A. weissii* 2298^T (CIP 110333^T)	3	2.70	2.62	0.38	0.77
(2) *A. weissii* 4321		3	2.55	0.69	0.84
(3) *A. weissii* 1656			3	0.72	0.43
(4) *A. bovis* DSM 43014^T				3	0.23
(5) *A. canis* DSM 15536^T					3

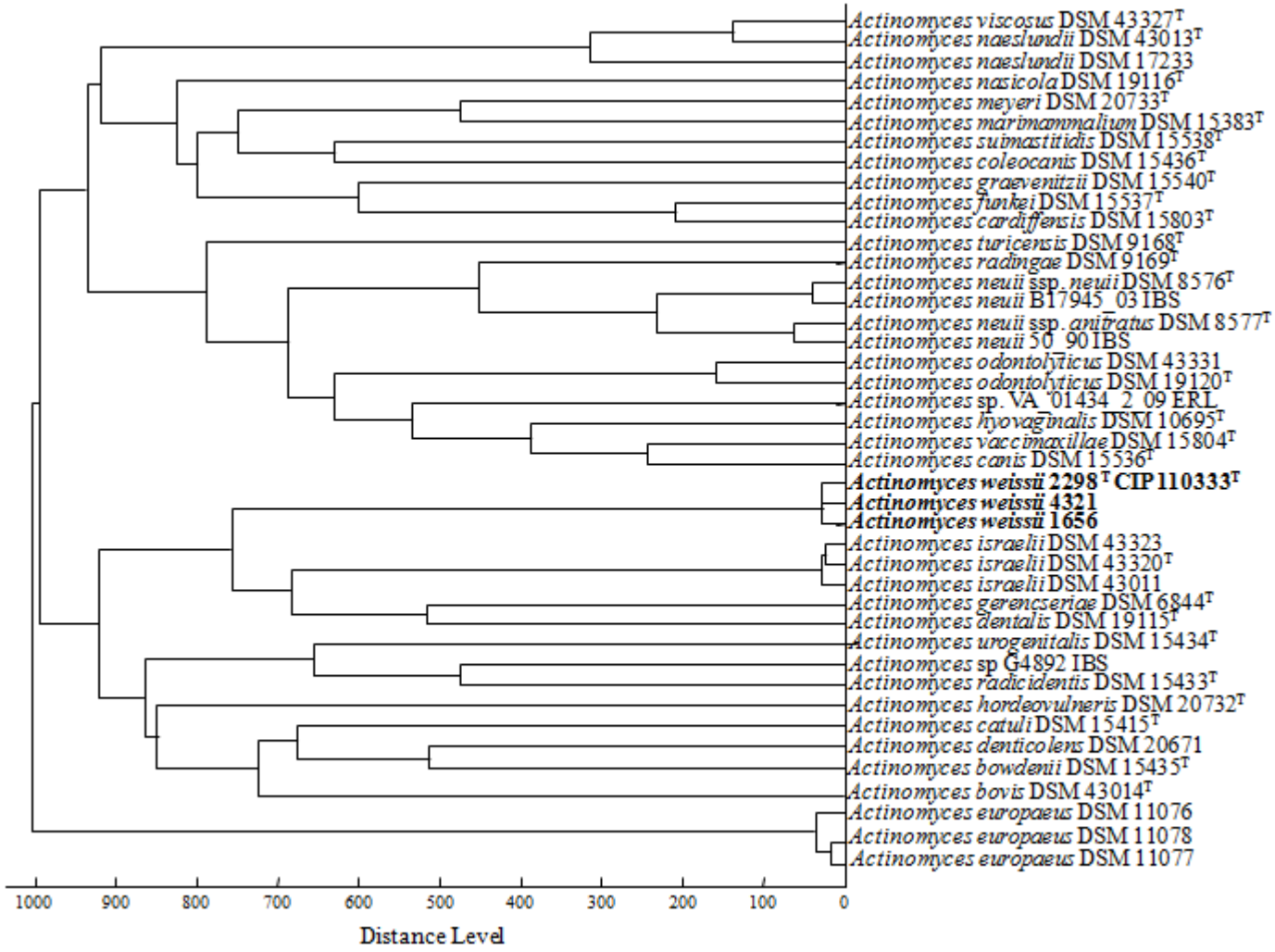


Fig. 38: A score oriented dendrogram of MALDI-TOF main spectra profiles of the *A. weissii* strains of the present (in bold) and other available strains of genus *Actinomyces* included in MALDI Biotyper 2.0 software package. For generating of the dendrogram see **Fig. 6**.

4.1.4.3 PCR-mediated identification of *A. weissii*

4.1.4.3.1 Amplification and sequencing of the 16S rDNA

The amplification of the 16S rDNA of the three *A. weissii* strains was performed using the oligonucleotide primers 16SUNI-L and 16SUNI-R as described (3.6.7). Using these oligonucleotide primers amplicons with an approximate size of 1400 bp (1403 bp expected size) could be detected for all three *A. weissii* strains (data not shown). Sequencing the 16S rDNA of the three strains *A. weissii* 2298^T (CIP 110333^T), *A. weissii* 4321 and *A. weissii* 1656 gave a continuous stretch of 1379 bp, 1347 bp and 1333 bp, respectively (GenBank accession numbers FN552454, FN552455 and FN610846), respectively. Alignment studies using Clustal W method (3.6.6) yielded an almost complete sequence identity of the three strains among each other **(Fig. 39)**. *A. weissii* 2298^T (CIP 110333^T), *A. weissii* 4321 and *A. weissii* 1656 had a sequence similarity between 99.7 % and 99.9 % among each other, to *A. bovis* DSM 43014^T a sequence similarity of 97.0 %, 97.2 % and 97.2 %, respectively and to *A. canis* DSM 15536^T a sequence similarity of 89.9 %, 89.7 % and 89.7 %, respectively. Sequence similarities to other selected species of genus *Actinomyces* were between 88.3 % and 96.6 % (data not shown) and to the nine species of genera *Arcanobacterium* and *Trueperella* between 61.1 % and 89.3 % **(Fig. 40)**. The 16S rDNA sequence similarity of the three *A. weissii* strains investigated in the present study differentiated the strains from *A. bovis*, *A. canis* and from other related bacterial species and clearly assigned the three strains as novel species of genus *Actinomyces*. A dendrogram analysis of the 16S rDNA sequencing results of the present study and some related bacterial species obtained from NCBI GenBank is shown in **Fig 41**.

```
                    1                                                    60

A. weissii 2298T    ACGGTGATGCCTGGCTTTTGCTGGGTGGATGAGTGGCGAACGGGTGAGTAACACGTGAGT
A. weissii 1656     ACGGTGATGCCCAGCTT--GCTGGGTGGATGAGTGGCGAACGGGTGAGTAACACGTGAGT
A. weissii 4321     ACGGTGATGCCTGGCTTTTGCTGGGTGGATGAGTGGCGAACGGGTGAGTAACACGTGAGT
                    61                                                   120
A. weissii 2298T    AACCTGCCCCCTTCTTCTGGATAACCGCCTGAAAGGGTGGCTAATACGGGATATTCTGGC
A. weissii 1656     AACCTGCCCCCTTCTTCTGGATAACCGCCTGAAAGGGTGGCTAATACGGGATATTCTGGC
A. weissii 4321     AACCTGCCCCCTTCTTCTGGATAACCGCCTGAAAGGGTGGCTAATACGGGATATTCTGGC
                    121                                                  180
A. weissii 2298T    CTGCTCGCATGGGTGGGTCTGGAAAGGTTTGTTTCTGGTGGGGGATGGGCTCGCGGCCTA
A. weissii 1656     CTGCTCGCATGGGTGGGTCTGGAAAGGTTTTTTTCTGGTGGGGGATGGGCTCGCGGCCTA
A. weissii 4321     CTGCTCGCATGGGTGGGTTTGGAAAGGTTT-TTTCTGGTGGGGGATGGGCTCGCGGCCTA
                    181                                                  240
A. weissii 2298T    TCAGCTTGTTGGTGGGGTGATGGCCTACCAAGGCTTTGACGGGTAGCCGGCCTGAGAGGG
A. weissii 1656     TCAGCTTGTTGGTGGGGTGATGGCCTACCAAGGCTTTGACGGGTAGCCGGCCTGAGAGGG
A. weissii 4321     TCAGCTTGTTGGTGGGGTGATGGCCTACCAAGGCTTTGACGGGTAGCCGGCCTGAGAGGG
```

```
                    241                                                 300
A. weissii 2298T    TGGACGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGA
A. weissii 1656     TGGACGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGA
A. weissii 4321     TGGACGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGA
                    301                                                 360
A. weissii 2298T    ATATTGCACAATGGGCGCAAGCCTGATGCAGCGACGCCGCGTGAGGGATGTAGGCCTTCG
A. weissii 1656     ATATTGCACAATGGGCGCAAGCCTGATGCAGCGACGCCGCGTGAGGGATGTAGGCCTTCG
A. weissii 4321     ATATTGCACAATGGGCGCAAGCCTGATGCAGCGACGCCGCGTGAGGGATGTAGGCCTTCG
                    361                                                 420
A. weissii 2298T    GGTTGTGAACCTCTTTCGCCAGTGAAGCAGGCCGCCTCCTTGGGGGGTGGTTGACGGTAG
A. weissii 1656     GGTTGTGAACCTCTTTCGCCAGTGAAGCAGGCCGCCTCCTTGGGGGGTGGTTGACGGTAG
A. weissii 4321     GGTTGTGAACCTCTTTCGCCAGTGAAGCAGGCCGCCTCCTTGTGGGGTGGTTGACGGTAG
                    421                                                 480
A. weissii 2298T    CTGGATAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCGAG
A. weissii 1656     CTGGATAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCGAG
A. weissii 4321     CTGGATAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCGAG
                    481                                                 540
A. weissii 2298T    CGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCTGGTCGCGTCTGTCGTGAA
A. weissii 1656     CGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCTGGTCGCGTCTGTCGTGAA
A. weissii 4321     CGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCTGGTCGCGTCTGTCGTGAA
                    541                                                 600
A. weissii 2298T    ATCCTCTGGCTTAACCGGGGGCTTGCGGTGGGTACGGGCCGGCTTGAGTGCGGTAGGGGA
A. weissii 1656     ATCCTCTGGCTTAACCGGGGGCTTGCGGTGGGTACGGGCCGGCTTGAGTGCGGTAGGGGA
A. weissii 4321     ATCCTCTGGCTTAACCGGGGGCTTGCGGTGGGTACGGGCCGGCTTGAGTGCGGTAGGGGA
                    601                                                 660
A. weissii 2298T    GACTGGAATTCCTGGTGTAGCGGTGGAATGCGCAGATATCAGGAGGAACACCGGTGGCGA
A. weissii 1656     GACTGGAATTCCTGGTGTAGCGGTGGAATGCGCAGATATCAGGAGGAACACCGGTGGCGA
A. weissii 4321     GACTGGAATTCCTGGTGTAGCGGTGGAATGCGCAGATATCAGGAGGAACACCGGTGGCGA
                    661                                                 720
A. weissii 2298T    AGGCGGGTCTCTGGGCCGTTACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGAT
A. weissii 1656     AGGCGGGTCTCTGGGCCGTTACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGAT
A. weissii 4321     AGGCGGGTCTCTGGGCCGTTACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGAT
                    721                                                 780
A. weissii 2298T    TAGATACCCTGGTAGTCCACGCCGTAAACGTTGGGCACTAGGTGTGGGGGCTCTTTCCGG
A. weissii 1656     TAGATACCCTGGTAGTCCACGCCGTAAACGTTGGGCACTAGGTGTGGGGGCTCTTTCCGG
A. weissii 4321     TAGATACCCTGGTAGTCCACGCCGTAAACGTTGGGCACTAGGTGTGGGGGCTCTTTCCGG
                    781                                                 840
A. weissii 2298T    GGTCTCCGCGCCGTAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCT
A. weissii 1656     GGTCTCCGCGCCGTAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCT
A. weissii 4321     GGTCTCCGCGCCGTAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCT
                    841                                                 900
A. weissii 2298T    AAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGCGGATTAATTCGAT
A. weissii 1656     AAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGCGGATTAATTCGAT
A. weissii 4321     AAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGCGGATTAATTCGAT
                    901                                                 960
A. weissii 2298T    GCAACGCGAAGAACCTTACCAAGGCTTGACATGGGGGTGGCGGGGCCGGAGACGGTCCTT
A. weissii 1656     GCAACGCGAAGAACCTTACCAAGGCTTGACATGGGGGTGGCGGGGCCGGAGACGGTCCTT
A. weissii 4321     GCAACGCGAAGAACCTTACCAAGGCTTGACATGGGGGTGGCGGGGCCGGAGACGGTCCTT
                    961                                                1020
A. weissii 2298T    CCCTTCGGGGCGCCCTCACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTT
A. weissii 1656     CCCTTCGGGGCGCCCTCACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTT
A. weissii 4321     CCCTTCGGGGCGCCCTCACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTT
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                    1021                                                1080
A. weissii 2298T    GGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCCGTGTTGCCAGCACGTAGTGGTGGGG
A. weissii 1656     GGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCCGTGTTGCCAGCACGTAGTGGTGGGG
A. weissii 4321     GGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCCGTGTTGCCAGCACGTAGTGGTGGGG
                    1081                                                1140
A. weissii 2298T    ACTCGCGGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGATGACGTCAAATCATCAT
A. weissii 1656     ACTCGCGGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGATGACGTCAAATCATCAT
A. weissii 4321     ACTCGCGGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGATGACGTCAAATCATCAT
                    1141                                                1200
A. weissii 2298T    GCCCCTTATGTCTTGGGCTTCACGCATGCTACAATGGCCGGTACAGAGGGCTGCGATACC
A. weissii 1656     GCCCCTTATGTCTTGGGCTTCACGCATGCTACAATGGCCGGTACAGAGGGCTGCGATACC
A. weissii 4321     GCCCCTTATGTCTTGGGCTTCACGCATGCTACAATGGCCGGTACAGAGGGCTGCGATACC
                    1201                                                1260
A. weissii 2298T    GTGAGGTGGAGCGAATCCCTTAAAGCCGGTCTCAGTTCGGATCGGTGTCTGCAACTCGAC
A. weissii 1656     GTGAGGTGGAGCGAATCCCTTAAAGCCGGTCTCAGTTCGGATCGGTGTCTGCAACTCGAC
A. weissii 4321     GTGAGGTGGAGCGAATCCCTTAAAGCCGGTCTCAGTTCGGATCGGTGTCTGCAACTCGAC
                    1261                                1302
A. weissii 2298T    ACCGTGAAGTTGGAGTCGCTAGTAATCGCAGATCAGCAACGC
A. weissii 1656     ACCGTGAAGTTGGAGTCGCTAGTAATCGCAGATCAGCAACGC
A. weissii 4321     ACCGTGAAGTTGGAGTCGCTAGTAATCGCAGATCAGCAACGC
```

Fig. 39: Sequence alignment of 16S rDNA (partial sequence) of three *A. weissii* strains of the present study using Clustal W method (3.6.6). The differences are marked.

Percent identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14		
1	■	99.9	99.8	97.0	89.9	62.0	88.9	86.5	88.0	87.8	88.5	88.6	89.3	89.2	1	*Actinomyces weissii* 2298^T (CIP 110333^T)
2	0.1	■	99.7	97.2	89.7	62.5	88.7	85.8	87.7	87.6	88.4	88.6	89.1	89.0	2	*Actinomyces weissii* 4321
3	0.2	0.3	■	97.2	89.7	61.1	88.6	85.9	87.8	87.5	88.3	89.0	88.3	88.9	3	*Actinomyces weissii* 1656
4	3.0	2.8	2.8	■	95.1	61.8	89.1	86.2	87.6	87.5	89.0	88.8	89.4	89.2	4	*Actinomyces bovis* DSM 43014^T
5	12.5	12.6	12.6	8.0	■	62.7	89.1	87.3	89.0	88.6	89.9	90.0	89.8	90.1	5	*Actinomyces canis* DSM 15536^T
6	12.5	51.6	54.3	52.9	51.0	■	67.5	65.7	66.1	66.3	65.7	65.9	65.9	65.5	6	*Arcanobacterium* (*Trueperella*) *pyogenes* DSM 20630^T
7	12.0	12.2	12.4	11.7	13.7	42.2	■	96.2	95.3	95.8	94.8	95.0	95.0	94.9	7	*Arcanobacterium* (*Trueperella*) *abortisuis* DSM 19515^T
8	15.6	47.5	51.9	15.2	11.0	47.5	3.9	■	93.0	93.2	91.9	92.1	92.5	92.3	8	*Arcanobacterium* (*Trueperella*) *bernardiae* DSM 9152^T
9	13.1	13.4	13.4	13.5	11.7	44.7	4.9	7.4	■	97.2	93.7	92.9	93.1	93.9	9	*Arcanobacterium* (*Trueperella*) *bialowiezense* DSM 17162^T
10	13.0	13.6	13.8	13.7	12.2	44.2	4.3	7.11	2.9	■	93.5	93.6	93.1	93.8	10	*Arcanobacterium* (*Trueperella*) *bonasi* DSM 17163^T
11	12.5	12.6	12.7	11.7	10.2	45.4	5.4	8.5	6.7	6.6	■	97.5	95.6	95.1	11	*Arcanobacterium haemolyticum* DSM 20595^T
12	12.0	12.0	12.4	11.7	10.7	44.5	4.8	8.0	6.4	7.1	2.1	■	96.1	95.0	12	*Arcanobacterium phocae* DSM 10003
13	11.0	11.9	12.7	11.4	10.5	45.1	5.2	7.9	7.2	7.3	4.5	4.8	■	94.9	13	*Arcanobacterium pluranimalium* DSM 13484^T
14	11.6	11.8	11.9	11.6	10.8	45.8	5.3	8.1	6.5	6.4	5.0	3.6	5.2	■	14	*Arcanobacterium hippocoleae* DSM 15539^T
	1	2	3	4	5	6	7	8	9	10	11	12	13	14		

Fig. 40: 16S rDNA sequence homology of the three *A. weissii* strains, *A. bovis* DSM 43014^T, *A. canis* DSM 15536^T and nine reference strains of genera *Arcanobacterium* and *Trueperella* using Clustal W method (3.6.6).

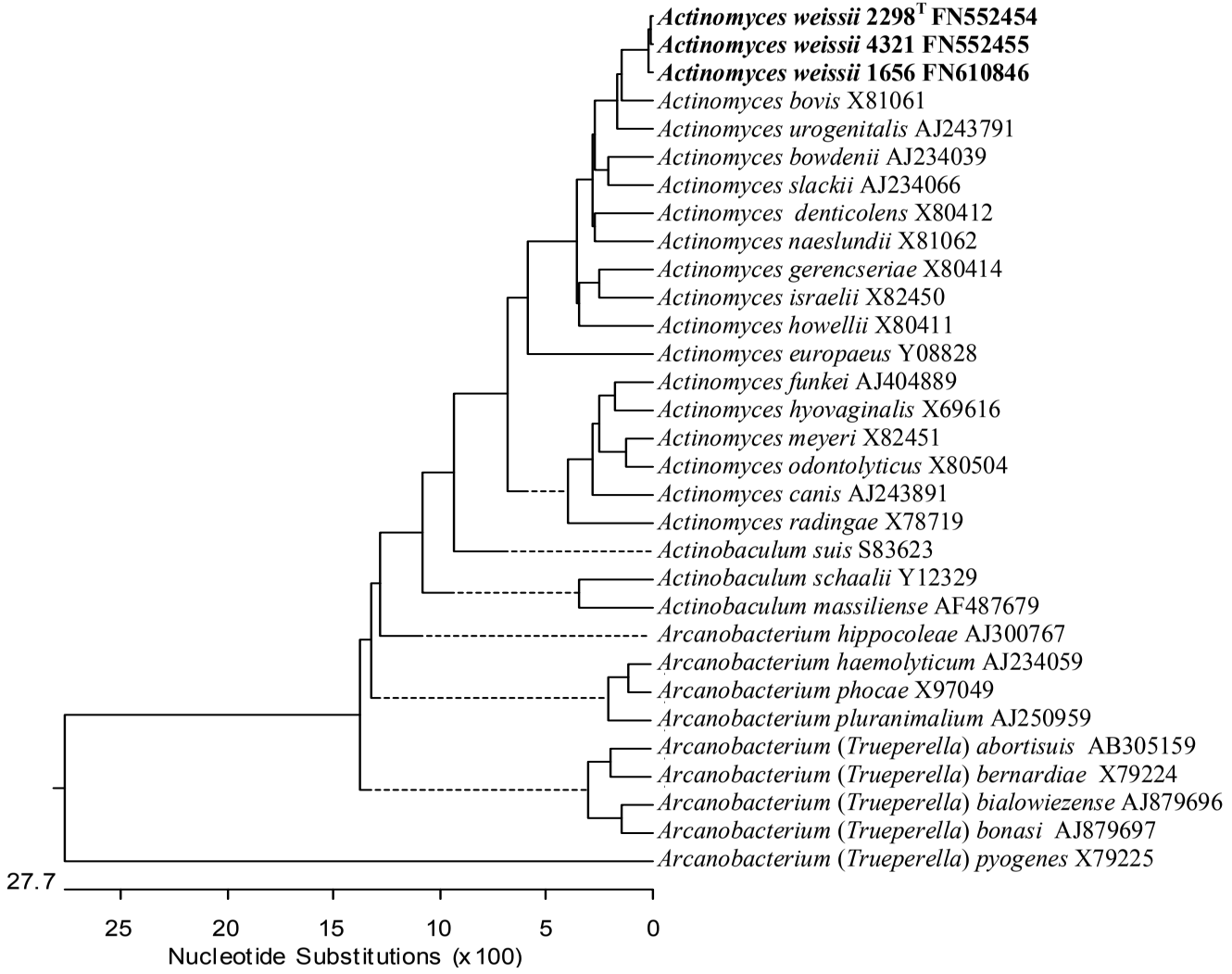

Fig. 41: Dendrogram analysis of 16S rDNA sequences of the three *A. weissii* strains of the present study (in bold) and some related bacterial species obtained from NCBI GenBank using Clustal W method (3.6.6).

4.1.4.3.2 Amplification and sequencing of the 23S rDNA

The amplification of the 23S rDNA of the three *A. weissii* strains and the two reference strains *A. bovis* DSM 43014^T and *A. canis* DSM 15536^T was performed using the oligonucleotide primers 23S-1 and 23S-2a as described (3.6.9). Using these oligonucleotide primers an amplicon with an approximate size of 700 bp (704 bp expected size) could be detected for all investigated strains (data not shown). Sequencing the 23S rDNA of *A. weissii* 2298^T (CIP 110333^T), *A. weissii* 4321, *A. weissii* 1656 and from the reference strains *A. bovis* DSM 43014^T and *A. canis* DSM 15536^T gave a continuous stretch of 704 bp, 683 bp, 669 bp, 687 bp and 655 bp (GenBank accession numbers FN995653, FN995654, FN995655, RF745892 and FR750379), respectively. Sequencing of the 23S rDNA of the three *A. weissii* strains yielded a complete sequence identity of all three *A. weissii* strains to each other with a sequence homology of 100 %, a sequence homology of 97.4 %, 97.3 % and 97.3 %, respectively to the 23S rDNA sequence of *A. bovis* DSM 43014^T and a sequence homology of 90.8 %, 90.8 % and 90.8 %, respectively to the 23S rDNA sequence of *A. canis* DSM 15536^T (**Fig 42)**. Comparing the sequence homology of the 23S rDNA of the three *A. weissii* strains to the sequence of the 23S rDNA of nine species of genera *Arcanobacterium* and *Trueperella* revealed a sequence homology between 87.5 % and 90.0 %. The 23S rDNA sequencing results clearly differentiated the three *A. weissii* strains from *A. bovis*, *A. canis* and from bacteria of genera *Arcanobacterium* and *Trueperella.* A dendrogram analysis of the 23S rDNA sequencing results of the present study and of nine reference strains of genera *Arcanobacterium* and *Trueperella* is shown in **Fig 43.**

Percent identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14		
1	■	100.0	100.0	97.4	90.8	88.8	88.8	88.4	87.9	88.4	89.3	89.9	89.3	88.5	1	*Actinomyces weissii* 2298^T (CIP 110333^T)
2	0.0	■	100.0	97.3	90.8	88.5	88.5	88.0	87.5	88.0	89.0	89.6	88.9	88.2	2	*Actinomyces weissii* 4321
3	0.0	0.0	■	97.3	90.8	89.1	89.1	88.6	88.2	88.6	89.4	90.0	89.5	88.9	3	*Actinomyces weissii* 1656
4	2.8	2.7	2.7	■	91.6	89.4	89.5	89.7	88.1	89.2	89.9	90.8	90.4	90.3	4	*Actinomyces bovis* DSM 43014^T
5	9.8	9.9	9.8	8.9	■	89.3	89.0	89.0	89.5	89.3	89.6	89.2	90.5	89.5	5	*Actinomyces canis* DSM 15536^T
6	12.8	12.3	12.0	11.6	11.6	■	98.8	99.0	97.7	97.7	95.2	96.8	97.7	96.6	6	*Arcanobacterium* (*Trueperella*) *pyogenes* DSM 20630^T
7	12.0	12.8	12.3	11.5	12.0	1.2	■	88.6	88.2	88.6	89.4	90.0	89.5	88.9	7	*Arcanobacterium* (*Trueperella*) *abortisuis* DSM 19515^T
8	12.6	13.4	12.9	11.3	12.0	1.0	0.4	■	97.8	97.8	94.9	96.8	96.9	96.6	8	*Arcanobacterium* (*Trueperella*) *bernardiae* DSM 9152^T
9	13.0	13.8	13.3	13.1	11.5	2.4	1.8	2.2	■	98.2	95.0	95.7	96.0	95.9	9	*Arcanobacterium* (*Trueperella*) *bialowiezense* DSM 17162^T
10	12.5	13.3	12.8	11.8	11.6	2.4	2.1	2.2	1.8	■	95.2	96.5	95.7	95.7	10	*Arcanobacterium* (*Trueperella*) *bonasi* DSM 17163^T
11	11.6	12.1	11.7	11.0	11.2	5.1	5.2	5.4	5.2	5.0	■	97.2	94.6	95.7	11	*Arcanobacterium haemolyticum* DSM 20595^T
12	10.9	11.4	11.0	10.0	11.8	3.3	3.2	3.3	4.4	3.6	2.8	■	96.3	97.2	12	*Arcanobacterium phocae* DSM 10003
13	11.4	12.2	11.8	10.4	10.2	2.4	3.2	3.2	4.1	4.4	5.7	3.8	■	97.5	13	*Arcanobacterium pluranimalium* DSM 13484^T
14	12.6	13.1	12.2	10.5	11.4	3.5	3.6	3.5	4.2	4.4	4.4	3.6	2.5	■	14	*Arcanobacterium hippocoleae* DSM 15539^T
	1	2	3	4	5	6	7	8	9	10	11	12	13	14		

Fig. 42: 23S rDNA sequence homology of the three *A. weissii* strains, *A. bovis* DSM 43014^T, *A. canis* DSM 15536^T and nine reference strains of genera *Arcanobacterium* and *Trueperella* using Clustal W method (3.6.6).

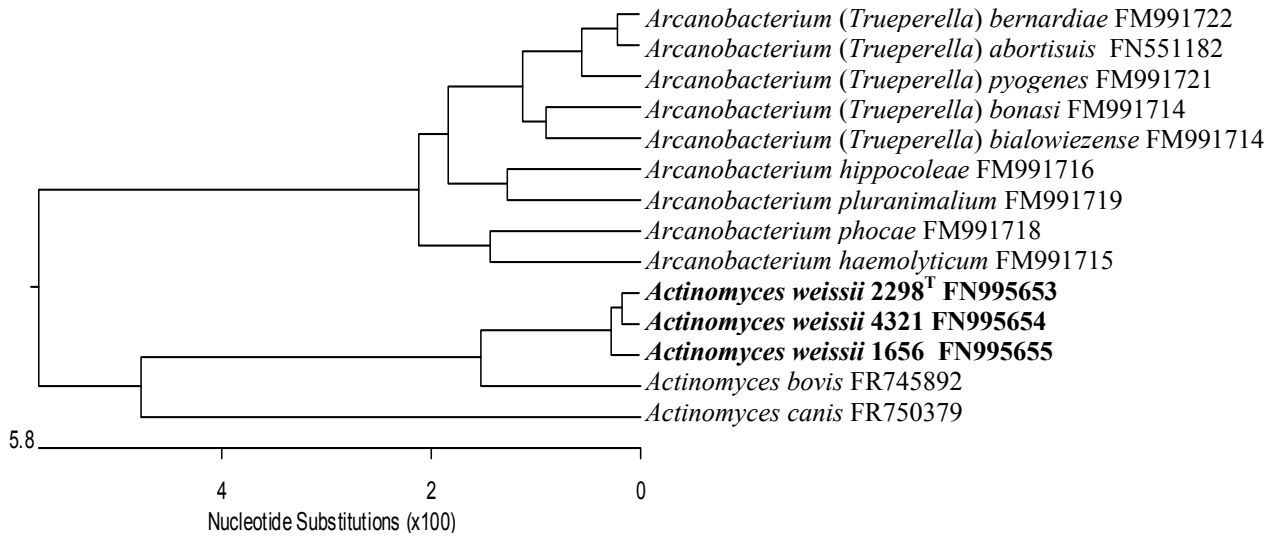

Fig. 43: Dendrogram analysis of 23S rDNA sequences of the three *A. weissii* strains (in bold), reference strains *A. bovis* DSM 43014^T and *A. canis* DSM 15536^T and nine reference strains of genera *Arcanobacterium* and *Trueperella* obtained from NCBI GenBank using Clustal W method (3.6.6).

4.1.4.3.3 Amplification and sequencing of chaperonin encoding gene *cpn*60

Amplification and sequencing of gene *cpn*60 of eight reference strains of genus *Arcanobacterium* and closely related genus *Trueperella* was performed as described in 3.6.10 and 3.6.6, respectively. Using the oligonucleotide primers HSP1-F and HSP1-R gene *cpn*60 of *A.* (*T.*) *abortisuis* DSM 19515^T, *A.* (*T.*) *bernardiae* DSM 9152^T, *A.* (*T.*) *bialowiezense* DSM17162^T, *A.* (*T.*) *bonasi* DSM 17163^T and *A. haemolyticum* DSM 20595^T was amplified with an amplicon of approximate size of 420 bp (419 bp expected size). Gene *cpn*60 of *A. phocae* DSM 10003 was amplified using the oligonucleotide primers HSP1-F and HSP2-R with an approximate amplicon size of 390 bp (385 bp, expected size). Moreover, the amplification of gene *cpn*60 of *A. hippocoleae* DSM 15539^T and *A. pluranimalium* DSM 13483^T was performed using the oligonucleotide primers HSP2-F and HSP2-R with an approximate amplicon size of 230 bp (230 bp, expected size) (data not shown). Sequencing of gene *cpn*60 of *A.* (*T.*) *abortisuis* DSM 19515^T, *A.* (*T.*) *bernardiae* DSM 9152^T, *A.* (*T.*) *bialowiezense* DSM17162^T, *A.* (*T.*) *bonasi* DSM 17163^T and *A. haemolyticum* DSM 20595^T, *A. hippocoleae* DSM 15539^T, *A. phocae* DSM 10003 and *A. pluranimalium* DSM 13483^T gave a continuous stretch of 369 bp, 411 bp, 407 bp, 412 bp, 341 bp, 223 bp, 345 bp and 191 bp, respectively (GenBank accession number FR681838, FR681839, FR681841,

FR681840, FR681842, FR681844, FR681843 and FR681845). The nine reference strains of genera *Arcanobacterium* and *Trueperella* had a *cpn*60 sequence similarity between 77.5 % and 91.9 %. *A*. (*T*.) *abortisuis* DSM 19515^T and *A*. (*T*.) *bernardiae* DSM 9152^T showed the highest similarity value (**Fig. 44)**.

Using the oligonucleotide primers HSP1-F and HSP2-R mentioned before (3.6.10) also allowed the amplification of gene *cpn*60 of the three *A. weissii* strains yielding amplicons with an approximate size of 390 bp (385 bp expected size). Sequencing gene *cpn*60 of *A. weissii* 2298^T (CIP 110333^T), *A. weissii* 4321 and *A. weissii* 1656 according to 3.7.6 revealed a continuous stretch of 345 bp, 350 bp and 345 bp (GenBank accession number FR682110, FR682111 and FR682112), respectively. Comparing the three *cpn*60 gene sequences yielded an almost complete sequence homology (between 99.4 % and 100 %) of the three strains among each other **(Fig. 44)**. Comparing the sequence homology of gene *cpn*60 of the three *A. weissii* strains to gene *cpn*60 of other species of genus *Actinomyces* obtained from *cpn*60 database (cpnDB, www.cpndb.ca) revealed a sequence homology between 67.7 % and 92.5 % (data not shown) and to gene *cpn*60 of bacteria of genera *Arcanobacterium* and *Trueperella* a sequence homology between 71.4 % and 82.6 % **(Fig. 44)**. The sequencing results of gene *cpn*60 clearly differentiated the three *A. weissii* strains from other species of genus *Actinomyces*. A dendrogram analysis of gene *cpn*60 of the three *A. weissii* strains, other species of genus *Actinomyces* obtained from *cpn*60 database and of nine reference strains of genera *Arcanobacterium* and *Trueperella* is shown in **Fig. 45**.

Percent identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12		
1		100.0	99.4	81.4	82.0	82.6	79.7	77.4	75.9	71.6	76.5	76.4	1	*Actinomyces weissii* 2298^T (CIP 110333^T)
2	0.0		99.4	81.4	81.4	82.3	79.4	78.0	76.1	71.4	77.0	76.8	2	*Actinomyces weissii* 4321
3	0.6	0.6		81.4	82.0	82.6	79.7	77.4	75.9	71.6	76.5	76.4	3	*Actinomyces weissii* 1656
4	21.3	21.3	21.3		89.7	91.5	86.5	83.3	81.5	80.3	78.8	80.1	4	*Arcanobacterium* (*Trueperella*) *pyogenes* DSM 20630^T
5	20.6	21.4	20.6	11.1	11	91.9	85.4	82.7	77.5	78.9	77.7	78.0	5	*Arcanobacterium* (*Trueperella*) *abortisuis* DSM 19515^T
6	19.8	20.2	19.8	9.0	8.6		88.9	85.6	80.4	80.7	78.6	80.1	6	*Arcanobacterium* (*Trueperella*) *bernardiae* DSM 9152^T
7	23.7	24.1	23.7	14.9	16.3	12.0		87.5	81.1	78.0	81.2	81.7	7	*Arcanobacterium* (*Trueperella*) *bialowiezense* DSM 17162^T
8	27.1	26.2	27.1	19.1	19.8	16.0	13.8		81.5	78.9	79.7	78.0	8	*Arcanobacterium* (*Trueperella*) *bonasi* DSM 17163^T
9	29.4	29.2	29.4	21.5	27.2	23.1	22.1	21.4		84.4	86.9	83.7	9	*Arcanobacterium haemolyticum* DSM 20595^T
10	36.1	36.3	36.1	23.0	25.2	22.6	26.5	25.1	17.6		79.4	78.5	10	*Arcanobacterium hippocoleae* DSM 15539^T
11	28.4	27.7	28.4	25.0	26.7	25.4	21.8	23.9	14.7	24.2		85.3	11	*Arcanobacterium phocae* DSM 10003
12	28.6	28.1	28.6	23.7	26.3	23.5	21.3	26.9	18.5	25.4	16.5		12	*Arcanobacterium pluranimalium* DSM 13484^T
	1	2	3	4	5	6	7	8	9	10	11	12		

Fig. 44: Gene *cpn*60 sequence homology of the three *A. weissii* strains and nine reference strains of genera *Arcanobacterium* and *Trueperella* using Clustal W method (3.6.6).

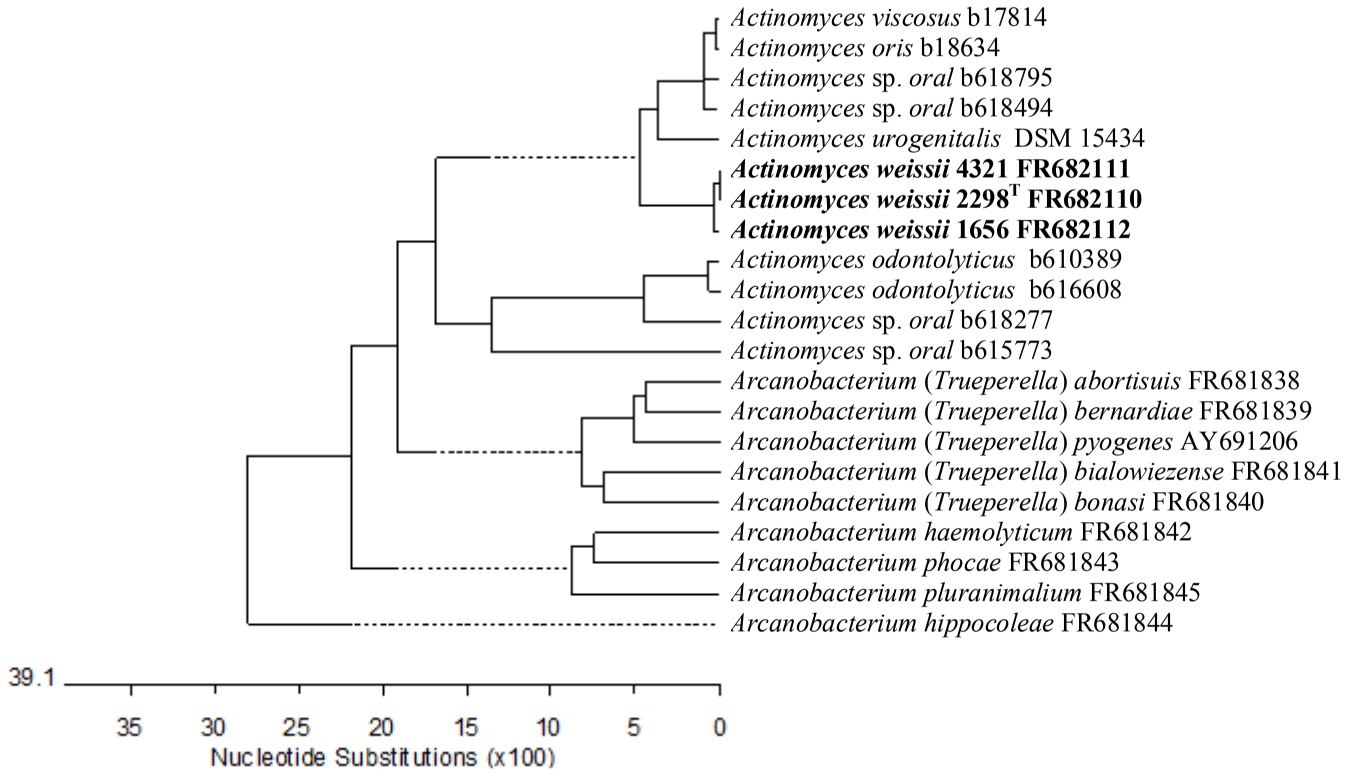

Fig. 45: Dendrogram analysis of gene *cpn*60 sequences of the three *A. weissii* strains (in bold) and additional species of genus *Actinomyces* obtained from *cpn*60 database (chaperonin ID) together with nine reference strains of genera *Arcanobacterium* and *Trueperella* obtained from NCBI GenBank using Clustal W method (3.6.6).

5 Discussion

5.1 Phenotypic characteristics, MALDI-TOF MS fingerprinting results and genotypic characteristics of *A.* (*T.*) *pyogenes*

Pyolysin (PLO) of *A.* (*T.*) *pyogenes* belongs to the cholesterol-dependent cytolysin (CDC) family of toxins (Billington et al., 1997). CDCs are membrane-damaging protein toxins. These hemolytic exotoxins are produced by various Gram-positive bacteria such as intermedilysin (ILY) from *S. intermedius*, listeriolysin O (LLO) from *L. monocytogenes*, perfringolysin O (PFO) from *C. perfringens*, pneumolysin (PLY) from *S. pneumonia* or streptolysin O (SLO) from *S. pyogenes* (Billington et al., 1997). They induce membrane damage after binding to cholesterol containing membranes (Billington et al., 2000; Palmer, 2001; Giddings et al., 2003). This membrane damage, visible by electron microscope and accompanied by circular-like and arc-like structured holes on red blood cells (RBCs), has been shown in several studies (Duncan and Schlegel, 1975; Smyth et al., 1975; Cowell et al., 1978; Smyth and Duncan, 1978; Rottem et al., 1982).

Pyolysin is a well characterized hemolysin from *A.* (*T.*) *pyogenes* which is responsible for the lyses of RBCs of humans and of a variety of animal species (Lovell, 1944; Lämmler and Blobel, 1988; Ding and Lämmler, 1996; Billington et al, 1997). This exotoxin was found to be the primary virulence factor of *A.* (*T.*) *pyogenes*, also involved in cytotoxic effects on a varying number of host cells such as polymorphonuclear leucocytes (PMNs) and macrophages (Ding and Lämmler, 1996; Billington et al., 1997; Jost et al., 1999). According to Lovell (1944), PLO was found to induce dermonecrotic and lethal effects in laboratory animals.

In the present study all 51 *A.* (*T.*) *pyogenes* and the two reference strains *A.* (*T.*) *pyogenes* DSM 20630^{T} and *A.* (*T.*) *pyogenes* DSM 20594 showed a complete zone of hemolysis after cultivation on sheep blood agar under microaerobic conditions in a candle jar. This comes in parallel with previous studies showing that PLO was produced by all investigated *A.* (*T.*) *pyogenes* and that this constant characteristic feature could be used as additional criteria for identification of this species (Ding and Lämmler, 1996; Billington et al., 1997; Ramos et al., 1997; Jost et al., 1999; Erta□ et al., 2005). The hemolysis of the *A.* (*T.*) *pyogenes* strains of the present study was not enhanced by cultivation on rabbit blood agar compared to sheep blood agar. An enhanced hemolysis after

cultivation on blood agar containing rabbit erythrocytes or erythrocytes from humans compared to sheep erythrocytes is a well known property of *A. haemolyticum* (Potel, 1982; Lämmler and Blobel, 1988) and to some extent for *A. hippocoleae* and *A. pluranimalium* (Ülbegi-Mohyla et al., 2009). Hemolytic properties on sheep blood agar were also described for all other species of genera *Arcanobacterium* and *Trueperella* (formerly known as genus *Arcanobacterium*) namely *A. haemolyticum, A.* (*T.*) *bernardiae, A. phocae, A. pluranimalium, A. hippocoleae, A.* (*T.*) *bialowiezense, A.* (*T.*) *bonasi* and *A.* (*T.*) *abortisuis* (Collins et al., 1982; Funke et al., 1995; Ramos et al., 1997; Lawson et al., 2001; Hoyles et al., 2002; Lehnen et al., 2006; Azuma et al., 2009).

Exotoxins of different bacterial species can exhibit hemolytic reactions synergistically and antagonistically. **C**hristie, **A**tkins and **M**unch-**P**etersen (1944) originally described the well known CAMP-reaction, which is a characteristic synergistic hemolytic reaction of CAMP-factor of *S. agalactiae* belonging to streptococci of serological group B, cultivated in the zone of incomplete staphylococcal β-hemolysin. This reaction proved to be a useful tool for presumptive identification of group B-streptococci. Comparable synergistic or CAMP-like hemolytic reactions could also be used for presumptive identification of bacteria of genus *Staphylococcus*, *R. equi*, *C. pseudotuberculosis* and for several other bacterial species (Frazer, 1964; Skalka et al., 1979; Linder, 1984; Hebert and Hancock, 1985; Lämmler and Blobel, 1987, 1988).

All 51 *A.* (*T.*) *pyogenes* investigated in the present study and the two *A.* (*T.*) *pyogenes* reference strains demonstrated synergistic CAMP-like reactions with *S. aureus* ß-hemolysin, *R. equi* and with bacteria of genus *Arcanobacterium*, namely *A. haemolyticum* and *A. phocae* as indicator strains. The *A.* (*T.*) *pyogenes* did not show a reverse CAMP reaction in the zone of staphylococcal ß-hemolysin. The results from the present study were in line with previous studies investigating synergistic hemolytic reactions of *A.* (*T.*) *pyogenes*. Fraser (1964) also described synergistic CAMP-like reactions of *A.* (*T.*) *pyogenes* cultivated on sheep and rabbit blood agar with *S. aureus* ß-toxin, with Equi-factor of *R. equi* and with *A. haemolyticum* as indicator strain. Lämmler and Blobel (1988) also described 42 *A.* (*T.*) *pyogenes* cultures from bovines and from other origin exhibiting synergistic CAMP-like reactions with *S. aureus* ß-toxin. In 2009, Ülbegi-Mohyla et al. investigated CAMP-like activities of bacteria of genus *Arcanobacterium* with various indicator strains. Results from this study showed 26 *A.* (*T.*) *pyogenes* strains isolated from milk samples of bovines displaying synergistic CAMP-like reactions with *S. aureus* ß-hemolysin

as well as with *R. equi* and with *A. haemolyticum* as indicator strain. However, Yassin et al. (2011) described a negative CAMP reaction of *A.* (*T.*) *pyogenes* DSM 20630^T (ATCC 19411^T) with ß-hemolysin of *S. aureus.* The results from these authors were contradictory to results of the present study and to previous studies (Lämmler and Blobel, 1988; Ülbegi-Mohyla et al., 2009).

No reverse CAMP reaction could be observed for *A.* (*T.*) *pyogenes*. On the other hand, among the nine species of genera *Arcanobacterium* and *Trueperella*, a reverse CAMP-reaction in the zone of staphylococcal ß-hemolysin appeared to be a characteristic property of *A. haemolyticum* (Fraser, 1964; Lämmler and Blobel, 1987) and *A. phocae* (Johnson et al., 2003; Ülbegi, 2010).

Thus, the synergistic or CAMP-like hemolytic reaction of *A.* (*T.*) *pyogenes* with various indicator strains shown in the present study might help to identify this bacterium, as well as to differentiate it from other species of genera *Arcanobacterium* and *Trueperella* in routine microbiological diagnostics.

Phenotypic identification and further characterization of the *A.* (*T.*) *pyogenes* strains investigated in the present study was performed by use of different biochemical tests and a serological test. Most of the 51 *A.* (*T.*) *pyogenes* strains investigated were found positive for the enzymes pyrrolidonyl arylamidase and alkaline phosphatase with percentages of 59 % and 82 %, respectively, while few strains showed a positive reaction for the enzyme α-mannosidase with a percentage of 20 %. Reference strains *A.* (*T.*) *pyogenes* DSM 20630^T and *A.* (*T.*) *pyogenes* DSM 20594 showed variable results for the enzyme pyrrolidonyl arylamidase, both strains were negative for the enzymes alkaline phosphatase and α-mannosidase. All three enzymes were determined with tablets containing the various substrates. The results for the two enzymes pyrrolidonyl arylamidase and alkaline phosphatase generally corresponded to other studies (Hoyles et al., 2002; Lehnen et al., 2006; Ülbegi, 2010). The enzyme α-mannosidase had been introduced as a rapid test for identification of *A. haemolyticum*, as well as to differentiate *A. haemolyticum* from *A.* (*T.*) *pyogenes* (Carlson and Kontiainen, 1994). Results from these authors showed that 99 % of the *A. haemolyticum* investigated (n = 139) were positive, while all *A.* (*T.*) *pyogenes* (n = 30) were α-mannosidase negative. However, according to the present study 20 % of the investigated *A.* (*T.*) *pyogenes* strains were positive and the remaining strains also including both *A.* (*T.*) *pyogenes* reference strains were negative for the enzyme α-mannosidase. According to the results from Ülbegi (2010) the reference strains of genus *Arcanobacterium*, namely *A. haemolyticum* as well as *A. phocae* and *A. pluranimalium*

were positive for enzyme α-mannosidase. Contradictory to Carlson and Kontiainen (1994), the results from the present study and from Ülbegi (2010) showed that detection of α-mannosidase could only be used as additional criterion for identification and differentiation of *A. haemolyticum.*

Further biochemical tests were performed using 4-methylumbelliferyl-conjugated substrates. This test was based on the generation of fluorescence visible under ultraviolet light (360 nm) when free 4-methylumbelliferone was released by enzymatic hydrolysis of the non-fluorescent 4-methylumbelliferyl-conjugated substrates. Using working solutions from the four substrates 4-methylumbelliferyl-β-D-glucuronide, 4-methylumbelliferyl-β-D-galactopyranoside, 4-methylumbelliferyl-α-D-glucoside and 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide (Sigma), all investigated *A.* (*T.*) *pyogenes* strains (n = 51) and the two *A.* (*T.*) *pyogenes* reference strains were found to produce these four enzymes. These results come in line with previous studies investigating the occurrence of these enzymes in *A.* (*T.*) *pyogenes* (Lawson et al., 2001; Hoyles et al., 2002; Johnson et al., 2003; Lehnen et al., 2006; Azuma et al., 2009). Results from Ülbegi (2010) showed that except for the two *A.* (*T.*) *pyogenes* reference strains and reference strain *A. hippocoleae* DSM 15539^T, none of the other species of genera *Arcanobacterium* and *Trueperella* showed positive results for all four enzymes namely, β-D-glucuronidase, β-D-galactosidase, α-D-glucosidase and N-acetyl-β-D-glucosaminidase.

The enzyme catalase was demonstrated with 3 % H_2O_2 on a microscopic slide. A positive reaction through a decomposition of H_2O_2 into water and gaseous oxygen was seen as bubble formation. All *A.* (*T.*) *pyogenes* investigated in this study, also including the two *A.* (*T.*) *pyogenes* reference strains, were catalase negative. A positive catalase reaction was limited to the two species *A. phocae* and *A. pluranimalium* (Ülbegi, 2010). These results corresponded with those reported by Collins et al. (1982b), Ding and Lämmler (1992), Ramos et al. (1997), Lawson et al. (2001), Hoyles et al. (2002), Johnson et al. (2003), Lehnen et al. (2006), Azuma et al. (2009) and Ülbegi (2010).

Generally, the investigation of enzymatic activities using tablets containing various substrates and 4-Methylumbelliferyl-conjugated substrates and the catalase test are fast and simple to perform tests and could help to identify *A.* (*T.*) *pyogenes* and other species of genera *Arcanobacterium* and *Trueperella*.

The investigation of the enzyme DNase was demonstrated using DNase test agar. According to Jeffries et al. (1957), the degradation of DNA could be detected by flooding the surface of the

incubated medium with 1 N HCl and observing clear zones in the medium surrounding bacterial growth. In the present study, all *A.* (*T.*) *pyogenes* strains investigated, also including the two *A.* (*T.*) *pyogenes* reference strains, were positive for this test. This was also comparable to previous studies which considered DNase as potential virulence factor associated with *A.* (*T.*) *pyogenes* infections (Collins et al., 1982b; Lämmler and Blobel, 1988; Ding and Lämmler, 1992; Lämmler and Hartwigk, 1995; Goyal et al., 2005; Jost and Billington, 2005; Parija et al., 2005). More recently, Ülbegi (2010) could show that except for *A.* (*T.*) *bialowiezense* and *A.* (*T.*) *bonasi* all other species of genera *Arcanobacterium* and *Trueperella* were DNase positive.

The detection of proteolytic enzymatic activities of the *A.* (*T.*) *pyogenes* strains of the present study was performed by investigating serolysis on Loeffler medium and by cultivation of the bacteria on casein agar. A groove formation in the Loeffler medium beneath the bacterial inoculation line was considered as positive reaction due to proteolytic activities. After flooding the casein agar with 10 % trichloroacetic acid any clear area around the grown culture indicated the breakdown of casein by the organism due to the production of caseinase. Similarly to the two *A.* (*T.*) *pyogenes* reference strains, all 51 *A.* (*T.*) *pyogenes* investigated in the present study displayed a liquefaction of Loeffler medium and a casein hydrolysis. These results were in line with previous studies showing that *A.* (*T.*) *pyogenes* produce gelatinase and caseinase (Lämmler and Blobel 1998; Narayanan 1998; Ülbegi, 2010). According to Hassan et al. (2009) seven *A. haemolyticum* isolated from infections of horses showed a moderate liquefaction of Loeffler medium. However, the liquefaction of Loeffler medium and caseinase activity are typical properties of *A.* (*T.*) *pyogenes* and could be used for identification of this species (Bisping and Amtsberg 1988; Lämmler and Hartwigk 1995; Takeuchi et al., 1995). An extracellular protease from *A.* (*T.*) *pyogenes* was described at first by Schaufuss et al. (1989) and had been further characterized to have gelatinase and caseinase activity by Takeuchi et al. (1995). According to Jost and Billington (2005) *A.* (*T.*) *pyogenes* proteases degrade host proteins making amino acids available as nutrients. The proteases may also degrade immunoglobulin A (IgA) which is involved in host defense, indicating that these extracellular proteases might play a role in *A.* (*T.*) *pyogenes* pathogenesis.

Detection of the enzyme hyaluronidase was carried out according to Winkle (1979). Using a mucoid growing *S. equi* subsp. *equi* strain as indicator, all 51 *A.* (*T.*) *pyogenes* and the two *A.* (*T.*) *pyogenes* reference strains appeared to be hyaluronidase negative. Ülbegi had already

shown in 2010 that all nine species of genera *Arcanobacterium* and *Trueperella* did not express this enzyme, indicating that this test should not be used for discriminating between different species of genera *Arcanobacterium* and *Trueperella.*

Testing of the *A.* (*T.*) *pyogenes* strains of the present study for the enzyme amylase revealed variable results. All *A.* (*T.*) *pyogenes* isolated from wild ruminants (n = 5), horse (n = 1) and rabbit (n = 1) produced amylase. Amylase production was less pronounced in *A.* (*T.*) *pyogenes* from pigs (62 %), small ruminants (60 %) and from dogs and cats (50 %). *A.* (*T.*) *pyogenes* from bovines (n = 19), rat (n = 1) and reptiles (n = 2) were amylase negative. The difference in the production of amylase between strains isolated from ruminants and pigs appeared to be significant (P = 0.047). Comparably to the negative amylase reaction of *A.* (*T.*) *pyogenes* obtained from bovines (n = 19), reference strain *A.* (*T.*) *pyog*enes DSM 20594 of bovine origin was also amylase negative, indicating that a negative amylase test might be used for differentiation of *A.* (*T.*) *pyogenes* isolated from bovines. Reference strain *A.* (*T.*) *pyogenes* DSM 20630^{T} originally isolated from a pig, produced amylase. However, the importance of the presence or absence of enzyme amylase for *A.* (*T.*) *pyogenes* pathogenesis remains to be elucidated.

As already described by Lämmler and Blobel (1988), a commercial streptococcal grouping kit could be used for tentative identification of *A.* (*T.*) *pyogenes*. A cross-reaction with streptococcal serogroup G-specific antiserum could be observed for all *A.* (*T.*) *pyogenes* investigated in the present study and the two *A. pyogenes* reference strains. Results from other investigators already demonstrated that this cross-reaction was considered as a typical characteristic of *A.* (*T.*) *pyogenes* and could be used for serological identification of this species (Lämmler and Blobel, 1988; Lämmler and Hartwigk, 1995). According to Ülbegi (2010) all other species of genera *Arcanobacterium* and *Trueperella* were negative in this test.

In the early beginning, Krishnamurthy et al. (1996), Claydon et al. (1996) and Holland et al. (1996) reported about the use of MALDI-TOF MS analysis of bacterial proteins for bacterial identification. This technique was used for bacterial identification to genus and species level and for some instances to subspecies or strain level (Lynn et al., 1999; Bernardo et al., 2002; Ruelle et al., 2004; Rupf et al., 2005; Murray, 2010). According to these studies, the identification was done through the comparison of the obtained unknown protein profiles or peaks with archived reference spectra or by co-analysis with spectra of known bacteria. For bacterial identification the reproducibility of the peptidic spectra is of importance. Several investigators reported about some

difficulties associated with the spectra reproducibility involved in MALDI-TOF MS analysis caused by bacterial storage, handling, or culturing over different time intervals prior to MALDI-TOF MS analysis (Arnold et al., 1999; Lay Jr, 2001; Vargha et al., 2006). However, conserved peaks in the peptidic spectra of a specific bacterium obtained under different experimental parameters had also been reported (Welham et al., 1998; Wang et al., 1998; Valentine et al., 2005).

In the present study, comparable cultivation conditions prior to MALDI-TOF MS analysis were applied for all investigated bacterial species. This included *A.* (*T.*) *pyogenes* (n = 51), *A.* (*T.*) *abortisuis* (n = 23), *A. haemolyticum* (n = 3), the novel species *A. weissii* (n = 3) and eleven reference strains representing nine species of genera *Arcanobacterium* and *Trueperella* and *A. bovis* DSM 43014^{T}. The strains were cultivated on sheep blood agar plates. Incubation of the inoculated culture media was carried out for 48 to 72 h at 37 °C under microaerobic conditions in a candle jar. In addition, using Bruker ethanol formic acid preparation procedure bacterial sample preparation for protein extraction was identical for all bacterial strains. These identical conditions prior to the MALDI-TOF MS fingerprinting analysis were perform to enhance the spectral reproducibility for each strain investigated in the present study.

All 11 reference strains representing nine species of genera *Arcanobacterium* and *Trueperella* could successfully be identified to the species level by matching against the newly acquired MSPs from the eleven reference strains of genera *Arcanobacterium* and *Trueperella* of the present study and against the reference library entries using the MALDI Biotyper 2.0 software package. The MALDI Biotyper 2.0 software calculates a similarity score (log (score)) by calculation of a value considering the proportion of matching peaks between the unknown spectrum and the comparable known spectra, the frequency of peaks in multiple measurements as well as the consistency of the peak intensities between these spectra. *Arcanobacterium* and *Trueperella* reference strains identified to the species level showed higher log (score) values when matched against the new MSPs obtained in the present study than by using reference library entries of MALDI Biotyper 2.0. The latter contained MSPs of eight species of genera *Arcanobacterium* and *Trueperella*, not including the newly described species *A.* (*T.*) *abortisuis*. According to Bruker Daltonik (Bremen, Germany) the MSPs of the eight species of genera *Arcanobacterium* and *Trueperella* (formerly known as genus *Arcanobacterium*) are part of the MALDI Biotyper 2.0 since 2008. These MSP entries were based on peptidic spectra obtained

from bacterial cells grown under different cultivation conditions compared to the present study. Moreover, the number and intensity of peptidic peaks of the MSP provided by MALDI Biotyper 2.0 were lower than those of the newly acquired MSPs of the present study. Because of this, the newly acquired MSPs were used together with the reference library entries of MALDI Biotyper 2.0 for identification of the *Arcanobacterium* and *Trueperella* species investigated in the present study.

The peak pattern of the raw spectra of the nine species of genera *Arcanobacterium* and *Trueperella*, respectively, showed major differences, also reflected with the help of the MALDI Biotyper 2.0 software package, by low log (score) values of the nine species among each other. However, the two reference strains *A.* (*T.*) *pyogenes* DSM 20630^T and *A.* (*T.*) *pyogenes* DSM 20594 had a log (score) value of 2.15 among each other could be identified to species level. Similarly, the two reference strains *A. phocae* DSM 10002^T and *A. phocae* DSM 10003 with a log (score) value of 2.86 among each other and could be identified to species level. As already described, log (score) values ≥ 2.0 are rated as identification of bacteria at species level, log (score) values ≥ 1.7 and < 2.0 rated as identification at genus level, log score values < 1.7 as not suitable for identification by the MALDI Biotyper (Mellmann et al., 2008; Barbuddhe et al., 2008; Ilina et al., 2009; Lartigue et al., 2009; Marklein et al., 2009; Nagy et al., 2009). A dendrogram analysis of the MSPs of 11 reference strains of genera *Arcanobacterium* and *Trueperella* could reliably differentiate all nine species of both genera.

As already described, Yassin et al. (2011) proposed that the formerly named species *A. pyogenes, A. abortisuis, A. bernardiae, A. bialowiezense* and *A. bonasi* should be reclassified in the newly established genus *Trueperella* as *T. pyogenes***,** *T. abortisuis*, *T. bernardiae*, *T. bialowiezensis* and *T. bonasi***.** The genus *Arcanobacterium* should be restricted to *A. haemolyticum, A. phocae, A. pluranimalium* and to the phylogenetic neighbour *A. hippocoleae*. Results from Ülbegi (2010) showing sequencing results of the 16S rDNA, ISR, 23S rDNA and the genes *sod*A and *rpo*B supported the proposal of Yassin and coauthors (2011) that the hitherto nine species of genus *Arcanobacterium* should be separated into two genera (data not shown). Comparing the Log (score) values of the nine species of genera *Arcanobacterium* and *Trueperella* among each other did not show any close relations except for *A.* (*T.*) *bernardiae* and *A.* (*T.*) *abortisuis* with a log score value of 1.77. All other species revealed log (score) values □ 1.70 among each other. The

findings using MALDI-TOF MS analyses did not correspond to the data obtained by 16S rDNA analyses (Ülbegi, 2010; Yassin et al., 2011).

MALDI-TOF MS fingerprint analysis allowed the identification of all 51 *A.* (*T.*) *pyogenes* strains investigated in the present study to species level. All 51 *A.* (*T.*) *pyogenes* strains matched against MSP of the reference strains *A.* (*T.*) *pyogenes* DSM 20630^T and *A.* (*T.*) *pyogenes* DSM 20594 of the present study and against reference library entries with log (score) values ranging from 2.11 to 2.63.

A comparison of typical raw spectra of *A.* (*T.*) *pyogenes* 1886/07 of bovine origin, *A.* (*T.*) *pyogenes* 1848/07 of pig origin, *A.* (*T.*) *pyogenes* 6122/08 of cat origin, *A.* (*T.*) *pyogenes* 734/03 of reptile origin and the two *A.* (*T.*) *pyogenes* reference strains, respectively, revealed minor differences in the peak pattern. The peak pattern similarities of the raw spectra reflected the strain identification to the species level with log (score) values ≥ 2.0. The dendrogram analysis of the MSPs of 20 selected *A.* (*T.*) *pyogenes* strains of the present study with eleven reference strains of genera *Arcanobacterium* and *Trueperella*, also including the two *A.* (*T.*) *pyogenes* reference strains investigated in the present study, come in line with this species identification.

A comparison of the log (score) values of the 51 *A.* (*T.*) *pyogenes* strains among each other yielded the lowest log (score) value of 1.89 to a maximal score of 2.63. The maximum log (score) value was between two strains isolated from a bearded dragon and a mountain reedbuck. *A.* (*T.*) *pyogenes* strains isolated from two pigs of the same farm domain matched in MALDI Biotyper analysis to each other with log (score) values between 2.14 and 2.37. This did not indicate any clonal relation of the strains isolated from same farm domain.

Furthermore, in the present study the MSPs of all 51 investigated *A.* (*T.*) *pyogenes* were matched to the MSPs of reference strains *A.* (*T.*) *pyogenes* DSM 20630^T and *A.* (*T.*) *pyogenes* DSM 20594. Based on two-way ANOVA, the mean of the log (score) values of all 51 *A.* (*T.*) *pyogenes* strains appeared to be significantly higher (P = 0.0001) using reference strain *A. pyogenes* DSM 20594 as matching partner than using *A.* (*T.*) *pyogenes* DSM 20630^T. This indicates that the MSP of *A. pyogenes* DSM 20594 of bovine origin could be used in future as a matching partner for MALDI-TOF MS-based *A.* (*T.*) *pyogenes* identifications. The inclusion of reference strain *A.* (*T.*) *pyogenes* DSM 20594 in the MALDI Biotyper 2.0 software package will improve a future MALDI-TOF MS-based identification of *A.* (*T.*) *pyogenes*. In addition, the mean log (score) values of *A.* (*T.*) *pyogenes* strains isolated from ruminants were significantly higher (P = 0.0059) than strains isolated

from non-ruminants using both *A.* (*T.*) *pyogenes* reference strains as matching partners. This indicates the high similarity of ruminant strains, including strains of bovines, to MSPs of both reference strains.

The present results give a first information using MALDI-TOF MS for identification of bacteria of genera *Arcanobacterium* and *Trueperella*. However, MALDI-TOF MS had been described by numerous authors as a fast and accurate method for identification of bacteria of various species (Bessède et at., 2011; Mellmann et al., 2008; Nagy et al., 2009; Seibold et al., 2010; Seng et al., 2009; Murray, 2010). According to the results of the present study, MALDI-TOF MS appears to be a promising tool for identification of all nine species of genera *Arcanobacterium* and *Trueperella* and could also be used to identify *A.* (*T.*) *pyogenes* strains of different animal origin.

A genotypic identification of the *A.* (*T.*) *pyogenes* of the present study was performed by investigating the ISR of *A.* (*T.*) *pyogenes*. It had been recognized that ISR sequence heterogeneity of various bacterial species could be used as a candidate region from which species-specific primers could be designed for bacterial identification (Barry et al, 1991; Tilsala-Timisjärvi and Alatossava, 1997). Amplification and sequencing of ISR of all nine species of genera *Arcanobacterium* and *Trueperella* by Hassan et al. (2009) and Ülbegi (2010) using the primer pair described by Kostman et al. (1995) and Chanter et al. (1997) resulted in a distinct separation of all nine species, indicating that ISR sequencing also appeared to be a useful target for genotypic characterization of bacteria of genera *Arcanobacterium* and *Trueperella*. The ISR sequences allowed the design of species specific oligonucleotide primers which could be used for PCR-mediated identification of *A.* (*T.*) *pyogenes*, *A.* (*T.*) *bernardiae*, *A.* (*T.*) *bialowiezense*, *A.* (*T.*) *bonasi*, *A. haemolyticum* and *A. hippocoleae* (Hassan et al., 2008; Ülbegi, 2010). According to the results of the present study the *A.* (*T.*) *pyogenes* ISR specific oligonucleotide primers described by Ülbegi (2010) could successfully be used to identify all 51 *A.* (*T.*) *pyogenes* investigated and both *A.* (*T.*) *pyogenes* reference strains.

Superoxide dismutase A encoding gene *sod*A had been shown by many investigators to have a discriminating power which could be used to differentiate several bacterial species (Poyart et al., 1998, 2000, 2001; Zolg and Philippi-Schulz, 1994). More recently, the universal oligonucleotide primer pair described by Zolg and Philippi-Schulz (1994) and a new primer pair designed by Ülbegi (2010) allowed the amplification and sequencing of gene *sod*A of eight species of genera *Arcanobacterium* and *Trueperella* (excluding *A.* (*T.*) *bialowiezense*). This allowed a distinct

differentiation of these eight species of genera *Arcanobacterium* and *Trueperella*. The *A.* (*T.*) *pyogenes sod*A specific oligonucleotide primers designed in the present study could subsequently be used for identification of all 51 investigated *A.* (*T.*) *pyogenes* strains and the two *A.* (*T.*) *pyogenes* reference strains with an approximate amplicon size of 200 bp. The ISR and *sod*A PCR-mediated identification of the *A.* (*T.*) *pyogenes* strains revealed no cross reactivity with the control strains representing eight other species of genera *Arcanobacterium* and *Trueperella*, indicating that both targets could be used for molecular identification of *A.* (*T.*) *pyogenes*.

A. (*T.*) *pyogenes* as an opportunistic pathogen possesses different virulence factors that contribute to its pathogenic potential (Jost and Billington, 2005). These virulence factors are important determinants associated with adherence and colonization of *A.* (*T.*) *pyogenes* and might cause host tissue damage associated with infection. In the present study, 51 *A. pyogenes* and two *A.* (*T.*) *pyogenes* reference strains were investigated for the presence of seven potential virulence factor encoding genes, namely *plo*, *cbp*, *nan*H, *nan*P, *fim*A, *fim*C and *fim*E by conventional PCR.

Gene *plo* encoding pyolysin, considered as a primary virulence factor of *A.* (*T.*) *pyogenes*, has a nucleotide sequence of 1,605 bp (Billington et al., 1997). Jost et al. (1999) showed that an *A.* (*T.*) *pyogenes plo* mutant was less virulent than the wildtype *A. pyogenes* and that providing *plo* gene *in trans* to the *plo* mutant *A.* (*T.*) *pyogenes* could restore its full virulence. This indicates, like other CDCs of additional Gram-positive bacteria, the importance of *A.* (*T.*) *pyogenes* gene *plo*, in the pathogenesis process. According to Ertaş et al. (2005), Jost and Billington (2005) and Silva et al (2008), all *A.* (*T.*) *pyogenes* examined expressed pyolysin encoding gene *plo.*

Using the oligonucleotide primers plo-F and plo-R gene *plo* with an amplicon size of approximately 700 bp could be detected for all 51 *A.* (*T.*) *pyogenes* and the two *A. pyogenes* reference strains investigated in the present study. Ertaş et al. (2005) and Silva et al. (2008) could amplify gene *plo* from *A.* (*T.*) *pyogenes* with oligonucleotide primers which differ from the oligonucleotide primers used in the present study. Results from these two previous studies come in line with results of the present study, indicating that gene *plo* encoding pyolysin could also be used for genotypic identification of *A.* (*T.*) *pyogenes*.

More recently, the arcanolysin encoding gene *aln* from *A. haemolyticum* (accession number ACV96715) was described within the complete genome sequence of *A. haemolyticum* DSM 20595^{T} (NCBI, reference sequence: NC_014218) (Yasawong et al., 2010). Ülbegi (2010)

described the phocaelysin encoding gene *phl* from the two reference strains *A. phocae* DSM 10002^{T} and *A. phocae* DSM 10003 (accession number FN999907 and FN999908, respectively). However, hemolytic properties on sheep blood agar were also described for all other species of genera *Arcanobacterium* and *Trueperella* including *A.* (*T.*) *bernardiae, A. pluranimalium, A. hippocoleae, A.* (*T.*) *bialowiezense, A.* (*T.*) *bonasi* and *A.* (*T.*) *abortisuis* (Funke et al., 1995; Ramos et al., 1997; Lawson et al., 2001; Hoyles et al., 2002; Lehnen et al., 2006; Azuma et al., 2009). At present nothing is known about the corresponding gene of these hemolytic bacterial species.

According to Esmay et al. (2003), *A.* (*T.*) *pyogenes* expresses the collagen-binding protein CbpA encoded by gene *cbp*A. A *cbp*A mutant of *A.* (*T.*) *pyogenes* exhibited a reduced adhesion to both HeLa (epithelial) and 3T6 (fibroblast) cell lines, as the introduction of *cbp*A into a naturally negative isolate enhanced the adhesion to both cell lines (Esmay et al., 2003). Moreover, Pietrocola et al. (2007) showed that CbpA bound to almost all collagen types including type I, II, III, IV, V, IX and XI, and that CbpA shows a second binding activity for fibronectin and to a lesser extent for fibrinogen. Results by Esmay et al. (2003) and Pietrocola et al. (2007) highlighted the importance of CbpA as virulence factor which promotes adhesion and subsequently colonization of *A.* (*T.*) *pyogenes* to collagen rich tissues.

Using the oligonucleotide primers cbp-F and cbp-R originally described by Ülbegi (2010), gene *cbp*A with an amplicon size of approximately 330 bp could successfully be amplified for 11 (22 %) of the 51 *A.* (*T.*) *pyogenes* strains investigated. Gene *cbp*A could rarely be detected in *A.* (*T.*) *pyogenes* of bovine origin (5 %), more frequently in *A.* (*T.*) *pyogenes* isolated from pigs (54 %), dogs and cats (25 %) and reptiles (100 %). *A.* (*T.*) *pyogenes* from small ruminants and wild ruminants, horse, rabbit and rat did not carry gene *cbp*A. The differences in the presence of gene *cbp*A between strains from ruminants and pigs appeared to be significant ($P = 0.0004$). Gene *cbp*A could also be found in reference strain *A.* (*T.*) *pyogenes* DSM 20630^{T}, but not in *A.* (*T.*) *pyogenes* DSM 20594. However, other investigators reported about different results concerning the presence of gene *cbp*A in *A.* (*T.*) *pyogenes*. According to the DNA dot blotting results of Esmay et al. (2003), *cbp*A was found in *A.* (*T.*) *pyogenes* of bovine (49 %), porcine (40 %) and avian (100 %) origin. Isolates from dogs and cats did not carry *cbp*A. Silva et al. (2008) could detect *cbp*A in all investigated *A.* (*T.*) *pyogenes* (n = 57) isolated from cows with uterine infections. However, the results of the present study corresponded to the findings of Santos et al. (2010) who

found *cbp*A only in 1.4 % of the *A.* (*T.*) *pyogenes* isolated from bovines. In contrast to Silva et al. (2008) and Santos et al. (2010), in the present study *cbp*A could also be detected in reference strain *A.* (*T.*) *pyogenes* DSM 20630^T (ATCC 19411^T), originally isolated from a pig. These differences are possibly explicable by the different oligonucleotide primer pairs used for amplification of this potential virulence gene. The importance of the distribution of CbpA which occurred rarely among *A.* (*T.*) *pyogenes* strains isolated from bovines and more pronounced among strains isolated from pigs remains to be elucidated.

A neuraminidase of *A. pyogenes* was first reported for two of 42 *A.* (*T.*) *pyogenes* cultures by Schaufuss and Lämmler (1989). The two *A. pyogenes* neuraminidase encoding genes *nan*H and *nan*P were previously characterized by Jost et al. (2001, 2002b). Bacterial neuraminidases were described to have an important role in promoting adhesion to host epithelial cells (Galen et al., 1992; Giebink, 1999; Tong et al., 2000). Similarly, a *nan*H-*nan*P double mutant of *A.* (*T.*) *pyogenes* completely lacked neuraminidase expression and impaired the ability to adhere to HeLa cells, indicating the importance of neuraminidase of *A.* (*T.*) *pyogenes* as adhesion mediated virulence factor (Jost and Billington, 2005). According to Jost and Billington (2005), all *A.* (*T.*) *pyogenes* strains carried *nan*H, 64.2% *nan*P. In the study of Silva et al. (2008) all investigated *A.* (*T.*) *pyogenes* strains carried *nan*H and *nan*P.

Using the oligonucleotide primers described by Silva et al. (2008) 47 (92 %) of the *A.* (*T.*) *pyogenes* strains of the present study were positive for *nan*H and 40 (78 %) isolates positive for *nan*P. However, some of the *A.* (*T.*) *pyogenes* strains of the present study were negative for the two virulence genes. This negative reaction occurred singly (n = 1, negative for *nan*H; n = 8, negative for *nan*P) or in combination (n = 3, negative for both *nan*H and *nan*P), with no significant differences among the *A.* (*T.*) *pyogenes* strains of various origins. The two *A.* (*T.*) *pyogenes* reference strains carried both genes *nan*H and *nan*P. According to Jost and Billington (2005) a *nan*H-*nan*P double mutant of *A.* (*T.*) *pyogenes* exhibited 53 % reduction in its ability to bind host epithelial cells, indicating that the host cells might also be recognized by different *A.* (*T.*) *pyogenes* adherence proteins such as CbpA and fimbriae.

Fimbriae as proteinaceous appendages which emerge from bacterial cell surface might serve for adherence of numerous bacterial pathogens. They are well known in Gram-negative bacteria mediating the adherence to host tissue (Beachey, 1981; Clegg and Gerlach; 1987), while only few

Gram-positive bacteria had been described to produce fimbriae (Wu and Fives-Taylor, 2001; Ton-That et al, 2004). In 2005, Jost and Billington preliminarily characterized fimbriae from *A.* (*T.*) *pyogenes*. They also might be involved in adhesion of *A.* (*T.*) *pyogenes* to host tissue.

In the present study gene *fim*A could be amplified using oligonucleotide primers described by Silva et al. (2008) with an amplicon size of approximately 610 bp for 50 (98 %) of the 51 *A.* (*T.*) *pyogenes* strains investigated. Comparable to the results of the present study, gene *fim*A was found by Jost and Billington (2005) in 94% of the investigated *A.* (*T.*) *pyogenes* isolates, according to Silva et al. (2008) in 100% of the strains and according to Santos et al. (2010) in 90.9% of the *A.* (*T.*) *pyogenes* strains isolated from metritis compared to 64.1% of the strains isolated from non metritic cows ($P \leq 0.01$). However, in contrast to the findings of Silva et al. (2008) and Santos et al. (2010) *A.* (*T.*) *pyogenes* DSM 20630^T (ATCC 19411^T) investigated in the present study appeared to be *fim*A negative.

In addition, gene *fim*C could be amplified with an amplicon size of approximately 850 bp for 37 (73 %) and gene *fim*E with an approximate amplicon size of 780 bp for 50 (98 %) of the 51 *A.* (*T.*) *pyogenes* strains. According to Silva et al. (2008) gene *fim*C and *fim*E were found in 100% and 98 % of the investigated strains, respectively. In line with the results of Silva et al. (2008) *A.* (*T.*) *pyogenes* DSM 20630^T (ATCC 19411^T) investigated in the present study appeared to be *fim*C and *fim*E positive.

Comparing the origin of the strains and the presence of fimbrial encoding genes revealed that only *fim*C showed significant differences in its distribution among *A.* (*T.*) *pyogenes* isolated from different animals. *fim*C appeared to be present in *A. pyogenes* of bovine origin (95 %) and in *A.* (*T.*) *pyogenes* strains from wild ruminants, horse, rat and reptiles (100 %), but less pronounced in *A.* (*T.*) *pyogenes* from pigs (46 %), small ruminants (40 %) and from dogs and cats (50 %). The *A.* (*T.*) *pyogenes* strain isolated from a rabbit did not carry gene *fim*C. The differences in the presence of *fim*C between *A.* (*T.*) *pyogenes* strains from ruminants and pigs appeared to be significant ($P = 0.011$). The differences in the presence of *fim*A and *fim*E between *A.* (*T.*) *pyogenes* strains isolated from different animal species so far appeared to be not significant ($P \leq 0.05$). Thus, meaning of the distribution of these virulence genes among *A.* (*T.*) *pyogenes* strains of different animal origin remains unclear. However, none of the *A.* (*T.*) *pyogenes* strains investigated in the present study was negative for all three fimbrial encoding

genes, indicating, as already described for Gram-negative and other Gram-positive bacteria (Beachey, 1981; Clegg and Gerlach; 1987; Wu and Fives-Taylor, 2001; Ton-That et al, 2004), the general importance of these virulence factors for the adhesion of the bacteria to host tissues. The results of the present study allowed a correct identification and further characterization of *A.* (*T.*) *pyogenes* of various origins using phenotypical properties, MALDI-TOF MS fingerprinting and by PCR-mediated amplification of various species specific targets. The synergistic CAMP-like reactivities with several bacterial species as indicator strains, the investigation of proteolytic enzymatic activities on Loeffler medium and casein agar and the cross-reaction with group G-specific antisera could be used for presumptive identification of *A.* (*T.*) *pyogenes*, as well as for differentiation of this species from other species of genera *Arcanobacterium* and *Trueperella*. In addition, the newly described MALDI-TOF MS protein fingerprinting methodology used in the present study appeared to be a fast and reliable technique for identification and classification of *A.* (*T.*) *pyogenes* and for other species of genera *Arcanobacterium* and *Trueperella*. Finally, the DNA level-based identification could be used for identification and further characterization of *A.* (*T.*) *pyogenes* isolated from a number of various animal species and might help to understand the process of infection with this bacterial pathogen.

5.2 Phenotypic characteristics, MALDI-TOF MS fingerprinting results and genotypic characteristics of *A.* (*T.*) *abortisuis*

A. (*T.*) *abortisuis* was described by Azuma and coauthors in 2009 as a novel species of genus *Arcanobacterium.* According to Yassin et al. (2011), this species has to be classified into the newly described genus *Trueperella*. The original species description was based on a single strain isolated from a placenta of a sow after an abortion.

In the present study 23 *A.* (*T.*) *abortisuis* isolated from samples of pigs and cows within a 12 years (1999 to 2010) and the reference strain *A.* (*T.*) *abortisuis* DSM 19515^{T} could be characterized phenotypically as well as by using MALDI-TOF MS fingerprinting and genotypically by PCR-mediated identification methods. The 23 *A.* (*T.*) *abortisuis* strains were mainly isolated from the urogenital tract of pigs and cows with varying clinical symptoms. Of the 23 *A.* (*T.*) *abortisuis*, 16 strains were recovered from pigs (n = 14) and cows (n = 2) with fertility problems and genital tract infections, three strains from cases of abortion of three pigs, one strain

from septicemia of a pig, one strain post mortem from a male pig with respiratory problems and two strains from pigs without any additional information. According to Azuma et al. (2009), *A.* (*T.*) *abortisuis* was isolated from a placenta of a pig following an abortion. However, in the present study only three *A.* (*T.*) *abortisuis* strains recovered from abortion were isolated together with various other bacteria, indicating that the role this species plays as causative agent of abortion remains to be elucidated. It was of interest that the present study gives a first description of two *A.* (*T.*) *abortisuis* strains recovered from samples of cows.

According to the present results growth of all 23 *A.* (*T.*) *abortisuis* strains and the reference strain *A.* (*T.*) *abortisuis* DSM 19515^T could be achieved by cultivation on blood agar under aerobic conditions, microaerobic conditions in a candle jar and under anaerobic conditions. A slightly enhanced growth could be recognized after cultivation of the bacterium under microaerobic conditions in a candle jar and by anaerobic conditions, compared to aerobic conditions. However, Azuma et al. (2009) described *A.* (*T.*) *abortisuis* as strictly anaerobic. All 23 *A.* (*T.*) *abortisuis* and the reference strain *A.* (*T.*) *abortisuis* DSM 19515^T produced a narrow zone of complete hemolysis on sheep and rabbit blood agar. Investigating the hemolysis after cultivation on rabbit blood agar compared to sheep blood agar resulted in no enhancement of the hemolysis on rabbit blood agar. An enhanced hemolysis on rabbit blood agar compared to sheep blood agar was described for *A. haemolyticum* (Lämmler and Blobel, 1988) and to some extent for *A. hippocoleae* and *A. pluranimalium* (Ülbegi-Mohyla et al., 2009). However, the degree of hemolysis of the 23 *A.* (*T.*) *abortisuis* and the reference strain *A.* (*T.*) *abortisuis* DSM 19515^T on sheep blood agar appeared to be slightly enhanced after cultivation under anaerobic conditions.

A synergistic hemolytic reaction with staphylococcal β-hemolysin, *R. equi, A. haemolyticum* and with *A. phocae* as indicator strains could be observed for all 23 *A.* (*T.*) *abortisuis* and reference strain *A.* (*T.*) *abortisuis* DSM 19515^T. The results from the present study were comparable to previous results from Ülbegi (2010) showing a synergistic hemolytic reaction of reference strain *A.* (*T.*) *abortisuis* DSM 19515^T with staphylococcal β-hemolysin and *R. equi* as indicator strain. However, Yassin et al. (2011) described a negative CAMP reaction of *A.* (*T.*) *abortisuis* with staphylococcal β-hemolysin. No reverse CAMP reaction could be observed for *A.* (*T.*) *abortisuis*. The synergistic hemolytic reactivities of *A.* (*T.*) *abortisuis* corresponded to synergistic hemolytic reactivities of the *A.* (*T.*) *pyogenes* strains of the present study, indicating that both bacterial

species may exhibit comparable exotoxin proteins reacting synergistically with the various known or hitherto unknown hemolytic exosubstances of the indicator strains.

For biochemical characterization of the 23 *A*. (*T*.) *abortisuis* strains and the reference strain *A*. (*T*.) *abortisuis* DSM 19515^T investigated in the present study the commercial identification system API Coryne was used. This test yielded 15 numerical profiles for the 24 *A*. (*T*.) *abortisuis* strains investigated. However, because *A*. (*T*.) *abortisuis* is not included in the database (version 1.2.1, 2003) the numerical profiles could not be identified by the API Coryne test system. In addition, the *A*. (*T*.) *abortisuis* strains were investigated with tablets containing various substrates, with 4-methylumbelliferyl conjugated substrates and with various other phenotypic tests.

Using the API Coryne system, tablets containing various substrates, 4-methylumbelliferyl conjugated substrates and various other phenotypic tests all 23 *A*. (*T*.) *abortisuis* strains and *A*. (*T*.) *abortisuis* DSM 19515^T showed a positive reaction for the enzymes pyrazinamidase, β-D-glucuronidase, β-D-galactosidase and α-D-glucosidase, for esculin hydrolysis and for degradation of D-glucose and D-maltose. These results corresponded to the results recognized by Azuma et al. (2009) and Ülbegi (2010) for reference strain *A*. (*T*.) *abortisuis* DSM 19515^T. Also comparable to Azuma et al. (2009) and Ülbegi (2010) were negative reactions by all 24 *A*. (*T*.) *abortisuis* strains of the present study, also including the reference strain, were observed for the enzymes N-acetyl-β-D-glucosaminidase, urease, gelatinase, α-mannosidase and catalase. However, a positive catalase test was described for the *A*. (*T*.) *abortisuis* strain investigated by Yassin et al. (2011). Variable results could be detected for the enzymes nitrate reductase, pyrrolidonyl arylamidase and alkaline phosphatase and for degradation of D-ribose, D-xylose, D-mannitol, D-lactose, D-saccharose and glycogen. However, corresponding with the results of Azuma et al. (2009) and Ülbegi (2010) degradation of D-lactose and D-saccharose but not of D-xylose and D-mannitol could be observed for reference strain *A*. (*T*.) *abortisuis* DSM 19515^T.

It was of interest that all *A*. (*T*.) *pyogenes* strains investigated in the present study and 28 *A*. (*T*.) *pyogenes* strains investigated by Ülbegi (2010) yielded positive reactions for the enzymes gelatinase and N-acetyl-β-D-glucosaminidase, indicating the usefulness of these two enzymes for phenotypical differentiation of the closely related species *A*. (*T*.) *pyogenes* and gelatinase and N-acetyl-β-D-glucosaminidase negative *A*. (*T*.) *abortisuis*. Comparable to Azuma et al. (2009) and Ülbegi (2010) all 24 investigated *A*. (*T*.) *abortisuis* strains produced the enzymes amylase and

DNase but not hyaluronidase. However, as mentioned before hyaluronidase enzyme activities are not suitable for discriminating between different species of genera *Arcanobacterium* and *Trueperella*.

In addition, all 24 *A.* (*T.*) *abortisuis* strains, also including the reference strain *A.* (*T.*) *abortisuis* DSM 19515^T, displayed neither liquefaction of Loeffler medium, caseinase activity nor any cross-reaction with streptococcal serogroup G-specific antiserum. These results also come in line with results obtained from Ülbegi (2010) investigating reference strain *A.* (*T.*) *abortisuis* DSM 19515^T. Both proteolytic enzymatic activities and a cross-reaction with streptococcal serogroup G-specific antiserum are well known as typical properties of *A.* (*T.*) *pyogenes* (Bisping and Amtsberg 1988; Lämmler and Blobel, 1988; Lämmler and Hartwigk 1995; Takeuchi et al., 1995). However, these proteolytic activity tests and the serological test could also be used as additional criteria to differentiate *A.* (*T.*) *pyogenes* and *A.* (*T.*) *abortisuis*.

Furthermore, MALDI-TOF MS allowed a correct identification of 22 *A.* (*T.*) *abortisuis* strains to the species level matching with log (score) values ranging from 2.36 to 2.69. This also included both *A.* (*T.*) *abortisuis* strains of bovine origin with log (score) values of 2.53 and 2.40, respectively. However, as already mentioned the newly described species *A.* (*T.*) *abortisuis* was not included in the database of the MALDI Biotyper 2.0 software. Therefore the MSP of reference strain *A. abortisuis* DSM 19515^T of the present study was used as matching partner for identification of the 23 *A.* (*T.*) *abortisuis* strains. The MALDI Biotyper (Bruker Daltonik) also allowed a comparison of the log (score) values of the 22 strains among each other with the lowest log (score) value of 2.138 (*A.* (*T.*) *abortisuis* strain 6320/08 of bovine origin to *A.* (*T.*) *abortisuis* strain 1672/02 of pig origin) to a maximal score of 2.83 (*A.* (*T.*) *abortisuis* strain 341/10 to *A.* (*T.*) *abortisuis* strain 343/10, both isolated from two different pigs of the same farm). Likewise strains of bovine origin matched in MALDI Biotyper analysis among themselves with a log (score) value of 2.83. These high and almost identical log (score) values of both pig and both bovine strains possibly indicate a clonal relationship of the strains which were isolated from one farm, respectively. Strain *A.* (*T.*) *abortisuis* P8609/00 matched against *A.* (*T.*) *abortisuis* DSM 19515^T with a log (score) value of 1.77. This strain could not be identified to species level but to genus level. However, synergistic CAMP-like reactions, phenotypic properties and sequencing of the 16S rDNA of strain P8609/00 indicated that this strain could be classified to the species *A.* (*T.*) *abortisuis.* Sequencing of the 16S rRNA of *A.* (*T.*) *abortisuis* P8609/00 (partial sequence, 1146

bp) revealed a sequence homology of 99.7 % to 16S rRNA gene of reference strain *A.* (*T.*) *abortisuis* DSM 19515^T. In addition, a dendrogram analysis of the 16S rRNA sequence of this strain together with 16S rRNA sequences of nine reference strains of genera *Arcanobacterium* and *Trueperella*, also including reference strain *A.* (*T.*) *abortisuis* DSM 19515^T obtained from NCBI GenBank allowed an unequivocal identification as *A.* (*T.*) *abortisuis*.

Moreover, sequencing the 16S-23S rDNA intergenic spacer region (ISR) allowed the design of *A.* (*T.*) *abortisuis* ISR specific oligonucleotide primers which could be used for PCR-mediated identification of all 24 *A.* (*T.*) *abortisuis* strains, also including reference strain *A.* (*T.*) *abortis*uis DSM 19515^T and *A.* (*T.*) *abortisuis* P8609/00. The *A.* (*T.*) *abortisuis* ISR specific PCR yielded no cross reaction with the control strains representing eight other species of genera *Arcanobacterium* and *Trueperella*, indicating the usefulness of this specific PCR for identification of the species *A.* (*T.*) *abortisuis*.

Obviously *A*. (*T.*) *abortisuis* P8609 represents a biological variation of this species. Inclusion of this strain into the MALDI reference database will enhance detection of *A*. (*T.*) *abortisuis* in future.

The results of the investigation of 23 *A.* (*T.*) *abortisuis* strains isolated from pigs and cows as well as the reference strain *A.* (*T.*) *abortisuis* DSM 19515^T presented here give a first detailed characterization of this newly described species. The cultural properties, the synergistic CAMP-like reactivities with several bacterial species as indicator strains and the phenotypic tests could possibly help veterinary diagnostic laboratories for presumptive identification of *A.* (*T.*) *abortisuis*. However, MALDI-TOF MS also seems to be a promising tool as fast and accurate method for identification of this newly described species. This could be supplemented genotypically using *A. abortisuis* ISR specific oligonucleotide primers which allowed a PCR-mediated identification of all 23 *A*. (*T.*) *abortisuis* strains and reference strain *A.* (*T.*) *abortisuis* DSM 19515^T of the present study. Comparable ISR specific oligonucleotide primers had already been used for PCR-mediated identification of *A.* (*T.*) *bialowiezense* and *A.* (*T.*) *bonasi* (Hassan et al., 2008), for the *A.* (*T.*) *pyogenes* strains and the *A. haemolyticum* strains of the present study and additional 28 *A.* (*T.*) *pyogenes* strains investigated by Ülbegi (2010) and seven *A. haemolyticum* strains investigated by Hassan et al. (2009).

5.3 Phenotypic characteristics, MALDI-TOF MS fingerprinting results and genotypic characteristics of *A. haemolyticum*

A. haemolyticum is well known as a human pathogen associated with pharyngitis and skin infections (MacLean et al., 1946; Banck et al., 1986; Waagner, 1991; Esteban et al., 1994). However, at present only few reports had been described about the isolation of *A. haemolyticum* from animal infections (Richardson and Smith, 1968; Roberts, 1969; Tyrrell et al., 2002). In 2009, Hassan et al. characterized phenotypically and genotypically seven *A. haemolyticum* isolated from infections of six horses. More recently, Bancroft-Hunt et al. (2010) characterized an *A. haemolyticum* strain isolated in pure culture from a necrotic fasciitis in a bull.

The results of the present study, identifying three *A. haemolyticum* strains isolated from different horse infections, come in line with the study of Hassan et al. (2009), and indicate again that this generally human pathogen sporadically also can be isolated from horses.

A. haemolyticum, originally known as *Corynebacterium haemolyticum* (MacLean et al., 1946) was, as proposed by Collins et al. (1982b) the first species of genus *Arcanobacterium*. According to Collins et al. (1982b), Lämmler and Hartwigk (1995), Goyal et al. (2005) and Tan et al. (2006), colonies of *A. haemolyticum* were, after 24-48 h incubation on sheep blood agar, surrounded by a zone of complete hemolysis. Accordingly all three *A. haemolyticum* strains isolated from infections of three horses investigated here and reference strain *A. haemolyticum* DSM 20595^T produced, after cultivation under microaerobic conditions in a candle jar, a narrow zone of complete hemolysis on sheep blood agar. Several authors had shown that hemolysis of *A. haemolyticum* was significantly increased after cultivation on rabbit and human blood agar media compared to hemolysis after cultivation on sheep blood agar (Lämmler and Blobel, 1988; Cummings et al., 1993; Lämmler, 1994). This enhanced hemolysis after cultivation on rabbit blood agar could also be demonstrated for all three *A. haemolyticum* strains and reference strain *A. haemolyticum* DSM 20595^T investigated in the present study. More recently, Ülbegi (2010) also described seven *A. haemolyticum* strains isolated from infections of horses displaying the phenomenon of an enhanced hemolysis on rabbit blood agar. Obviously, the hemolytic property on rabbit blood agar compared to sheep blood agar appeared to be a typical property of *A. haemolyticum* and could be used for presumptive identification of this species (Lämmler and Blobel, 1988; Hassan et al., 2009; Ülbegi-Mohyla et al., 2009).

Several previous studies had investigated synergistic or CAMP-like and antagonistic hemolytic reactions of *A. haemolyticum*. Frazer (1964) described synergistic CAMP-like reactions of *A. haemolyticum* cultivated on sheep blood agar in the presence of *S. agalactiae* and *R. equi*. In 2009, Ülbegi et al. described a novel CAMP-like reaction observed between *P. phenylpyruvicus*, isolated from a sample of a harbour seal and *A. haemolyticum* and *A. phocae* of genus *Arcanobacterium*. Moreover, Fraser (1964) described exosubstances of *A. haemolyticum* that exhibited an antagonistic effect on staphylococcal β-hemolysin leading to a reverse CAMP reaction. The exosubstances of *A. haemolyticum* responsible for the inhibition of staphylococcal β-hemolysin were identified as phospholipase D (Souckova and Soucek, 1972). A reverse CAMP-reaction in the zone of staphylococcal β-hemolysin appeared to be a characteristic property of *A. haemolyticum* and *A. phocae* (Fraser, 1964; Linder, 1997; Johnson et al., 2003; Ülbegi-Mohyla et al., 2009; Yassin et al., 2011).

In the present study, all three *A. haemolyticum* strains and reference strain *A. haemolyticum* DSM 20595^T demonstrated synergistic CAMP-like reaction with *S. agalactiae, R. equi, P. phenylpyruvicus* and with bacteria of genera *Arcanobacterium* and *Trueperella*, namely *A.* (*T.*) *pyogenes, A.* (*T.*) *abortisuis, A. hippocoleae* and *A. pluranimalium* as indicator strains and a reverse CAMP reaction in the zone of staphylococcal β-hemolysin. These results come in line with several previous studies (Lämmler and Blobel, 1988; Lämmler, 1994; Linder, 1997; Lämmler and Hartwigk, 1995; Almuzara et al., 2002; Goyal et al., 2005; Parija et al., 2005; Tan et al., 2006; Hassan et al., 2009; Ülbegi-Mohyla et al., 2009). According to Ülbegi (2010) the results investigating synergistic or CAMP-like reaction and antagonistic hemolytic reactions of *A. phocae* (n = 43) corresponded to the results obtained for *A. haemolyticum* (n = 7), indicating that this test could not be used for differentiation of these two closely related species of genus *Arcanobacterium*.

The biochemical characterization of the three *A. haemolyticum* strains investigated in the present study and reference strain *A. haemolyticum* DSM 20595^T with the commercial identification system API-Coryne-test was generally in agreement with the results of other authors (MacLean et al., 1946; Jobanputra and Swain, 1975; Lämmler and Blobel, 1988; Ding and Lämmler, 1992; Carlson et al., 1994; Lawson et al., 2001; Hoyles et al., 2002; Johnson et al., 2003; Goyal et al., 2005; Parija et al., 2005; Hassan et al., 2009; Lehnen et al.; 2006). Using API-Coryne database the seven-digit codes obtained for the three *A. haemolyticum* strains yielded an identity of

99.9 % as *A. haemolyticum*. The accuracy of the API Coryne test system for identification of *A. haemolyticum* had already been demonstrated by Freney et al. (1991) and Gavin et al. (1992). Additional enzymatic investigations for the *A. haemolyticum* strains of the present study had been performed using tablets containing various substrates, with 4-methylumbelliferyl conjugated substrates and various other phenotypic tests. Using API Coryne system, tablets containing various substrates and 4-methylumbelliferyl conjugated substrates all *A. haemolyticum* strains investigated in the present study, also including reference strain *A. haemolyticum* DSM 20595^T, showed a positive reaction for the enzymes pyrazinamidase, β-D-galactosidase, α-D-glucosidase and N-acetyl-β-glucosaminidase and for degradation of D-glucose, D-ribose, D-maltose and D-lactose. Moreover, enzyme α-mannosidase could be detected for all investigated *A. haemolyticum* strains, indicating, as described by Carlson and Kontiainen (1994), that a positive α-mannosidase test appeared to be typical and could be used for identification of *A. haemolyticum*. Negative reactions could be observed for nitrate reductase, esculin hydrolysis, urease, gelatinase and for degradation of D-xylose, D-mannitol, D-saccharose and glycogen. These results were generally in agreement to results obtained by several authors (Lämmler and Blobel, 1988; Lawson et al., 2001; Hoyles et al., 2002; Hassan et al., 2009; Azuma et al., 2009; Ülbegi, 2010, Yassin et al., 2011).

In addition, two of the three *A. haemolyticum* strains isolated from horses displayed a moderate liquefaction of Loeffler medium and a hydrolysis of casein. These results were comparable to the results of Hassan et al. (2009) investigating seven *A. haemolyticum* strains isolated from infections of horses. However, the extracellular substance causing the moderate serum liquefaction of the two *A. haemolyticum* strains of the present study and the seven *A. haemolyticum* strains described by Hassan et al. (2009) is not known. As described before liquefaction of Loeffler medium is a typical property of *A.* (*T.*) *pyogenes* and could be used for preliminary identification of this species (Bisping and Amtsberg 1988; Lämmler and Blobel, 1988; Lämmler and Hartwigk 1995). In the present study, all three *A. haemolyticum* strains and reference strain *A. haemolyticum* DSM 20595^T were DNase positive, but catalase and hyaluronidase negative. These results were in agreement with previous results of Hassan et al. (2009). In contrast to reference strain *A. haemolyticum* DSM 20595^T starch hydrolysis could be observed for all three *A. haemolyticum* field strains investigated in the present study. Furthermore, all three *A. haemolyticum* strains and reference strain *A. haemolyticum* DSM 20595^T did not show a cross-reaction with group G-

specific antiserum. However, as described before a cross-reaction with group G-specific antiserum was considered as typical characteristic of *A.* (*T.*) *pyogenes*.

Use of MALDI-TOF MS allowed a correct identification of the three *A. haemolyticum* strains isolated from infections of three horses to the species level matching against reference strain *A. haemolyticum* DSM 20595^T with log (score) values between 2.41 to 2.44. In addition, the MALDI Biotyper software (Bruker Daltonik) allowed a comparison of the log (score) values of the three *A. haemolyticum* strains among each other with log (score) values between 2.60 to 2.70. Comparable to the results of the *A.* (*T.*) *pyogenes* and the *A.* (*T.*) *abortisuis* strains investigated in the present study the MSPs of the three *A. haemolyticum* strains showed that MALDI-TOF MS also allowed a correct identification of this generally human pathogenic species of genus *Arcanobacterium* isolated from infections of horses.

Using the primer pair described by Kostman et al. (1995) and Chanter et al. (1997) for amplification and sequencing of ISR all three *A. haemolyticum* strains investigated in the present study could be identified genotypically. The ISR sequences of the three *A. haemolyticum* strains yielded an almost complete sequence identity from 99.0 % to 99.8 % to the corresponding sequence of reference strain *A. haemolyticum* DSM 20595^T obtained from GenBank database. Furthermore, a dendrogram analysis of the ISR sequences of the three *A. haemolyticum* strains and reference strain *A. haemolyticum* DSM 20595^T together with eight other reference strains of genera *Arcanobacterium* and *Trueperella* showed this identity.

Sequencing of the ISR had already been shown to be useful target for genotypic characterization of all nine species of genera *Arcanobacterium* and *Trueperella* (Hassan et al., 2009; Ülbegi, 2010). As already mentioned before ISR sequencing of species of genera *Arcanobacterium* and *Trueperella* also allowed the design of species specific oligonucleotide primers which could be used for PCR-mediated identification of *A.* (*T.*) *pyogenes* and *A.* (*T.*) *abortisuis* investigated in the present study and for the species *A.* (*T.*) *bernardiae*, *A.* (*T.*) *bialowiezense*, *A.* (*T.*) *bonasi*, *A. haemolyticum* and *A. hippocoleae* (Ülbegi, 2010). According to the results of the present study the *A. haemolyticum* ISR-23S rDNA specific oligonucleotide primers described by Ülbegi (2010) could successfully be used to identify the three *A. haemolyticum* strains and reference strain *A. haemolyticum* DSM 20595^T investigated in the present study.

The species identity of the *A. haemolyticum* strains investigated in the present study could also be confirmed by PCR-mediated amplification of a species-specific region of gene *pld* encoding phospholipase D of *A. haemolyticum*. The phospholipase D encoding gene *pld* from *A. haemolyticum* was cloned and sequenced and showed a DNA sequence homology of 65 % to the corresponding genes of *Corynebacterium pseudotuberculosis* and *Corynebacterium ulcerans* (Cuevas and Songer, 1993; McNamara et al, 1995). Recently, Lucas et al. (2010) had characterized *A. haemolyticum* gene *pld*. According to Lucas et al. (2010), gene *pld* is found on a genome region of reduced G + C content, suggesting the recent horizontal acquisition. The importance of gene *pld* as a virulence factor of *A. haemolyticum* for disease pathogenesis through enhancing bacterial adhesion and promoting the host cell necrosis following invasion was also described by Lucas et al. (2010). Species specific parts of gene *pld* of *C. pseudotuberculosis* had previously been shown to be a useful target for molecular identification of this species (Pacheco et al., 2007). In 2009, *A. haemolyticum pld* specific oligonucleotide primers described by Hassan et al. (2009) had been successfully used for genotypic identification of seven *A. haemolyticum* strains isolated from infections of horses.

In the present study, this *A. haemolyticum pld* specific oligonucleotide primer pair described by Hassan et al. (2009) also allowed a PCR-mediated identification of all three *A. haemolyticum* strains.

An additional PCR-mediated identification of *A. haemolyticum* could be achieved in the present study by amplification of *A. haemolyticum* specific regions of CAMP factor family protein encoding gene *cfa.* Originally, the well known CAMP-reaction is a characteristic synergistic hemolytic reaction of *S. agalactiae* cultivated in the zone of incomplete staphylococcal β-hemolysin (Christie et al., 1944). The protein responsible for the CAMP reaction was described as sphingomyelinase from *S. aureus* and CAMP factor, a protein secreted by *S. agalactiae* (Brown et al., 1974). Sphingomyelinase initially hydrolyzes sphingomyelin to ceramide which makes erythrocytes susceptible to the lytic activity of CAMP factor (Lang and Palmer, 2003). The CAMP factor genes of *S. agalactiae*, *Streptococcus uberis* and *Streptococcus pyogenes* have been cloned in *E. coli*, and their sequences were found to be highly homologous among each other (Gase et al., 1999).

The *A. haemolyticum cfa* specific oligonucleotide primer pair designed in the present study could successfully be used for identification of all three investigated *A. haemolyticum* strains and

reference strain *A. haemolyticum* DSM 20595^T with an approximate amplicon size of 650 bp. However, at present, nothing is known about properties of CAMP factor of *A. haemolyticum* and its potential role in the pathogenesis of this species.

All three species specific PCR revealed no cross reactivity with the control strains representing eight other species of genera *Arcanobacterium* and *Trueperella*, indicating the usefulness of all three species specific oligonucleotide primers for genotypic identification of this species.

Generally, cultural properties, the enhanced hemolysis after cultivation on rabbit blood agar compared to sheep blood agar, the synergistic CAMP-like reactivities with several bacterial species as indicator strains, the reverse CAMP reaction in the zone of β-hemolytic *S. aureus* and the phenotypic tests including the API Coryne test system allow a preliminary identification of *A. haemolyticum*, as well as a differentiation of this species from the closely related species *A. phocae.* In addition, MALDI-TOF MS correctly identified the *A. haemolyticum* to the species level, indicating that this newly described technique could also be used for identification of this originally human pathogenic species of genus *Arcanobacterium* isolated from animals. Genotypically, identification of the *A. haemolyticum* strains of the present study could be confirmed by amplification and sequencing of the genome segment ISR and by PCR-mediated amplification of species specific regions of three different targets. Comparable to Hassan et al. (2009) the results of the present study might help to elucidate the importance of this well-known human pathogenic bacterium as causative agent of infections of horses and possibly of other animals. However, at present nothing is known about a possible role the horse owner might play in the infection process.

5.4 Phenotypic characteristics, MALDI-TOF MS fingerprinting results and genotypic characteristics of *A. weissii*

In the present study three bacterial strains which were isolated together with several other bacterial species from infections of the oral cavity of three dogs suffering from a wound infection caused by a foreign body, a gingivitis and a periodontitis, respectively could be identified by conventional tests, by MALDI-TOF MS and by sequencing the 16S rDNA and additional target genes as new species belonging to genus *Actinomyces*, for which the name *Actinomyces weissii* sp. nov. was proposed.

Genus *Actinomyces* is one of the largest genera within the class *Actinobacteria* and comprises a broad spectrum of anaerobic or facultatively anaerobic, asporogenous, non-motile, non-acid fast, Gram-positive, filamentous or diphtheroid, rod shaped organisms with a high G + C content (Schaal, 1986). Branching rods are common (10-50 µm, long) and may occur singly or in pairs (Holt et al., 1994). Several species of genus *Actinomyces* are long-established as pathogens of humans and animals (Schaal, 1986; Holt et al., 1994; Hoyles et al., 2001; Lawson et al., 2001). However, in recent years various new species of genus *Actinomyces* such as *Actinomyces ruminicola* (An et al., 2006), *Actinomyces oris* and *Actinomyces johnsonii* (Henssge et al., 2009), *Actinomyces massiliensis* (Renvoise et al., 2009), *Actinomyces timonensis* (Renvoise et al., 2010) and *Actinomyces hominis* (Funke et al., 2010) had been described. At present, genus *Actinomyces* comprises 44 species (http://www.bacterio.cict.fr/a/actinomyces.html). According to Holt et al. (1994) and Hoyles et al. (2001) members of genus *Actinomyces* occur mainly in the oral cavity and on mucous membranes of humans and animals. Bacteria of this genus commonly cause in association with concomitant bacteria various pyogenic infections.

Schaal (1986), Holt et al. (1994), Collins et al. (2000), Hoyles et al. (2001), Lawson et al. (2001) and Renvoise et al. (2009) described that bacteria of genus *Actinomyces* could be cultivated on agar containing sheep or horse blood under an optimum growth temperature between 35 ^{o}C and 37 ^{o}C. However, some studies reported about hemolytic activities of *Actinomyces* species. This was reported for *Actinomyces neuii* subsp. *anitratus* (Funke et al., 1994), *Actinomyces europaeus* (Funke et al., 1997) and *Actinomyces turicensis* and *Actinomyces radingae* (Vandamme et al., 1998).

In the present study, the cultivation of the *A. weissii* strains was performed on sheep blood agar for 48 to 72 h at 37 ^{o}C under microaerobic conditions in a candle jar. The colonies showed after 72 h incubation a white-greyish appearance with a diameter of 1 mm and a weak zone of hemolysis. However, growth and hemolysis were less pronounced after cultivation under aerobic and under anaerobic conditions. No enhanced hemolysis could be observed after cultivation on rabbit blood agar compared to sheep blood agar. As mentioned before an enhanced hemolysis on rabbit blood agar compared to sheep blood agar was described for *A. haemolyticum* (Lämmler and Blobel, 1988; Hassan et al., 2009) and to some extent for *A. hippocoleae* and *A. pluranimalium* (Ülbegi-Mohyla et al., 2009). The morphological description of members of genus *Actinomyces* mentioned before (Schaal, 1986; Holt et al., 1994) come in line with the

results obtained for the three *A. weissii* strains of the present study. According to microscopical investigations the *A. weissii* strains appeared to be Gram-positive, rod-shaped and non-spore forming. Using light microscopy and phase contrast microscopy *A. weissii* 2298^T (CIP 110333^T) and *A. weissii* 4321 appeared with branches also forming a hyphae-like structure. However, *A. weissii* 1656 was rod shaped and occurred singly or in pairs without hyphae structure. Comparable to other bacterial species of genus *Actinomyces* (Holt et al., 1994; Renvoise et al., 2009) the three *A. weissii* strains of the present study were non-motile.

As already mentioned before the *A.* (*T.*) *pyogenes*, *A.* (*T.*) *abortisuis* and the *A. haemolyticum* strains investigated in the present study displayed synergistic or CAMP-like hemolytic activities on sheep blood agar in the presence of various indicator strains and *A. haemolyticum* a reverse CAMP reaction in the presence of *S. aureus* β-hemolysin. These are typical properties of bacteria of genera *Arcanobacterium* and *Trueperella* (Ülbegi-Mohyla et al., 2009).

However, the three *A. weissii* strains of the present study also displayed synergistic CAMP-like activities with *S. agalactiae, R. equi, P. phenylpyruvicus, A.* (*T.*) *pyogenes, A.* (*T.*) *abortisuis, A. hippocoleae* and *A. pluranimalium* as indicator strain and a reverse CAMP reaction in the zone of *S. aureus* β-hemolysin. The reference strains *A. bovis* DSM 43014^T and *A. canis* DSM 15536^T studied for comparative purposes were found to be negative for CAMP-like hemolytic reactions and for the reverse CAMP reaction. Funke et al. (1994; 2010) described a positive CAMP reaction for *A. neuii* and *A. hominis*. However, the results of the present study give a first description of a reverse CAMP-reaction of bacteria of genus *Actinomyces*.

It was of interest that the synergistic or CAMP-like hemolytic activities and the reverse CAMP reaction of the *A. weissii* strains displayed on sheep blood agar in the presence of various indicator strains were comparable to the corresponding results obtained for the *A. haemolyticum* strains investigated in the present study and for *A. phocae* (Ülbegi-Mohyla et al., 2009; Ülbegi, 2010), indicating that the three bacterial species of closely related taxa may exhibit comparable exotoxin proteins reacting synergistically and antagonistically with the various known or hitherto unknown hemolytic exosubstances of the indicator strains.

A biochemical characterization of the *A. weissii* strains and the reference strains *A. bovis* DSM 43014^T and *A. canis* DSM 15536^T was performed with the commercial identification system API Coryne, with tablets containing various substrates, with 4-methylumbelliferyl conjugated substrates and with various other phenotypic tests. The results of the API Coryne test system

yielded the two numerical profiles 0570167 and 0530167 for the three *A. weissii* strains. Using the API Coryne database both numerical profiles did not allow a species identification of the hitherto unknown species *A. weissii*. In addition, *A. bovis* DSM 43014^T and *A. canis* DSM 15536^T yielded the two numerical profiles 2470163 and 0430767, respectively which were also not included in the API Coryne database. However, the numerical profiles of *A. bovis* and *A. canis* of the present study are of particular value in distinguishing both species from *A. weissii*.

The phenotypic characteristics of the three *A. weissii* strains revealed that all three strains displayed almost identical properties. Differences among the three strains could be observed for esculin hydrolysis, α-mannosidase and DNase. Comparable to the reference strains *A. bovis* DSM 43014^T and *A. canis* DSM 15536^T the three *A. weissii* strains displayed neither liquefaction of Loeffler medium nor hydrolysis of casein. The phenotypic properties of the reference strains *A. bovis* DSM 43014^T and *A. canis* DSM 15536^T of the present study were generally in agreement with several previous studies (Pine et al., 1960; Collins et al., 2000; Hoyles et al., 2000; Hoyles et al., 2001).

Investigating the three *A. weissii* strains by MALDI-TOF MS also did not allow an identification of the hitherto unknown bacterial species to species level. Using the MALDI Biotyper 2.0 software package the MSPs of the three strains matched with log (score) values 1.70 against reference library entries and with the newly acquired MSP from reference strain *A. bovis* DSM 43014^T. The *A. weissii* strains matched with log (score) values between 0.38 and 0.72 to *A. bovis* DSM 43014^T and with log (score) values between 0.43 and 0.84 to *A. canis* DSM 15536^T. However, the software program allowed a comparison of the log (score) values of the three *A. weissii* strains isolated from three dogs among each other with log (score) values between 2.55 to 2.70, indicating that the three strains could be classified to one species. *A. bovis* DSM 43014^T and *A. canis* DSM 15536^T investigated comparatively matched to each other with a log (score) value of 0.23. Moreover, a dendrogram analysis of the MSPs of the three *A. weissii* strains together with different other species of genus *Actinomyces* obtained from Bruker reference library clearly separated the novel species *A. weissii* from other species of genus *Actinomyces*.

As already described, the 16S rRNA gene is well known as a universal target among bacteria and large enough for informatic purposes. This gene had emerged among the most informative targets as rDNA-based molecular identification tool for bacterial phylogeny and to ascertain the degree of genetic relatedness of different microorganisms (Weisburg et al., 1991; Patel, 2001; Cai et al.,

2003; Ludwig, 2010). However, as mentioned before no universal definition for bacterial species identification using 16S rDNA sequencing exists, but several investigators had proposed acceptable criteria for establishing a species and genus identification cutoff. A value of ≥ 99 % similarity of 16S rDNA sequence was assigned as suitable cutoff for bacterial species identification and ≥ 97 % for bacterial identification at genus level (Drancourt et al., 2000). However, 16S rDNA sequencing results had already facilitated the assignment of several new bacterial species to genus *Actinomyces* and considered valuable when phenotypic classification are of limited value (Ramos et al., 1997; Henssge et al., 2009).

To ascertain the phylogenetic classification of the *A. weissii* strains investigated in the present study, their 16S rRNA genes were sequenced and subjected to a comparative analysis doing alignment studies. The almost complete 16S rDNA sequences of *A. weissii* 2298^T, *A. weissii* 4321 and *A. weissii* 1656 with a continuous stretch of 1379 bp, 1347 bp and 1333 bp, respectively yielded an almost complete sequence identity with 99.7 % to 99.9 % sequence similarity of the three strains among each other. Sequence similarities to other selected species of genera *Actinomyces* were between 88.3 % and 97.2 %. The highest sequence similarity could be observed for *A. bovis* DSM 43014^T (97.2 % similarity). According to these sequencing results the hitherto unknown species *A. weissii* sp. nov. with the type strain 2298^T (CIP 110333^T) should be classified to genus *Actinomyces*. Dendrogram analysis of the 16S rDNA sequences of the three *A. weissii* strains of the present study and some related bacterial species obtained from NCBI GenBank also reflected the high similarity of the *A. weissii* strains and identified them as members of genus *Actinomyces*.

As mentioned before the 23S rRNA gene contains a large number of sequence variations which also allow phylogenetic studies (Lewin, 1998). This gene had already been used for molecular identification of an *A. pluranimalium* strain isolated from pyoderma of a dog and for differentiation of all nine species of genera *Arcanobacterium* and *Trueperella* (Ülbegi-Mohyla et al., 2010).

In the present study the 23S rRNA gene of *A. weissii* 2298^T, *A. weissii* 4321, *A. weissii* 1656 and for reference strains *A. bovis* DSM 43014^T and *A. canis* DSM 15536^T was amplified and sequenced revealing a continuous stretch of 704 bp, 683 bp, 669 bp, 687 bp and 655 bp, respectively. Alignment studies of the obtained partial sequences of the 23S rDNA of the *A. weissii* strains showed a complete sequence identity of the three strains among each other (100

% similarity), to *A. bovis* DSM 43014^T a sequence similarity between 97.3 % and 97.4 % and to *A. canis* DSM 15536^T a sequence similarity of 90.8%. Sequence similarities of the 23S rDNA sequences of *A. weissii* to 23S rDNA sequences of bacteria of genera *Arcanobacterium* and *Trueperella* were between 87.5 % and 90.0 %. These results also indicated that the three *A. weissii* strains represented a single species and should be classified to genus *Actinomyces*. This could also be demonstrated by hierarchical cluster analysis of 23S rRNA gene sequences of the three *A. weissii* strains, *A. bovis* DSM 43014^T, *A. canis* DSM 15536^T and nine reference strains of genera *Arcanobacterium* and *Trueperella*.

According to Goh et al. (1996) and Hill et al. (2004), gene *cpn*60 encoding the 60 kDa chaperonin protein or heat shock protein (HSP60) appeared to be present in all bacteria. This universal nature of gene *cpn*60 could be used for molecular identification of microorganisms as well as for phylogenetic studies which resulted in the introduction of a *cpn*60 database (cpnDB, www.cpndb.ca) (Hill et al., 2004). More recently, sequencing gene *cpn*60 were shown to be useful for molecular identification of *Lactobacillus* spp. (Blaiotta et al., 2008), *Aeromonas* spp. (Miñana-Galbis et al., 2009) and several other bacterial species (Schellenberg et al., 2011; Verbeke et al., 2011).

Using the oligonucleotide primer pairs described in the present study the hitherto unknown gene *cpn*60 of bacteria of genera *Arcanobacterium* and *Trueperella* was amplified and sequenced. *A.* (*T.*) *abortisuis* DSM 19515^T, *A.* (*T.*) *bernardiae* DSM 9152^T, *A.* (*T.*) *bialowiezense* DSM 17162^T, *A.* (*T.*) *bonasi* DSM 17163^T and *A. haemolyticum* DSM 20595^T, *A. hippocoleae* DSM 15539^T, *A. phocae* DSM 10003 and *A. pluranimalium* DSM 13483^T yielded a continuous stretch of 369 bp, 411 bp, 407 bp, 412 bp, 341 bp, 223 bp, 345 bp and 191 bp, respectively. The *cpn*60 gene sequence of *A.* (*T.*) *pyogenes* was obtained from NCBI GenBank (accession number AY691206). Alignment studies of partial sequences of gene *cpn*60 showed a sequence similarity between 77.5 % and 91.9 % among the nine species of genera *Arcanobacterium* and *Trueperella*. The highest sequence similarity could be observed between *A.* (*T.*) *abortisuis* DSM 19515^T and *A.* (*T.*) *bernardiae* DSM 9152^T (91.9 % similarity). According to Ülbegi (2010) dendrogram analysis of 16S rDNA, ISR, 23S rDNA and the genes *sod*A and *rpo*B of bacteria of genus *Arcanobacterium* and genus *Trueperella* supported the proposal from Yassin and coauthors (2011) that the nine species should be separated into the two closely related but distinct genera *Arcanobacterium* and *Trueperella*. It was of interest that dendrogram analysis of gene *cpn*60 of

nine reference strains of both genera of the present study also come in line with the results from Ülbegi (2010) and Yassin et al. (2011). This dendrogram differentiated *A.* (*T.*) *pyogenes, A.* (*T.*) *abortisuis, A.* (*T.*) *bernardiae, A.* (*T.*) *bialowiezense* and *A.* (*T.*) *bonasi*, which are affiliated to the newly described genus *Trueperella*, from genus *Arcanobacterium*, which is restricted to the species *A. haemolyticum, A. phocae* and *A. pluranimalium* and the phylogenetic neighbour *A. hippocoleae*.
However, gene *cpn*60 was additionally amplified and sequenced for the three *A. weissii* strains of the present study. A partial sequence of 345 bp, 350 bp and 345 bp were obtained for *A. weissii* 2298^T (CIP 110333^T), *A. weissii* 4321 and *A. weissii* 1656, respectively. These sequencing results revealed an almost complete sequence homology (between 99.4 % and 100 %) of the three strains among each other, a sequence homology (between 67.7 % and 92.5 %) to gene *cpn*60 of various other species of genus *Actinomyces* obtained from *cpn*60 database and (between 71.4 % and 82.6 %) to the corresponding gene of bacteria of genera *Arcanobacterium* and *Trueperella*. These results also reflected the high similarity of the three *A. weissii* strains among each other, and as mentioned before for 16S rDNA and 23S rDNA, allowed a classification of the three strains as novel species belonging to genus *Actinomyces*. Dendrogram analysis of the *cpn*60 sequencing results of the three *A. weissii* strains, other species of genus *Actinomyces* and the nine reference strains of genera *Arcanobacterium* and *Trueperella* also assigned this status as novel species.
According to the present results, cultural properties, Gram staining, investigation of cell morphology, hemolytic properties, the synergistic CAMP-like reactivities with several bacterial species as indicator strains, the reverse CAMP reaction in the zone of β-hemolytic *S. aureus* and the phenotypical properties allowed a preliminary identification of the three *A. weissii* strains as one species and a differentiation of *A. weissii* from other closely related species of genera *Actinomyces*, *Arcanobacterium* and *Trueperella*. MALDI-TOF MS could not identify the hitherto unknown species *A. weissii* because it is not included in the Biotyper software to the species level. This reflects that there are no false-positive identification results by MALDI Biotyper software. However, this technique showed that the three *A. weissii* strains belong to one species. A further update of the reference database library would allow a correct identification of the novel species *A. weissii* also by MALDI-TOF MS technique.
The species identity of the three *A. weissii* strains as novel species of genus *Actinomyces* could be determined genotypically by 16S rRNA, 23S rRNA and *cpn*60 gene sequencing.

Comparable to the isolation of other members of genus *Actinomyces* from infections of the oral cavity and mucous membranes of humans and animals (Holt et al., 1994; Hoyles et al., 2001) *A. weissii* was isolated together with various other bacteria from infections of the oral cavity of dogs, indicating that the pathogenic importance of this newly described species as causative agent for pyogenic infections in dogs, other animals and possibly in humans remains to be elucidated.

5.5 Conclusion

Throughout the last three decades *A*. (*T*.) *pyogenes* together with eight other species were assigned to genus *Arcanobacterium*. However, genus *Arcanobacterium* was exposed to a taxonomic revision by Yassin et al. (2011). These authors proposed that genus *Arcanobacterium* should be divided into one group with *A. haemolyticum*, *A. phocae*, *A. pluranimalium* and *A. hippocoleae* as phylogenetic neighbour, and a second robust group consisting of *A. abortisuis*, *A. bernardiae*, *A. bialowiezense*, *A. bonasi* and *A. pyogenes* which should be reclassified in a new genus, for which the name *Trueperella* was proposed.

A. (*T*.) *pyogenes* is commonly known as a pathogen of economically important livestock and of a large number of various other animal species, causing mastitis, abortion and a variety of pyogenic infections (Lämmler and Hartwigk, 1995; Jost and Billington, 2005; Moore et al., 2010). However, at present little is known about the role the eight other species of genera *Arcanobacterium* and *Trueperella* play in infections of animals.

The present study provided detailed phenotypic and genotypic characteristics of the bacterial species *A*. (*T*.) *pyogenes*, *A*. (*T*.) *abortisuis* and *A*. *haemolyticum* using conventional microbiological analysis, using MALDI-TOF MS fingerprinting and using PCR-mediated techniques. MALDI-TOF MS analyses were firstly described as a promising tool for reliable identification and differentiation of bacteria of genera *Arcanobacterium* and *Trueperella*. This technique had a comparable discriminating power as tests on DNA level.

In addition, during the course of the present study, three bacterial strains isolated from the oral cavity of three dogs could be identified by conventional tests and by 16S rDNA sequencing as novel *Actinomyces* species, for which the name *A. weissii* with the type strain *A. weissii* 2298^T (CIP 110333^T) was proposed.

However, the phenotypic characteristics, the MALDI-TOF MS fingerprinting results and the genotypic characteristics of bacteria of genera *Arcanobacterium*, *Trueperella* and *Actinomyces* shown in the present study might improve a future diagnosis and could help to elucidate the role these species play in infections of animals and humans.

6 Summary

In the present study 51 *A.* (*T.*) *pyogenes* isolated from various animal origins, 23 *A.* (*T.*) *abortisuis* isolated from pigs and cows, three *A. haemolyticum* isolated from horses and three strains representing a novel species of genus *Actinomyces*, for which the name *Actinomyces weissii* was proposed, together with 13 reference strains representing nine species of genera *Arcanobacterium* and *Trueperella* and two species of genus *Actinomyces* could be identified phenotypically, by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) fingerprinting and by genotypic techniques.

The strains were characterized by determination of cultural properties, their hemolysis, by detection of synergistic and antagonistic hemolytic reactions, by determination of various biochemical properties and by investigating their peptidic profiles by MALDI-TOF MS. The latter appeared to be a novel technique which allowed a rapid and reliable identification and characterization of all strains investigated in the present study. A molecular approach was performed by 16S rDNA analyses, by sequencing of the 16S-23S rDNA intergenic spacer region (ISR), the 23S rDNA and gene *cpn*60 encoding heat shock protein or chaperonin CPN60. The sequencing results allowed the design of species specific oligonucleotide primers which could be used for molecular identification of all investigated strains of genera *Arcanobacterium* and *Trueperella* isolated from various origins.

The additionally performed PCR-mediated amplification of the seven known and putative virulence factor encoding genes *plo*, *cbpA*, *nanH*, *nanP*, *fimA*, *fimC* and *fimE* of the *A.* (*T.*) *pyogenes* investigated in the present study allowed an individual strain characterization. This might help to clarify the role such putative virulence factors play in infections caused by this bacterial pathogen.

The 23 *A.* (*T.*) *abortisuis* strains isolated from samples of pigs and cows in a period of 12 years were mainly isolated together with various other bacteria from the urogenital tract of pigs and cows with varying clinical symptoms. Among these strains only three *A.* (*T.*) *abortisuis* were isolated from cases of abortion, indicating that the role this species plays as causative agent for abortion remains to be elucidated.

The three *A. haemolyticum* strains investigated in the present study were isolated together with various other bacterial species from infections of three horses, indicating that this well-known human

pathogenic bacterium could also be isolated from infections of horses possibly caused by a cross infection between the horse owner and the horse.

The three additionally investigated *A. weissii* strains of the present study were isolated from the oral cavity of three dogs. These strains, displaying synergistic CAMP-like activities with various indicator strains and a reverse CAMP-reaction in the zone of staphylococcal ß-hemolysin, could be characterized by conventional tests, by MALDI-TOF MS fingerprinting and by 16S rDNA, 23S rDNA and *cpn*60 gene sequencing as novel species of genus *Actinomyces*, for which the name *A. weissii* with the type strain *A. weissii* 2298^{T} (CIP 110333^{T}) was proposed.

7 Zusammenfassung

In der vorliegenden Arbeit wurden 51 *A*. (*T*.) *pyogenes*, isoliert von verschiedenen Tierarten, 23 *A*. (*T*.) *abortisuis*, isoliert von Schweinen und Kühen, 3 *A*. *haemolyticum*, isoliert von Pferden und 3 Stämme einer neuen Spezies der Gattung *Actinomyces*, für die der Name *Actinomyces weissii* gegeben wurde, zusammen mit 13 Referenzstämmen, von 9 Spezies der Gattungen *Arcanobacterium* und *Trueperella* sowie von zwei Spezies der Gattung *Actinomyces* mittels phänotypischer Methoden, durch Matrix-unterstützte Laser-Desorption/Ionisations Flugzeit-Massenspektrometrie (MALDI-TOF MS) Fingerprinting und durch genotypische Methoden identifiziert.

Die Stämme wurden aufgrund ihrer kulturellen Eigenschaften, ihrer Hämolyse, ihrer synergistischen und antagonistischen Hämolysereaktionen, verschiedener biochemischer Eigenschaften sowie durch Nachweis unterschiedlicher Peptidspektren durch MALDI-TOF MS identifiziert. Letzteres stellt eine neue Methode dar, die eine schnelle und zuverlässige Identifizierung und Charakterisierung aller in dieser Arbeit untersuchten Bakterienstämme erlaubte. Eine molekulare Untersuchung wurde durch 16S rDNA-Analysen, durch Sequenzierung der 16S-23S rDNA Intergenic spacer region (ISR), der 23S rDNA und des Hitzeschockprotein- oder Chaperonin (CPN60)-kodierenden Gens *cpn*60 durchgeführt. Aufgrund der Sequenzierungsdaten ließen sich speziesspezifische Oligonukleotidprimer erstellen die eine molekulare Identifizierung sämtlicher Stämme der Gattungen *Arcanobacterium* und *Trueperella* unterschiedlicher Herkunft ermöglichten.

Eine zusätzliche PCR-vermittelte Amplifizierung der 7 bereits bekannten bzw. mutmaßlichen Virulenzfaktor-kodierenden Gene *plo*, *cbp*A, *nan*H, *nan*P, *fim*A, *fim*C und *fim*E von *A*. (*T*.) *pyogenes* erlaubte eine individuelle Charakterisierung der jeweiligen Stämme. Dies könnte dazu beitragen die Rolle dieser mutmaßlichen Virulenzfaktoren bei einer Infektion mit diesem Krankheitserreger zu verstehen.

Die 23 *A*. (*T*.) *abortisuis* Stämme wurden überwiegend zusammen mit verschiedenen anderen Bakterienarten aus dem Urogenitaltrakt von Schweinen und Kühen mit unterschiedlichen Symptomen in einem Zeitraum von 12 Jahren isoliert. Unter diesen Stämmen wiesen lediglich 3 *A*. (*T*.) *abortisuis* einen Zusammenhang mit Aborten auf, wobei die Beteiligung von *A*. (*T*.) *abortisuis* als Aborterreger bei Schweinen noch eingehender untersucht werden muss.

Die drei untersuchten *A. haemolyticum* Stämme der vorliegenden Arbeit wurden zusammen mit verschiedenen anderen Bakterienarten von Infektionen von Pferden isoliert. Dies wies darauf hin, dass diese überwiegend humanpathogene Bakterienspezies auch bei Infektionen von Pferden isoliert werden kann. Möglicherweise deutet dieses Vorkommen auf eine Kreuzinfektion zwischen Pferdebesitzer und Pferd hin.

Die drei zusätzlich untersuchten *A. weissii* Stämme, die aus der Maulhöhle von drei Hunden isoliert wurden, zeigten synergistische CAMP-ähnliche Aktivitäten mit verschiedenen Indikatorstämmen und eine reverse CAMP-Reaktion der Staphylokokken-β-Hämolysinzone. Diese drei Stämme konnten anhand konventioneller Tests, durch MALDI-TOF-Fingerprinting sowie durch Sequenzierungen der 16S rDNA, 23S rDNA und des Gens *cpn*60 als eine neue Spezies der Gattung *Actinomyces* identifiziert und charakterisiert werden, wobei für sie der Name *A. weissii* mit dem Typstamm *A. weissii* 2298^T (CIP 110333^T) vorgeschlagen wurde.

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