Phenotypic and genotypic characteristics and epidemiological relation of *Trueperella pyogenes* isolated from various origins

INAUGURAL-DISSERTATION

zur Erlangung des Grades eines

Dr. med. vet.

beim Fachbereich Veterinärmedizin

der Justus-Liebig-Universität Gießen

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eingereicht von

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Tierarzt aus Kairo, Ägypten

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„I find I'm so excited, I can barely sit still or hold a thought in my head. I think it's the excitement only a free man can feel, a free man at the start of a long journey whose conclusion is uncertain. I hope I can make it across the border. I hope to see my friend and shake his hand. I hope the Pacific is as blue as it has been in my dreams. I hope.“

(Shawshank Redemption, Stephen King)

For my Parents and Family

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Frequently used abbreviations and units

A.	*Arcanobacterium*
A.	*Actinomyces*
aqua bidest.	Aqua bidestillata
aqua dest.	Aqua destillata
bp	Base pair
C	Cytosine
CAMP -Test	Christie-Atkins-Munch-Petersen-Test
Da	Dalton, unit of molecular mass
DNA	Deoxyribonucleic acid
dNTP	Desoxynucleosidtriphosphat
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
et al.	And others
Fig.	Figure
FT-IR spectroscopy	Fourier Transform Infrared Spectroscopy
g	Gram
G	Guanine
gen. n.	Genus novus or new genus
h	Hour
ILY	Intermedilysin from *Streptococcus intermedius*
ily	*Streptococcus 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	*Listeria 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
MLSA	Multilocus sequence analysis
MLST	Multilocus Sequence Typing
mol	Amounts of a chemical substance
MSP	Main spectrum (reference spectrum of the peptidic raw spectra)
N	Normality
NCBI	National Center for Biotechnology Information
p	Pico (10^{-12})
PCR	Polymerase chain reaction
PFO	Perfringolysin O from *Clostridium perfringens*
pfo	*Clostridium perfringens* perfringolysin encoding gene
PLO	Pyolysin from *Trueperella pyogenes*
plo	*T. pyogenes* pyolysin encoding gene
PLY	Pneumolysin from *Streptococcus pneumoniae*
ply	*Streptococcus pneumoniae* pneumolysin encoding gene
pmol	Picomole
RNA	Ribonucleic acid
s	Second
SLO	Streptolysin O from *Streptococcus pyogenes*
slo	*Streptococcus pyogenes* streptolysin encoding gene
sodA	superoxid dismutase A encoding gene
T.	*Trueperella*
Tab.	Table
U	Unit (international unit)
UV	Ultraviolet
V	Volt
°C	Degree Celsius
µg	Microgram

µl	Microliter
µm	Micrometer

1 Introduction

Bovine mastitis is an endemic disease affecting dairy herds worldwide (Halasa et al., 2007; Miller et al., 1993). The economic impact is mainly due to the continuous decrease in milk production and the increase of the control costs (Seegers et al., 2003). Mastitis is an inflammation of mammary gland parenchyma, which is associated with a wide range of physical and chemical changes of the milk and pathological changes in the udder tissue (Radostits et al., 2007). Mastitis is generally classified as clinical or subclinical mastitis depending on the degree of inflammation in the mammary gland. The changes that can be observed in bovine clinical mastitis are accompanied with clots in milk, discoloration of milk and high numbers of leukocytes in the affected milk. Furthermore, clinical signs observed in bovine mastitis are swelling, heat, redness and pain in the udder. Mastitis is usually caused by two groups of bacterial pathogens which can be classified as contagious pathogens which comprise *Streptococcus agalactiae*, *Staphylococcus aureus* and *Mycoplasma bovis,* as well as environmental pathogens which comprise species of genus *Streptococcus* (*Streptococcus uberis* and *Streptococcus dysgalactiae*), Gram-negative coliform bacteria (*Escherichia coli*, *Klebsiella* spp., *Citrobacter* spp. and *Enterobacter* spp.), *Enterococcus faecalis*, *Enterococcus faecium*, other Gram-negative bacteria such as *Serratia marcescens*, *Pseudomonas* spp. and *Proteus* spp. and the Gram-positive bacterium *Trueperella pyogenes* (formerly *Arcanobacterium pyogenes*). *T. pyogenes* causes a generally complete dysfunction of the affected quarters (Radostits et al., 2007).

T. pyogenes is a worldwide known bacterium of genus *Trueperella* belonging to the family *Actinomycetaceae* which causes mastitis, abortion and various other pyogenic infections in domestic animals like ruminants and pigs (Lämmler and Hartwigk, 1995; Hirsh and Biberstein, 2004; Moore et al., 2010). *T. pyogenes* was also isolated from other animal species, like antelopes, bison, camels, chicken, deer, elephants, gazelles, horses, macaws, reindeer, turkeys and wildebeest (Al-Tarazi et al., 2001; Jost and Billington, 2005). In addition, *T. pyogenes* could be recovered from feline otitis externa, canine cystitis and from reptiles (Billington et al., 2002; Ülbegi-Mohyla et al., 2010). Infections of human patients with *T. pyogenes* are rare (Gahrn-Hansen and Frederiksen, 1992; Kavitha et al., 2010).

The aim of the present study was to identify and further characterize *T. pyogenes* isolated from clinical mastitis of dairy cattle, from bovine genital tract and from fatal infections of

three grey slender lorises (*Loris lydekkerianus nordicus*). The bacteria were investigated, together with reference strains of genera *Trueperella* and *Arcanobacterium,* for cultural characteristics and for further phenotypical properties, by Matrix-assisted laser desorption ionization-time of flight mass spectrometry and Fourier Transform Infrared Spectroscopy and by genotypic methods. The latter were performed by studying various species-specific targets, by determination of putative virulence factor encoding genes and by investigating the epidemiological relationship of the *T. pyogenes* among each other.

2 Review of the literature

2.1 History and taxonomy of *T. pyogenes*

The species name of *T. pyogenes* was derived from the Latin word "*pyum*"- pus and the word "*gennaio*"- produce to describe the pus inducing bacterium. The taxonomy of *T. pyogenes* was not clear for many years. *T. pyogenes* was named formerly as *Bacillus liquefaciens pyogenes* and was mentioned as one of the pathogenic microorganisms causing suppurative inflammation in cattle (Lucet, 1893). Following that, other authors reported about *T. pyogenes* infections in cattle, swine and from cow mastitis (Grips, 1898; Glage, 1903; Künnemann, 1903). In 1903, Glage suggested the name *Bacillus pyogenes* (Glage, 1903). In 1903, Künnemann reported about a similar organism in cattle and called it *Bacillus pyogenes bovis* and found that both isolates from pigs and bovines were similar and suggested the name *B. pyogenes*. In 1918, the name *Bacillus pyogenes bovis* was changed to *Corynebacterium pyogenes* (Eberson, 1918). Barksdale et al. (1957) proposed that *C. haemolyticum* is a mutant form of *C. pyogenes* according to phenotypic and cell wall structure analysies for both species. Furthermore, other researchers had recognized that *C. pyogenes* had similar phenotypic properties and cell wall structure as *C. haemolyticum* and taxonomically both species should be reclassified to genus *Streptococcus* (Cummins and Harris, 1956; Barksdale et al., 1957). In 1974, *C. pyogenes* was assigned, according to the 8th edition of Bergey's Manual of Determinative Bacteriology, to genus *Corynebacterium* as *Corynebacterium pyogenes*. This was based on the similarity to the type species *Corynebacterium diphtheriae* and related animal pathogenic Corynebacteria (Rogosa et al., 1974). Later on, several phylogenetic studies distinguished *C. haemolyticum* and *C. pyogenes* from each other, which led to a new classification of *C. pyogenes* within the genus *Actinomyces* as *Actinomyces pyogenes* (Jones 1975; Goodfellow et al., 1976; Minnikin et al., 1978; Schofield and Schaal, 1981; Collins and Jones, 1982a; Reddy et al., 1982). Furthermore, another reclassification was introduced leading to renaming *Corynebacterium haemolyticum* to *Arcanobacterium haemolyticum*. That depended on the composition of cell wall fatty acids and the type of peptidoglycan of this microorganism which differed from species of genus *Corynebacterium* (Collins et al., 1982b). Another study reported that *Actinomyces pyogenes* and *Actinomyces bernardiae* should be 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). In 2011, came another divarication

opinion by Yassin et al. (2011) based on the compostion of 16S rRNA signature nucleotides, menaquinones and phospholipids. These researchers proposed another reclassification of genus *Arcanobacterium*. They proposed that the name *Arcanobacterium* should be limited for *A. haemolyticum*, *A. hippocoleae, A. phocae* and *A. pluranimalium*. Later on, a polyphasic taxonomic study was performed on an unidentified *Arcanobacterium*-like Gram stain positive bacteria isolated from otitis externa of a dog and another two bacterium isolated from harbour seals, respectively. Comparative 16S rRNA gene sequencing showed that both bacteria belonged to genus *Arcanobacterium* and were most closely related to the type strains of *A. haemolyticum* (97.2%), *Arcanobacterium hippocoleae* (96.5%) and *Arcanobacterium phocae* (96.4%). Based on these tests, it was proposed that the unknown bacteria should be classified as the novel species *Arcanobacterium canis* (Hijazin et al., 2012c), *Arcanobacterium phocisimile* (Hijazin et al., 2013) and *Arcanobacterium pinnipediorum* (Sammra et al., 2015), respectively. The remaining five species of genus *Arcanobacterium*, also including *A. pyogenes,* were reclassified under a new genus with the name *Trueperella*, as *Trueperella pyogenes, Trueperella abortisuis*, *Trueperella bernardiae*, *Trueperella bialowiezensis* and *Trueperella bonasi*. Based on 16S rRNA gene sequence analysis species of the genus *Trueperella* clustered together with sequence similarities ranging from 95.3 to 98.6% (Yassin et al., 2011). The analysis of the phosphoglycolipid composition also divided the formerly named genus *Arcanobacterium* into two distinct groups (Yassin et al., 2011). An unknown phosphoglycolipid was detected in genus *Trueperella* but not in genus *Arcanobacterium* (Yassin et al., 2011). In addition, the analysis of the menaquinone patterns showed that genus *Trueperella* shared the same pattern and could be distinguished from *Arcanobacterium* (Yassin et al., 2011). Genus *Trueperella* was named after Hans Georg Trüper, a German microbiologist (Yassin et al., 2011). The current classification of genus *Trueperella* is also shown in Tab. 1.

Tab. 1: Current classification of the family *Actinomycetaceae*, genera *Arcanobacterium* and *Trueperella* according to the National Center for Biotechnology Information and the German Collection of Microorganisms and Cell Cultures (Yassin et al., 2011; Sammra et al., 2015).

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

2.2 Clinical manifestations of *T. pyogenes* infections

T. pyogenes is characterized in the medical literature as an obligate inhabitant of various mucosal surfaces of domestic animals and an opportunistic pathogen responsible for infections of a variety of organs, including skin, joints and visceral organs in a large number of animal species (Schaal, 1986; Nattermann and Horsch, 1977; Lämmler and Hartwigk, 1995; Narayanan et al., 1998; Trinh et al., 2002; Jost and Billington, 2005). *T. pyogenes* is commonly associated with abscess formation in cattle (Narayanan et al., 1998; Ertaş et al., 2005; Lin et al., 2010). *T. pyogenes* is also considered as a major cause of endometritis and was isolated from the intrauterine perfusion fluid of a Holstein dairy cow (Kaneko et al., 1997). It has been observed that *T. pyogenes* plays an important role in the pathogenesis of acute putrid endometritis (Huszenicza et al., 1999), puerperal uterine infections (Silva et al., 2008) and pneumonia (Gagea et al., 2006). *T. pyogenes* was also isolated in 41.3% of cows with endometritis and from 3.5% of cows without endometritis (Petit et al., 2009). In 2011, Zambrano-Nava et al. showed that *T. pyogenes* was one of the predominant Gram-positive bacteria of vaginal microbiota of healthy Criollo Limonero cows (Zambrano-Nava et al., 2011). In 1992, Madsen et al. showed notably a higher percentage of *T. pyogenes* in Pyogenes mastitis in contrast to summer mastitis in cattle (Madsen et al., 2011). Differences

were also noted in the combination of bacteria in the mastitic secretions. The majority of Pyogenes mastitis secretions yielded either pure cultures of *T. pyogenes*, or mixed cultures of *T. pyogenes* and *Peptostreptococcus indolicus*, or of *T. pyogenes*, *P. indolicus* and microaerophilic cocci. The summer mastitis appeared to be more complex, yielding a broader range of bacterial combinations and with more frequent involvement of Bacteroidaceae and *Streptococcus dysgalactiae*. *S. dysgalactiae* occurred more frequently in summer mastitis secretions than in Pyogenes mastitis secretions, mostly together with other organisms although few cases yielded pure cultures (Madsen et al., 1992). Furthermore, *T. pyogenes* could also be recovered from mastitic cultures in summer mastitis cases, which were associated with loss of the mammary gland function (Quinn et al., 2002; Gröhn et al., 2004). However, *T. pyogenes* was isolated alone or in combination with other pathogens, mostly occurring in non-lactating pregnant cows, at parturition, newly calved cattle and rare in lactating cows (Hillerton, 1987). In 2009, Vasil', reported about *T. pyogenes, Streptococcus* spp. and coagulase-negative staphylococci as main mastitis pathogens in lactating dairy cattle (Vasil', 2009). *T. pyogenes* was reported in pigs in cases of suppurative pneumonia (Falk et al., 1991), polyarthritis (Hariharan et al., 1992) and abscessation (Ohba et al., 2007) and could be isolated from 39% of porcine stomachs (Jost et al., 2002a). *T. pyogenes* was mentioned as important bacterial pathogen with negative economic impact on pig production (Martínez et al., 2007). *T. pyogenes* was also described by Azawi et al. (2007; 2010) among buffaloes together with various other Gram-positive and Gram-negative bacteria. *T. pyogenes* was also isolated from cases of pneumonia and arthritis in camels (Al-Tarazi et al., 2001), from pneumonia and arthritis of camel calves (Bani Ismail et al., 2007), from thoracic abscess in an alpaca (Adolf et al., 2001), from foot infections in a fallow deer (Lavín et al., 2004), septicemic infections in captive blackbucks (Portas and Bryant, 2005), from intracranial abscessation in a white-tailed deer (Karns et al., 2009), from septicemia in a southern pudu (Twomey et al., 2010) and from septicemic infections of a bearded dragon and a gecko (Ülbegi et al., 2010). Billington and coauthors reported in 2002, about a case of feline otitis externa associated with pure cultures of *T. pyogenes* and from a dog case with urinary tract infection in which *T. pyogenes* was the predominant bacterial species (Billington et al., 2002). A synergism was demonstrated between *T. pyogenes* and *Fusobacterium necrophorum* resulting in severe infections (Roberts, 1967; Ruder et al., 1981; Roeder et al., 1989; Seimiya et al., 2004; Williams et al., 2005). The synergistic effect between these two bacteria is thought to be caused by a leucocidal toxin produced by *F. necrophorum* that inhibited the

phagocytosis and encouraged the *T. pyogenes* growth, which will be reflected on the growth of *F. necrophorum* providing it with catalase and necessary growth factors (Ruder et al., 1981). In 1992, Gahrn-Hansen and Frederiksen reported about the isolation of *T. pyogenes* in Denmark from 11 human cases in the time period from 1986 to 1992 without any possible animal contact information (Gahrn-Hansen and Frederiksen 1992). In 1998, Lynch et al. described a case of septic arthritis and osteomyelitis in a diabetic farmer, Ide et al. (2006) reported about a case of spondylodiscitis in a veterinary surgeon (Ide et al., 2006) and Plamondon et al. (2007) mentioned an endocarditis case in a human (Plamondon et al., 2006). In 2009, Levy et al. observed another human case in Brazil caused by *T. pyogenes* (Levy et al., 2009) and in 2010 Kavitha et al. isolated *T. pyogenes* from wound infections in a human (Kavitha et al., 2010). In 2013, Kaneko et al. mentioned that *T. pyogenes* infusions in nonlactating Holstein cows affect the ovarian function (Kaneko et al., 2013). The gene expression of virulence genes in *T. pyogenes* were investigated in a mouse model and a significant difference in virulence gene expression between virulent and non-virulent isolates was reported (Zhao et al., 2013a). *T. pyogenes* was reported as primary pathogen of abscesses in forest musk deer (Zhao et al., 2013b). In 2014, Machado et al. evaluated subcutaneous vaccine formulations containing different combinations of proteins from *T. pyogenes* and of other bacteria which prevented dairy cows against puerperal metritis during the first lactation (Machado et al., 2014). Results from Zhang et al. revealed a correlation between drug resistance and the *nanH* expression in *T. pyogenes* (Zhang et al., 2014). *T. pyogenes* infections in pigs were described in association with several organs (Jarosz et al., 2014). In 2015, Ribeiro et al. evaluated the epidemiological and clinical aspects of *T. pyogenes* in 144 cases of infections among domestic animals between 2002 and 2012 (Ribeiro et al., 2015). *T. pyogenes* could also be recovered from a cranial abscess disease of a white-tailed deer (Belser et al., 2015). Finally, the minimum inhibitory concentrations of selected antimicrobials against *E. coli* and *T. pyogenes* were estimated in bovine intrauterine bacterial infection cases (de Boer et al., 2015).

2.3 Morphology and growth properties of *T. pyogenes*

T. pyogenes is a 1 to 2 μm by 0.2 to 0.5 μm, small, non-motile and non-spore-forming bacterium (Lämmler and Hartwigk, 1995). The species was described as facultative anaerobic (Collins and Jones, 1982; Reddy et al., 1982; Schaal, 1986; Lämmler and Hartwigk, 1995;

Narayanan et al., 1998). *T. pyogenes* is Gram-positive after staining from fresh cultures and appear as single or in pairs arranged in V- or T-shapes (Reddy et al., 1982). Bacteria of genus *Trueperella* are characterized by their capability of facultative growth under anaerobic or microaerobic conditions, enhanced in CO_2-enriched atmosphere after 24 to 48 h incubation at 37°C in a candle jar. Growth of the bacteria occurs on ordinary media but is enhanced on media containing blood or serum. Colonies of *T. pyogenes* on sheep blood agar were described as pinpoint, convex, slightly translucent and circular and were surrounded by a zone of β-hemolysis (Schaal, 1986; Lämmler and Hartwigk, 1995). According to Collins and Jones (1982), *T. pyogenes* could be inactivated at 60 °C within 15 min (Collins and Jones, 1982).

2.4 Biochemical characteristics of *T. pyogenes*

T. pyogenes has 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; Plamondon et al., 2007). *T. pyogenes* has 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 *T. pyogenes* has also proteolytic properties, which which can be shown as serolysis on Loeffler medium and the ability to hydrolyze gelatin (Hartwigk and Marcus, 1962; Gahrn-Hansen and Frederiksen, 1992; Narayanan et al., 1998; Lämmler and Hartwigk, 1995). A further identification and characterization of the species *T. pyogenes* was performed by Ülbegi-Mohyla et al. (2010), Hijazin et al. (2011) and Hijazin (2012a). It was reported that *T. pyogenes* strains DSM 20630^T and DSM 20594 induce hemolysis on sheep blood agar and were negative in the reverse CAMP-like reaction. Both *T. pyogenes* reference strains were negative for the enzymes pyrazinamidase, α-galactosidase, β-glucosidase, esculin, urease, catalase, α-mannosidase and for nitrate reduction. However, both *T. pyogenes* strains were positive for the enzymes pyrrolidonyl arylamidase, β-glucuronidase, β-galactosidase, α-glucosidase, N-acetyl- β-glucosaminidase, gelatinase, caseinase, D-glucose, D-ribose, D-xylose, D-maltose and D-lactose. However, it was reported that *T. pyogenes* DSM 20630^T was positive for glycogen degradation and starch hydrolysis but *T. pyogenes* DSM 20594 was negative (Ülbegi-Mohyla et al., 2009; Hijazin et al., 2011; Hijazin, 2012a). The peptidoglycan of the *T.*

pyogenes cell wall is based the on amino acid lysine and the fatty acid analyses revealed a straight chain of saturated and monounsaturated acid (Reddy et al., 1982).

2.5 Genome of *T. pyogenes*

The genomic DNA of *T. pyogenes* containes between 56 to 58 mol% Guanine and Cytosine (G + C) (Collins and Jones, 1982; Reddy et al., 1982). In May 2014, the complete genome sequence of *T. pyogenes* was announced from a field isolate from the uterus of a dairy cow affected with metritis. The complete circular genome of *T. pyogenes* strain TP6375 encompassed 2,338,390 bp, with a GC content of 59.5 mol% and with 2,082 predicted genes. The genome contained 1,984 coding sequences (CDS) encoding 1,981 proteins with several known and putative virulence factors like adhesion factors (1 collagen adhesion and 4 fimbrial proteins) and toxins (pyolysin, cytotoxin and one other toxin). The annotated chromosome sequence of *T. pyogenes* TP6375 has been deposited in GenBank under the accession number CP007519 (Machado et al., 2014). Later on, another genome sequence project was announced in January 2015. The genome sequence project was reported for *T. pyogenes* strain TP8, which was recovered from the abscess of forest musk deer (Zhao et al., 2011). The complete circular genome of *T. pyogenes* TP8 encompassed 2,272,494 bp, with a GC content of 59.6 mol% and with 2,091 predicted genes. The genome contained 2,001 coding sequences (CDS) encoding 1996 proteins. The annotated chromosome sequence of *T. pyogenes* strain TP8 has been deposited in GenBank under the accession number CP007003 (Zhao et al., 2014). Currently, another genome sequence project is running with *T. pyogenes* strain MS249, an isolate from the infected uterus of a postpartum cow with metritis. The primary announced results mentioned that the *T. pyogenes* MS249 genome contains 2,236,677 bp, with a GC content of 59.8 mol%. The genome was predicted to contain 2,095 protein-coding sequences (CDSs). The annotated chromosome sequence of *T. pyogenes* MS249 has been deposited in GenBank under the accession number JALQ00000000 (Goldstone et al., 2014).

2.6 Putative virulence factors of *T. pyogenes*

2.6.1 Pyolysin

The hemolysin of *T. pyogenes* (Pyolysin) is a primary virulence factor, which is heat-labile, oxygen-stable protein and sensitive for the proteolytic enzymes trypsin and amylase (Ding and Lämmler, 1996; Funk et al., 1996). Its activity was not affected by treatment with oxidizing agents (Funk et al., 1996). Further studies described *T. pyogenes* pyolysin (PLO) as an extracellular toxin (Ding and Lämmler, 1996; Billington et al., 1997; Jost et al., 1999). PLO is an extracellular cytotoxin that binds to a varying number of eukaryotic cell membranes including that of macrophages (Ding and Lämmler, 1996; Billington et al., 1997; Jost et al., 1999), forming pores and thus causes cell lysis (Billington et al., 2000). PLO protein has a molecular weight of 57.9 kDa encoded by a 1,605 bp nucleotide sequence (Billington et al., 1997). PLO also exposes similarity to other members of the cholesterol dependent cytolysin family. The amino acid sequence similarity percentage of PLO is 30-40% 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) (Billington et al., 1997). PLO is insensitive to reducing compounds and not susceptible to thiol-blocking agents (Billington et al., 2000). Thiol-activated cytolysins are cholesterol-dependent cytolysins (CDCs) indicating that their cytolytic ability through pore formation in eukaryotic cell membranes is restricted to cholesterol containing target membranes (Giddings et al., 2003). Although cholesterol is the major target cell receptor for these toxins and small amounts of free cholesterol can inhibit lytic activity, it was mentioned that cholesterol had no effect on PLO activity (Funk et al., 1996). The lethal effect of PLO was demonstrated by using a 1% solution of purified toxin at different doses through intravenous inoculation (20 µl - 33 µl) into mice and by intradermal injection (25 µl - 30 µl) into Guinea pigs (Lovell, 1944).

In 1997, Billington used specific antibodies against purified PLO, which neutralized the hemolysin activity of *T. pyogenes* and protected mice from death after infection indicating that PLO is important in the pathogenesis process (Billington et al., 1997). In 1999, Jost et al. induced an insertional mutation in the *plo* gene which disabled the expression of functional cytolytic PLO molecule. This is confirming that PLO plays a role for in vivo survival of *T. pyogenes* and perhaps is protecting the bacteria during the early stage of infection from host

immune defense (Jost et al., 1999). Moreover, this mutagenesis showed a significant reduction in virulence of *T. pyogenes*. In a mouse model, this mutant was unable to establish intraperitoneal infections in mice. In the opposite direction, Jost et al. (1999) reported that the *T. pyogenes* mutant could be restored to its full virulence by adding the intact *plo* gene *in trans* from of a wild-type *T. pyogenes*. This shows that the failure in PLO production leads to a reduction in virulence.

PLO had a cytolytic effect on phagocytic cells and neutrophils (Jost et al., 1999). PLO is produced by all *T. pyogenes* isolates examined to date and its production is restricted to stationary phase cultures (Ding and Lämmler, 1996; Billington et al., 1997; Jost et al., 1999; Silva et al., 2008). The PLO production in *T. pyogenes* is controlled in such manner that in commensal phase it is down regulated in order to prevent inflammatory response of the host. During the infection phase, it is up regulated expressing its virulence (Rudnick et al., 2003). The position of gene *plo* on a genomic insertion between two essential genes may play a role in the maintenance of this important virulence gene during its commensal phase on the mucosal membrane of its host (Rudnick et al., 2008).

2.6.2 Neuraminidases

Neuraminidases (sialidases) belong to a class of glycosyl hydrolases that cleave terminal N-acetylneuraminic (sialic) acid residues from glycoproteins, glycolipids and polysaccharides and make them available as carbon source for bacteria (Roggentin et al., 1993). They are key enzymes of sialic acid catabolism, hydrolysing the glycosidic linkage between sialic acid molecules and glycoconjugates. Neuraminidase enzymes had been detected in a variety of microorganisms such as protozoa, bacteria, fungi and viruses. Neuraminidases play a significant role in the pathogenesis of infectious diseases, whose etiologic agents produce the enzyme by cleaving sialic acids in infected tissues to facilitate the spread of the disease (Galen et al., 1992; Giebink, 1999; Tong et al., 2000). In 1989, Schaufuss and Lämmler first reported about the presence of a *T. pyogenes* neuraminidase for two of 42 *T. pyogenes* isolates (Schaufuss and Lämmler, 1989). In 2001, Jost et al. characterized the activity of the first neuraminidase encoding gene *nanH* (3,009 bp) from a *T. pyogenes* isolate from a bovine abscess (Jost et al., 2001). In 2002, Jost et al. succeeded to identify and characterize a second neuraminidase in a *nanH* negative mutant. *T. pyogenes* isolate BBR1, exhibited neuraminidase activity and the encoding gene was *nanP* (5,112 bp) encoding a protein with a

molecular weight of 186.8 kDa (Jost et al., 2002b). Moreover, these authors showed that the absence of both *nanH* and *nanP* in *T. pyogenes* led to total deficiency of neuraminidase activity associated with an incompetence to adhere to HeLa cells. This mutation could be reversed by providing *nanP* in *trans* on a replicating plasmid, indicating that neuraminidases play an important role as virulence factor in adhesion to host epithelial cells (Jost et al., 2002b). The percentage of the presence of both genes *nanH* and *nanP* in *T. pyogenes* was variable. Jost et al. (2001) reported that all 53 *T. pyogenes* isolates from different animal origin showed neuraminidase activity. However, the percentage of gene *nan*P was lower in contrast to *nanH*. Gene *nan*P was only present in 64.2% of the 53 isolates investigated by Jost et al. (2002b). In 2012, Hijazin reported about the presence of genes *nanH* and *nanP* in *T. pyogenes* of different animal origin and found that the presence of *nanH* (92 %) is higher in contrast to gene *nan*P (78 %) (Hijazin, 2012a).

2.7 Identification and classification of bacteria using alternative methods

2.7.1 Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) for bacterial identification

The Matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI TOF MS) was first introduced in 1987 by Michael Karas in Münster and was honored with a shared Nobel Prize in 2002. MALDI-TOF MS has been successfully applied as an identification procedure in clinical microbiology for bacteria or fungi and detection of antibiotic resistance. MALDI-TOF MS can be used for identification of microorganism to genus and species levels and for some instances to subspecies or strains levels and currently is widely used in routine laboratory practice because of its economical and diagnostic benefits. (Lynn et al., 1999; Bernardo et al., 2002; Ruelle et al., 2004; Rupf et al., 2005; Murray, 2010; Bizzini et al., 2011; Clark et al., 2013; Hrabák et al., 2013). MALDI-TOF MS allows bacterial identification through soft ionization of proteins, which allows the analysis of large biomolecules including ribosomal proteins with sizes measured up to100 kDa. This could be conducted with little or no sample pretreatment in few minutes (Karas et al., 1987; Tanaka et al., 1988; Hillenkamp and Karas, 1990; Albrethsen, 2007). Ribosomal proteins obtained from whole bacterial cells were found to be responsible in generating of different mass spectra that are unique to their respective bacterial taxon (Suh et al., 2005; Maier and Kostrzewa, 2007). MALDI-TOF MS is a very fast developing method in chemical and biological fields. In 1996,

Claydon et al. showed the use of MALDI-TOF MS as a rapid tool for identification of intact microorganisms using a single colony of bacteria directly from the sample plate (Claydon et al., 1996). In 1996, Holland et al. used MALDI-TOF MS for analysis of the spectral patterns of proteins obtained directly from intact bacterial cells through a chemotaxonomic approach. This in turn helped in identifying different strains of bacteria through comparing of the obtained unknown spectra with a database of reference spectra of known bacteria (Holland et al., 1996). In the same direction, Krishnamurthy et al. evaluated that MALDI-TOF MS methods in describing specific biomarkers obtained from protein extracts of individual bacterial species, which could be used to permit the identification, as well as distinguishing pathogenic species and to differentiate it from the corresponding non-pathogenic species (Krishnamurthy and Ross, 1996; Krishnamurthy et al., 1996). Later on, a more extensive MALDI-TOF MS based analysis of genus *Bacillus* was developed by using a database based on ribosomal protein markers (Hotta et al., 2011). The MALDI-TOF MS is based on ”a mild” ionization technique using the formed ions with low internal energy, allowing the observation of ionized molecules with little or no fragmentation. The following step is the biomolecular detection stage of the ionized molecules and the transformation of signals into spectra and peaks of the target bacteria followed by matching these with those reproducible spectra or peaks, which are stored in respective database libraries by using specific software. It is important to standardize the culturing conditions of the targeted microorganisms to generate reproducible and accurate spectra in order to make analyses with other species (Marvin et al., 2003; Seng et al., 2009). However, other studies have reported the difficulties in identification of several microorganisms associated with the spectra reproducibility involved in MALDI-TOF MS analysis. This misidentification by MALDI-TOF MS and non-identical spectra could be attributed to the bacterial growth, bacterial storage, handling, or culturing over different time intervals, as well as different time intervals associated with removing and analyzing of the bacterial cells (Arnold et al., 1999; Lay Jr, 2001; Vargha et al., 2006). In 1998, Welham et al. showed that there are margins of conserved peaks in the spectra obtained under different experimental conditions (Welham et al., 1998). According to several other authors, MALDI-TOF MS appeared to be an accurate method for bacterial identification, even with changing conditions such as culture medium, pH and temperature and provided a good interlaboratory comparison with minimal usage of reagents (Valentine et al., 2005; Wunschel et al., 2005; Liu et al., 2007; Mellmann et al., 2008). However, Wang et al. reported that spectral reproducibility of the MALDI-TOF MS analysis can be affected by the

used bacterial protein extraction protocol and the targeted protein spectra may have various numbers of relatively reproducible peaks (Wang et al., 1998). Sample preparation is technically simple and reproducible. This successful technique can be easily used by most medical and research laboratory scientists in microbial determination and identifications with the aid of associated software. Several studies showed the possibility to identify various types of bacteria using MALDI-TOF technique either directly from precultured whole bacterial cells (Holland et al., 1996; Krishnamurthy and Ross, 1996) or by using protein extracts (Cain et al., 1994; Krishnamurthy et al., 1996). The direct method is simple in which the steel MALDI target plate is loaded with biological specimens from single colonies and then is overlaid with the MALDI matrix. The indirect method uses the extraction of ribosomal protein with alpha-Cyano-4-hydroxycinnamic acid (HCCA) which facilitates the measurement automation (Lay Jr, 2001; Maier and Kostrzewa, 2007). The variant sample preparation protocols can lead to different spectra but they are still very similar in their peak patterns (Maier and Kostrzewa, 2007). The MALDI-TOF MS analysis is done by placing sample-matrix crystal on the surface of the metal plate which then is subjected to a UV laser beam (N_2 laser beam with a wave-length of 337 nm is utilized in commercial instruments). This is done for a short time in order to prevent excess overheating, which may deteriorate or degradate the biological specimen sample embedded in the matrix. The laser beam is focused on a small spot on the matrix-clinical sample crystalline surface (typically 0.05 to 0.2 mm in diameter). The uptake of energy from the laser beam triggers evaporation of the matrix into a gas phase, which is directly followed by the ionization of the protein content (soft ionization). The ionized proteins are accelerated by an electric field to flow into the flight tube of the mass spectrometer. In this stage the ions pass through a field free drift region where the only force affecting the ionic movement is the kinetic energy from the acceleration step, in which the ions are separated according to their different velocity (i.e. a function of ion mass and charge). The lighter ions travel faster and reach the detector in less time than the heavier ions. MALDI-TOF MS is a diagnostic tool with high discriminatory power for species and strain level and able to identify several Gram-positive and Gram-negative pathogens such as *Streptococcus* spp. (Kumar et al., 2004), *Salmonella* spp. (Leuschner et al., 2004), *Campylobacter* spp. (Mandrell et al., 2005), *Enterococcus* sp. (Reynaud af Geijersstam al., 2007), *Listeria* spp. (Barbuddhe et al., 2008), *Clostridium* spp. (Grosse-Herrenthey et al., 2008), *Corynebacterium* spp. (Konrad et al., 2010), 32 genera obtained from blood cultures (Stevenson et al., 2010), *Legionella* spp. as respiratory human pathogen (Gaia et al., 2011), methicillin-resistant

Staphylococcus aureus (Wolters et al., 2011) and bacteria of genera *Arcanobacterium* and *Trueperella* (Hijazin et al., 2012a, 2012d). In veterinary field, MALDI-TOF MS was used to identify subclinical mastitis pathogens obtained from milk (Barreiro et al., 2010), the *Staphylococcus intermedius* group (SIG) (Decristophoris et al., 2011) and for *Streptococcus* spp. (Hinse et al., 2011). Also, in environmental microbiology MALDI-TOF MS allowed the identification of a wide spectrum of bacterial species (Ruelle et al., 2004; Munoz et al., 2011).

2.7.2 Fourier Transform Infrared Spectroscopy (FT-IR spectroscopy) for bacterial identification

William Herschel had discovered the radiation beyond the visible red light in the 1800s (Penn, 2014). The first application of infrared spectrophotometry to biological materials was introduced in 1949 by Barer, Cole and Thompson (Barer et al., 1949). Then it was widely applied to differentiate and identify bacteria in the 1950‘s (Thomas and Greenstreet, 1954, Riddle el al., 1956, Kenner et al., 1958). This technique showed a reproducible infrared spectrum of intact and entire bacteria as finger printing that are unique for individual strains. Later on the FT-IR spectroscopy technique was reintroduced by developing the basic instrumental and sample parameters for today‘s microbial analysis by FT-IR spectroscopy (Helm el al., 1991b; Naumann et al., l99l). In evaluating a new method for identification of microorganisms, there are many parameters which must be considered. It should be simple and not consume much time in the analytical procedures, it should be accurate and specific in its results. We can find this requirement in Fourier transform infrared spectroscopy (FT-IR spectroscopy) combined with artificial neural networks (ANNs) (Schmitt et al., l998). Using FT-IR spectroscopy unknown microorganisms can be identified very easily and quickly depending on an extensive database of spectral reference library. The infrared spectrum of the isolate under observation is compared with all spectra present in the reference library and it will be matched with the library strain whose spectrum is similar (Naumann et al., l99l). This technique has a high discrimination capability that can differentiate to species or even strain level together with simple usage and with high reproducibility and comparability of results in the long-term perspective. Also it is characterized by the high rapidity, less effort and low costs. All previous characteristics nominated the FT-IR to be applied for routine identification and diagnosis in industrial, clinical or food laboratories as well as research institutes (Naumann et al., l99l).

FT-IR spectroscopy technique is based on the fact that the atoms of a molecule can be excited with specific frequencies which will constantly vibrate around their equilibrium position (Fig. 1). The frequency of this oscillation depends on their mass, the type and nature of the chemical bonds as well as other effects such as hydrogen bonds, hydrophobic and electrostatic interactions. Furthermore, molecular bonds can be excited to vibration via the absorption of energy quanta of impinging infrared radiation. The infrared spectrum is a result of fraction absorption of the incident radiation which is at a particular energy after exposure the sample to infrared radiation. The absorbed energy at the used frequencies leads to the molecular mode of vibration which is corresponding to the molecule or chemical group in the sample (Stuart, 2004).

The peaks in an absorption spectrum are corresponding to the frequency of vibration of a part of a sample molecule. The unit wavenumber is commonly used instead of the wavelength since the former is directly proportional to the radiation energy. The IR radiation commonly embraces the electromagnetic radiation whose frequency is between 14,300 and 20 cm^{-1}. The fundamental operation of the FT-IR spectroscopy is shown in Fig. 2. The radiation from the IR source is directed onto a beam splitter, where it is splitted by two ways moving mirror and the fixed mirror. The two beams reunited again at the beam splitter producing interference, by using a Michelson interferometer that encodes the initial frequencies into modulated IR beam form to be read by the detector. The modulated IR beam is then reflected and passed through the sample compartment then to the detector compartment (Naumann, 2000). Before converting and translating the spectra data, the results should be transformed in order to minimize variability and to amplify the chemically-based spectral differences. For interpretation the results**,** the absorption spectrum raw data (interferogram) must be processed in order to turn the raw data (light absorption for each mirror position) into the desired result (light absorption for each wavelength). The processing required turns out to be a common algorithm called the Fourier transformer (hence the name, "Fourier transform spectroscopy").

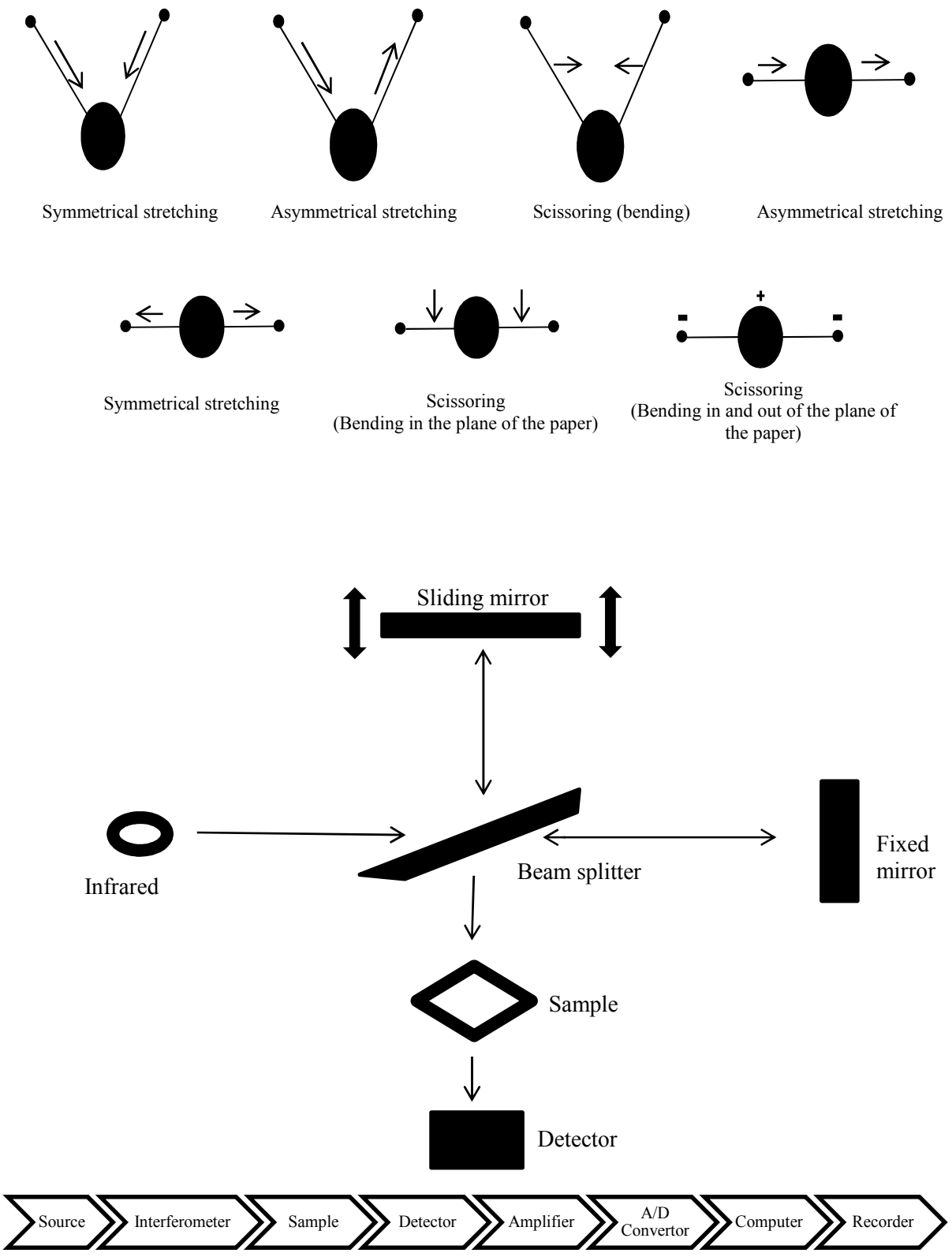

Fig. 2: The fundamental operations of the Fourier transform infrared spectroscopy (FT-IR) (modified according to Naumann, 2000).

The infrared radiation regions are divided into the near, mid and far infrared region. The mid-infrared region covers the wavelengths 4000-500 cm^{-1}. FT-IR spectra of microorganisms show broad and complex spectra rather than distinct peaks as in MALDI-TOF MS. The spectra represent complex images of the total chemical composition due to the using of the whole cells and they reveal the characteristic features of all cellular components, such as fatty acids, membrane proteins, intracellular proteins, polysaccharides and nucleic acids of the cell. Owing to the multitude of cellular compounds, broad and superimposed absorbance bands are observed throughout the entire spectral range (Helm et al., 1991a,b) and most of the structural information is hidden beneath the shape of the spectrum. Some of the IR bands are considered as marker bands of a distinct chemical structure or microbial composition such as poly-beta-hydroxybutyric acid granules, which are frequently found in genera *Bacillus*, *Clostridium*, *Acetobacter*, *Legionella* and *Pseudomonas* (Naumann, 2000).

However, the information content of the spectra is not yet fully understood. There are identified five spectral frequency ranges, which contain the significant spectral information (Naumann et al., 1991). Identifying and selection of the used frequency ranges relevant for the differentiation of microorganisms is an important task in the course of the establishment of a validated reference library (Helm et al., 1991a,b). FT-IR spectroscopy is a promising technique for rapid and reliable identification of bacterial microorganisms. The FT-IR spectroscopy has been reported for identification and classification of microorganisms for some species of the genera *Bacillus* (Beattie et al., 1998, Lin et al., 1998), *Lactobacillus* (Curk et al., 1994), *Listeria* (Holt et al., 1995, Lefier et al., 1997), and *Streptococcus* (Goodacre et al., 1996) and for urinary tract infection bacteria (Goodacre et al., 1998). A detailed FT-IR library for the identification of various fermentative yeasts has been already established to be used for the routine identification of a wide variety of yeast isolates from different habitats (Kümmerle et al., 1998). FT-IR spectroscopy has been established as a method for identification of several bacteria, yeasts and other microorganisms (Naumann et al., 2000; Wenning et al., 2014), also including *Actinomycetales* (Oberreuter et al., 2002, Contzen et al., 2011), *Thermomonosporaceae*, *Streptosporangiaceae*, *Micromonosporaceae* and *Streptomycetaceae* and *Corynebacteriaceae* (Haag et al., 1996). Additionally, FT-IR spectroscopy was a valuable tool for rapid screening of environmental isolates and microorganism (Tindall et al., 2000). FT-IR spectroscopy had already been used as tool for classification of *Listeria* and *Yersinia* species (Janbu et al., 2008; Kuhm et al., 2009; Wortberg et al., 2012), coryneform bacteria (Oberreuter et al., 2002) and for a large number

of other clinically relevant pathogens (Samuels et al., 2009; Contzen et al., 2011; Grunert et al., 2013). This spectroscopic technique had also been approved to investigate the most common mastitis-inducing bacteria from genera *Staphylococcus* (Spohr et al., 2011) and *Streptococcus* (Horlacher et al., 2009; Schabauer et al., 2011; Schabauer et al., 2014) and to determine the predominant bacterial flora in raw milk (Fricker et al., 2011). According to the preliminary results of Prunner et al. (2013) FT-IR spectroscopy could also be used to detect *T. pyogenes* in the uterus of cows of Austrian dairy farms.

2.7.3 Molecular targets for PCR-mediated identification

2.7.3.1 16S rRNA gene

Recently, due to the wide steps of development in researches and the widespread use of PCR, cloning and DNA sequencing, the 16S rRNA gene sequencing has played a pivotal role in the accurate identification of bacterial isolates and the discovery of novel species. The 16S rRNA gene is important for bacterial identification because it of its presence almost in all types of bacteria. Not only the presence of the 16S rRNA gene in all bacteria but also the large size, candidates it as a universal target for bacterial identification and offers a wide scope of analysis (Patel et al., 2001). In addition, 16S rRNA gene sequencing is a fast method for identification of unusual phenotypic bacteria or slow growing bacteria. Moreover, it also helps clinicians in identification and choosing antibiotics and in determining the duration of treatment and infection control procedures (Woo et al., 2008). Since 1980s, the 16S rRNA gene is another important landmark in the study of the evolution and classification of living organisms. Because of the wide spread of the 16S rRNA gene, it has served as base molecular identification tool for study of evolutionary relationships among groups of bacteria (Weisburg et al., 1991; Patel, 2001; Cai et al., 2003). Bacteria have 70S ribosomes, which are consisting of a small subunit (30S) and a large subunit (50S). The 16S ribosomal RNA (or 16S rRNA) is a component of the 30S small subunit of prokaryotic ribosomes (consisting of 1,543 nucleotides). The 50S is the larger subunit of the 70S ribosome of prokaryotes including the 5S ribosomal RNA (120 nucleotides), 23S ribosomal RNA (2,906 nucleotides). The S stands for Svedberg unit which refers to the molecular weight of each part after centrifugation and measuring the size of the sedimentation rate (Fig. 3) (Gürtler and Stanisich, 1996).

The sequencing of 16S rRNA gene has been widely used for bacterial identification using the rRNA gene sequence databases at GenBank and at RDP-II, which facilitate the identification

of unknown bacteria up to the genus or species level, and the generating of information on phylogenetic relation between different bacteria (Sacchi et al., 2002; Cai et al., 2003; Song et al., 2005). Gürtler and Stanisich suggested region two of the 16s rRNA gene (nt 1390-1407) as the regions of choice for the construction of primers because its high level of sequence conservation among the analysed species (Gürtler and Stanisich, 1996). Unfortunately, the use of 16S rRNA gene sequencing for species identification is not without limitations. Several authors had suggested criteria of the identification cutoff for a species and genus. Several studies of diverse taxa showed that the majority of the identified species that have been examined to date differ in their 16S rRNA gene sequences from related species of the same genus in at least 1% of the sequence positions and typically by more (Song et al., 2005). According to Palys et al. (1997) and Snydman et al. (2002) there is no definite cutoff value of 16S rRNA sequence similarity for species definition. However, Drancourt et al. (2000) defined the cutoff values of 16S rRNA-based bacterial identification. A value of ≥ 99% similarity of 16S rRNA gene sequence should be a suitable cutoff for bacterial species identification and ≥ 97% for bacterial identification at genus level. Moreover, Cai et al. (2003) showed that full 16S rRNA gene sequencing (about 1,540 nt) provides more accurate species identification for clinical bacteria from veterinary origin. These authors also mentioned that the sequencing of the species-specific variable regions (1, 2, and 3) within the 16S rRNA gene could be used as a low-cost preliminary identification tool (Cai et al., 2003). The Bergey's Manual of Systematic Bacteriology which is the most widely used and authoritative reference, classified 16S rRNA gene sequence analysis as the backbone tool in bacterial taxonomy (Garrity and Holt, 2001). The role of 16S rRNA gene as single gene as tool in evolution and classification of living organisms had been estimated by several researchers in comparison with molecular targets such as RNA polymerase subunits and heat shock protein. Construction phylogenetic trees based on the 16S rRNA gene is highly similar to the phylogenetic trees based on protein sequences of target genes (Goh et al., 1996; Ghebremedhin et al., 2008). The 16S rRNA gene sequence is used to study bacterial phylogeny and taxonomy of different microorganisms and resemble the most primary common genetic marker (Case et al., 2007). However, 16S rRNA gene sequencing has low discrimination power at the species level and poor discriminatory power for some genera (Bosshard et al., 2004; Mignard et al., 2006).

Fig. 3: Schematic representation of the bacterial rRNA operon (modified according to Gürtler and Stanisich, 1996).

2.7.3.2 16S-23S rDNA intergenic spacer region (ISR)

The ISR is located between the 16S and the 23S rRNA genes and displayes variable lengths in bacterial species. The size of the ISR appears to be important for different species (Condon et al., 1995). The ISR contains several functional units as transfer RNA (tRNA) genes and its length is depending on functional units in it. In Gram-negative bacteria like *E. coli* the ISR contains different tRNA genes including those of $tRNA^{Ala}$ (alanine), $tRNA^{Ile}$ (isoleucine) and $tRNA^{Glu}$ (glutamine) (Condon et al., 1995). The ISR of *Bacillus subtilis* contains both $tRNA^{Ala}$ and $tRNA^{Ile}$ genes (Loughney et al., 1982). In another study, the ISR of *Streptococcus pneumoniae* contains $tRNA^{Ala}$ but not $tRNA^{Ile}$ (Bacot et al., 1991). The constant regions of the ISR between the 16S and the 23S rRNA genes give the chance of designing universal oligonucleotide primers for PCR amplification of these DNA regions (Whiley et al., 1995). The first description of a universal ISR primer pair was developed in 1995 from conserved regions of the 16S and 23S ribosomal RNA genes, amplifying the ISR of different bacterial pathogens including *S. aureus*, *Enterococcus faecium*, *E. coli* and *Enterobacter* spp. (Kostman et al., 1995). That primer pair could be used later for identification of a wide variety of bacterial species of genus *Streptococcus* (Chanter et al., 1997).

The ISR is a good nominated region from which species-specific primers could be designed for various bacterial species (Barry et al., 1991; Tilsala-Timisjärvi and Alatossava, 1997). The ISR sequence of reference species of genera *Arcanobacterium* and *Trueperella* was amplified by Hassan et al. (2008), Ülbegi (2010) and Hijazin et al. (2010; 2012b) using the primer pair described by Kostman et al. (1995) and Chanter et al. (1997). Sequencing ISR also allowed a molecular identification of *A. haemolyticum* isolated from infections of horses (Hassan et al., 2009), *A. haemolyticum* from human origin (Sammra et al., 2014b) and *A. pluranimalium* isolated from a dog (Ülbegi-Mohyla et al., 2010). The design of ISR species-specific oligonucleotide primers could also be used for PCR-mediated identification of

T. pyogenes, *T. bernardiae*, *T. bialowiezensis*, *T. bonasi*, *A. haemolyticum* and *A. hippocoleae* (Hassan et al., 2008; Ülbegi, 2010).

2.7.3.3 23S rRNA gene

The 23S rRNA gene is 2,904 nt long in *E. coli*. It is longer and contains more sequence variations compared to the 16S rRNA gene (Lewin, 1998). Comparative taxonomic and phylogenetic studies showed that the 23S rRNA gene could also be used for bacterial identification. These variable areas of the 23S rRNA gene have a high discriminatory power and facilitated the design of species-specific primers which can be used for *Actinobacillus actinomycetemcomitans* (Preus et al., 1992), *Campylobacter* spp. (Eyers et al., 1993; Konkel et al., 1994) and *Pasteurella multocida* (Miflin and Blackall, 2001). According to Ülbegi-Mohyla et al. (2010) sequencing of the 23S rRNA gene also allowed a molecular identification of *A. pluranimalium* isolated from of a dog and a clear separation of all other *Arcanobacterium* and *Trueperella* species (Ülbegi-Mohyla et al., 2010). However, Jost et al. (2004) characterized ribosomal mutations within the 23S rRNA gene, which were associated with macrolide resistance in *T. pyogenes* (Jost et al., 2004).

In 1996, Gürtler and Stanisich suggested region ten of the 23S rRNA gene (nt 456-474) or with combination of the region seven of the 23S rRNA gene (nt 188-208) and region 2 of the 16s rRNA gene (nt 1,390-1,407) for primer construction due to its highly conserved sequence identity in most of the investigated species (Gürtler and Stanisich, 1996). However, Gürtler and Stanisich did not recommended the primer combinations involving region five of the 23s rRNA gene (nt 21-38) due to the relatively poor sequence conservation of this region. The view in using of 23S rRNA gene for comparative taxonomic and phylogenetic studies is changing over time. The 23S rRNA has lost advantage over 16S rRNA in phylogenetic analysis and taxonomic classification due to its large sequence. This is because of the inability to design broad-range sequencing primers and sequencing larger genes with older sequencing technology. However, there is a new interest in use of the 23S rRNA gene, due to the new generation DNA sequencing technology (454 sequencing) and it is used in the Roadmap Initiative in the Human Microbiome Project (http://nihroadmap.nih.gov/hmp/) (Pei et al., 2010). The 23S rRNA genes has more discriminatory power in phylogenetic analysies due to length, unique insertions and/or deletions and more sequence variability in comparison to 16S rRNA genes (Ludwig and Schleifer, 1994). In the same direction, another study showed that

23S rRNA genes contain highly conserved regions for designing broad-range primers with a similar degree of universality as the broad-range primers for 16S rRNA genes (Hunt et al., 2006; Pei et al., 2010).

2.7.3.4 Superoxide dismutase A encoding gene *sodA*

Superoxide dismutase enzyme displays antioxidant defense activity against superoxide free radical (O^-) thus protecting the cell from superoxide toxicity. Superoxide dismutase was previously a group of metalloproteinases with unknown function. The first description of the superoxide dismutase purified from bovine erythrocytes was mentioned in 1969 by McCord and Fridovich (1969). The superoxide is released when oxygen (O_2) becomes partially reduced to superoxide free radical (Fridovich, 1978). Free radical results in damage to lipids, proteins and DNA. It is proposed that the bacteria may decrease such toxicity by destroying free radical and utilize it (Imlay and Linn, 1988). There is another group of microorganisms which can live in fortified aerobic medium, and is able to detoxify superoxide into oxygen and hydrogen peroxide (H_2O_2) using enzyme catalase ($2\ O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$).

There are several forms of superoxide dismutases: they are proteins cofactored with copper and zinc (Cu/Zn-SOD), or manganese (Mn-SOD), iron (Fe-SOD), or nickel (Ni-SOD) (Clements et al., 1999). The major families of superoxide dismutase, depending on the metal cofactor are: Cu/Zn (which binds both copper and zinc), Fe and Mn types (which bind either iron or manganese) and the Ni type, which binds nickel. Mn-SOD and Fe-SOD are synthesized in prokaryotes (Martin et al., 1986; Clements et al., 1999). The manganese-dependent superoxide dismutase encoding gene *sodA* is widely applied as target for identification and differentiation of different bacterial species such as *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). A universal oligonucleotide primer pair was designed which allowed the amplification, sequencing and identification of gene *sodA* of genera *Arcanobacterium* and *Trueperella* (not *T. bialowiezensis*) (Ülbegi, 2010). The *sodA* sequencing facilitated a clear separation of species of genus *Arcanobacterium* from those of genus *Trueperella* (Hijazin, 2012).

2.7.3.5 Translation elongation factor Tu

Translation elongation factor Tu is one of the prokaryotic elongation factors (EF-Tu, EF-Ts and EF-G), which binds all elongator aminoacyl–transfer RNAs (aatRNAs) for their delivery to the ribosome during protein synthesis (LaRiviere et al., 2001). In the ribosome, they facilitate translational elongation of the nascent amino acid chain, from the formation of the first peptide bond to the formation of the last one. Translation elongation factor Tu is activated upon binding with GTP and forms a complex with transfer RNA (t-RNA) that links individual amino acids together and forms the protein chain. Then comes the role of the messenger RNA (mRNA) which carries a codon that codes for each amino acid which is exposed in the ribosomal A-site. The ribosome creates a protein chain by following the mRNA code and selecting the next t-RNA and its amino acid. Translation elongation factor Tu is recycled into another GTP active form by the nucleotide-exchange factor EF-Ts. Finally, EF-G helps in the translocation of tRNAs and the mRNA by exactly one codon on the ribosome (Andersen et al., 2003). Translation elongation factor Tu is composed of two distinct parts: a nucleotide-binding protein, EF-1α and a nucleotide exchange protein complex EF-βγ. Translation elongation factor Tu usually occurs in different molecular forms depending on the different amounts of EF-1α and EF-βγ. The molecular mass varies from 50 kDa up to several thousand kDa. The molecular mass of EF-β is 26 kDa and EF-γ is 46 kDa. EF-1α consists of three domains. Domain I or the G domain plays a role in binding to GTP or GDP. Both domain II and domain III are connected together thus, likely to act as one functional unit (Andersen et al., 2003). The translation elongation factor Tu encoding gene *tuf* is widely applied in identification and differentiating different bacterial species such as *Lactobacillus* spp. and *Bifidobacterium* spp. (Ventura et al., 2003), *Yersinia* spp. (Isabel et al., 2008) and Gram-positive cocci of the genra *Enterococcus*, *Streptococcus*, *Staphylococcus*, and *Lactococcus* (Li et al., 2012).

2.7.3.6 RNA polymerase encoding gene *rpoB*

The *rpoB* gene encodes the β subunit of bacterial RNA polymerase which plays the main role in the majority of the catalytic function of the RNA polymerase (Adékambi et al., 2009). The *rpoB* gene is widely used for an accurate representation of microbial communities like species of genus *Corynebacterium* (Khamis et al., 2004), *S. aureus* (Rowland et al., 1993), *Enterobacteriaceae* (Mollet et al., 1997) and genus *Mycobacterium* (Adékambi et al., 2006).

The *rpoB* gene was also useful in differentiating species of genera *Arcanobacterium* and *Trueperella* (Ülbegi, 2010). In some non-tuberculosis mycobacteria, the *rpoB* gene provided a better phylogenetic resolution better than the 16S rRNA gene (Adékambi et al., 2009).

2.7.3.7 DNA gyrase subunit A encoding gene *gyrA*

DNA gyrase is composed of two subunits GyrA and GyrB. GyrA consists of two domains: an N-terminal 64 kDa domain (GyrA64) and a C-terminal 33 kDa domain (GyrA33) (Williams et al., 2001). DNA gyrase is an essential enzyme for DNA supercoiling and DNA-wrapping, which is required for DNA replication and gene transcription (Gellert et al., 1976; Williams et al., 2001). Gene *gyrA* was successfully used for identification and to study the taxonomic relationship of *Klebsiella pneumonia* (Brisse and Verhoef, 2001), *Campylobacter jejuni* and *Campylobacter coli* (Ragimbeau et al., 2014) and *Actinomyces naeslundii* and *Actinomyces oris* (Henssge et al., 2009).

2.7.3.8 Glyceraldehyde-3-phosphate dehydrogenase encoding gene *gap*

The glyceraldehyde 3-phosphate dehydrogenase is a glycolytic enzyme responsible for the glycolysis step and helped in breakdown of glucose for energy and carbon molecules (Lodish et al., 2004). The application value of gene *gap* as biological marker for identification and for taxonomical species analysis of different bacterial genera was confirmed in previous studies (Yugueros et al., 2000, Nawrotek et al., 2009: Sammra et al., 2014a).

2.8 Identification and classification of bacteria using DNA fingerprinting methods

Many biomolecular methods have been developed for identifying and subtyping bacteria at species level like pulsed-field gel electrophoresis, restriction fragment length polymorphism PCR, arbitrarily primed PCR/random amplification of polymorphic DNA, repetitive sequencing-based PCR, denaturing gradient gel electrophoresis, high-resolution melting analysis, multilocus sequence typing and analysis, sequencing of 16S-23S rRNA gene internal transcribed spacer, whole genome sequencing and DNA hybridization (Li et al., 2009). The subtyping using serological and bacteriological methods cannot be assigned for all bacterial species and the phenotypic characteristics under different environmental and culture

conditions may not be stably expressed (Farber, 1996; Olive and Bean, 1999). Typing of bacteria is a fast growing research field, which is a combination of older methods with developing, advanced and new methods. The bacterial typing research field is directed now from the phenotypic approaches towards DNA-based genotypic subtyping methods (Olive and Bean, 1999). These subtyping methods differ in the usage, results, resolution, interpretation and cost. DNA-based molecular typing methods depend on a combination of two elements: genetic loci that own regions of high conservation and can be targeted in all isolates and loci that also provide highly variable regions that will be the basis for differentiation among the isolates.

2.8.1 Genomic fingerprinting using repetitive sequence-based polymerase chain reaction (rep-PCRs)

The genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction is based on the presence of oligonucleotide primers complementary to interspersed repetitive sequence elements, which will enable the amplification of differently sized DNA fragments from the respective bacterial genome depending on the sequences lying between these elements. These multiple DNA amplicons of different sizes can be fractionalized by electrophoresis in to a band pattern, which is regarded as a DNA fingerprint of the bacterial strain under investigation. This fingerprint is specific for each individual bacterial clone, which can clearly distinguish between different eubacterial species and strains. Either purified genomic DNA or crude cell lysate can be used directly for PCR analysis (Versalovic et al., 1994; Versalovic et al., 1998).

There are dispersed repetitive sequences separating longer single-copy DNA sequences in both prokaryotic and eukaryotic genomes (Versalovic et al., 1998). The noncoding repetitive elements are present in high copy numbers relative to the larger repeated elements which contain coding sequences (Yanofsky et al., 1981; Lawther et al., 1987; Versalovic et al., 1994). These families of short intergenic repeated sequences have been described in enteric bacteria and in Gram-negative and Gram-positive bacteria representing several different genera (Versalovic et al., 1991; Versalovic et al., 1994). The interspersed repetitive DNA sequence size is variable in length between 15 and several hundred base pairs (bp)' (Lupski and Weinstock, I992). Various classes of repeated DNA sequences have been described in diverse prokaryotic genomes such as BOX (Martin et al., 1992), ERIC (IRU)

(Hulton et al., 1991; Sharples and Lloyd, 1990; Versalovic et al., 1991) and REP (PU) (Higgins et al., 1982; Stern et al., 1984; Gilson et al., 1984; Versalovic et al., 1991).

The BOX elements are repetitive DNA sequences, which have a differentially conserved subsequence nature and were firstly described in Gram-positive organism (*Streptococcus pneumoniae*) (Martin et al., 1992). The BOX elements consists of three different subunits; boxA (57 bp), boxB (43 bp) and boxC (50 bp) (Martin et al., 1992). The boxA-like subunit sequence appears to be highly conserved and in high copy numbers among different bacteria like *S. pneumoniae*, but is not present in other streptococcal organisms such as *Streptococcus pyogenes* and *Streptococcus agalactiae.* Also, multiple copies of boxA-like subunit subsequences were found in Gram-negative organisms such as *E. coli* and *Salmonella* Typhimurium scattered in their respective genomes. The boxB and boxC subunits were only found in *S. pneumonia* (Versalovic et al., 1994).

The repetitive extragenic palindromic (REP) elements are 38 bp palindromic nucleotide units, which contain a 5 bp variable loop in the proposed stem-loop structure (Higgins et al., 1982; Stern et al., 1984; Gilson et al., 1984). The enterobacterial repetitive intergenic consensus (ERIC) (IRU) elements have a size of 126 bp and are characterized by central conserved nucleotide sequences (Hulton et al., 1991; Sharples and Lloyd, 1990). The REP and ERIC sequences were described in the Gram-negative enteric bacteria, *E. coli* and *S.* Typhimurium. Also these elements were described in related Gram-negative enteric bacteria and various unrelated bacteria from multiple phyla (Versalovic et al., 1991; de Bruijn, 1992). Another interspersed element called direct repeat (DR) was recently described in *Mycobacterium bovis* (Doran et al., 1993). The rep-PCR methods are depending on the diversity of insertion sites of interspersed repetitive elements and do not need prior DNA sequence information about these sites (Versalovic et al., 1998). These genotyping methods can be universally used for most Gram-positive and Gram-negative bacteria but differ in their discriminatory power depending on the taxonomic level they are used (Fig. 4). The rep-PCRs based typing methods represent the first-line molecular typing in clinical microbiology laboratories because it is an easy tool to use as well as rapid, requiring less hands-on time than PFGE typing. The genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction analysis is not informative at the taxonomic level of the genus or family. However, it has a high discriminatory power at the taxonomic level of the species, subspecies and strain (Savelkoul et al., 1999).

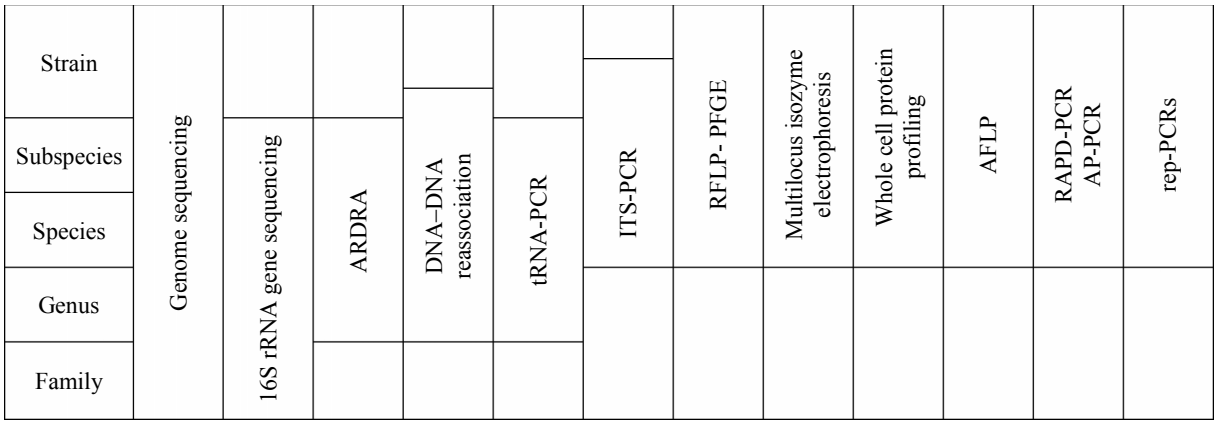

Fig. 4: Comparison between relative applicability of various fingerprinting and DNA techniques at different levels of taxonomic resolution. ARDRA: amplified ribosomal DNA restriction analysis; tRNA-PCR: tRNA intergenic spacer region PCR; ITS-PCR: 16S-23S rRNA intergenic spacer region PCR; RFLP: Restriction fragment length polymorphism; PFGE: pulsed-field gel electrophoresis; AFLP: amplified fragment length polymorphism; AP-PCR, arbitrarily primed PCR. (modified according to Savelkoul et al., 1999)

2.8.2 Genomic fingerprinting using randomly amplified polymorphic DNA polymerase chain reaction (RAPD-PCR)

The randomly amplified polymorphic DNA PCR (RAPD-PCR) is another genomic fingerprinting method developed in 1990 by Williams (Williams et al., 1990). The RAPD-PCR is different from traditional PCR analysis, in that it does not require any specific knowledge of the target DNA sequence of the organism (Welsh and McClelland, 1990). This technique is used to establish genetic maps in a variety of species which will lead to find the genetic variation, relationship between species and development of organism. The RAPD fingerprinting method is based on the use of single arbitrarily chosen primers with a length of about 10 nt under relatively low annealing temperature. These primers will amplify targeted genomic DNA. These leads to the amplification of several genomic DNA fragments with different sizes and resulting in sequences and different amplified DNA band patterns in electrophoresis. In previous studies, RAPD-PCR has been successfully used for genetic fingerprinting and molecular typing of various species, including *Campylobacter jejuni* (Payne et al., 1999), *Streptococcus thermophilus, Enterococcus faecalis,* and *Enterococcus faecium* (Moschetti et al., 2001), *Mycobacterium tuberculosis* (Singh et al., 2006) and *E. coli* (Salehi et al., 2008).

2.8.3 Genomic fingerprinting using multilocus sequence typing (MLST) and multilocus sequence analysis (MLSA)

In the last century, bacterial taxonomy was based on different approaches including both phenotypic and genotypic results with 16S rRNA gene sequence analysis. Later on, the DNA–DNA hybridization was used and is considered as a gold standard for distinguishing species and genotypic characterization for descriptions of novel species (Vandamme et al., 1996; Coenye et al., 2005). However, some disadvantages of DNA–DNA hybridization were recorded and criticized (Stackebrandt et al., 2002; Schouls et al., 2003). The ad hoc committee for re-evaluation of the species definition in Bacteriology suggested the multilocus sequence analysis (MLSA) as a method of great promise for prokaryotic systematics (Stackebrandt et al., 2002). The multilocus sequence typing (MLST) and the multilocus sequence analysis (MLSA) are molecular biology techniques used for typing and analysis of multiple loci in a bacterial genome. Both methods depend on characterizing isolates of microbial species by sequencing the DNA of multiple housekeeping genes to establish evolutionary and phylogenetic relationships between closely related species. MLSA was recently considered as the best method in studying and finding phylogenetical relationships between closely related species and to discriminate clonal lineage of the same species. MLSA and MLST depend on sequencing different fragments of housekeeping genes (400 to 600 bp in length), using an automated DNA sequencer. In MLST, the sequences of internal fragments are assigned as distinct allele numbers. The alleles at each of the loci define the allelic profile or sequence type (ST) for each isolate (Maiden, 2006). In MLSA, the actual DNA and protein-coding sequences are used directly in the downstream analyses to describe the evolutionary and phylogenetic relationship between microorganisms (Gevers et al., 2005).

In 1998, the first MLST scheme was published for *Neisseria meningitides* (Maiden et al., 1998). Several studies have used the sequences from housekeeping genes for identification on the species level (Wertz et al., 2003; Zeigler, 2003), as well as for evolutionary population genetics and taxonomy (Stepkowski et al., 2003; Vinuesa et al., 2005a, b; Bailly et al., 2006). MLST is also used as a biomolecular technique for molecular epidemiology and population genetic studies of several bacterial pathogens (Cooper and Feil, 2004; Maiden et al., 1998; Maiden, 2006; Turner and Feil, 2007; Urwin and Maiden, 2003). MLSA is based on partial sequencing of well-chosen protein-coding housekeeping genes. These housekeeping genes should be universally distributed in different microbes, present as single copies and located at

distinct chromosomal loci (Gevers et al., 2005; Kämpfer and Glaeser, 2012). The housekeeping protein coding genes are essential in normal and patho-physiological conditions for supporting the maintenance of normal basic cellular functions and are highly expressed under all conditions of the microbe (Zhu et al., 2008). MLSA as a rapid and robust tool could be applied using a universal set of genes, which would allow a hierarchical classification of all prokaryotes.

MLSA is depending on sequencing several universal genes that are present within single copy at least in the taxon of the bacteria under study. Then, comes the next step, which does not use the assigned alleles, but instead concatenated the sequences of the multiple genes to construct a phylogenetic tree (Gevers et al., 2005). The phylogenetic tree of multiple concatenate gene sequences is used to overcome the limitations of single gene phylogenies. The genetic relatedness between the investigated microbes was measured by various genetic markers like the phylogenetic analysis based on DNA nucleotide sequence, GC content, the pairwise comparison, amino acid sequences, the analysis of the amino acid compositions and amino acids composition similarity.

3 Materials and methods

3.1 Bacterial strains and isolates

A total of 96 bacterial strains and isolates were investigated in the present study. The cultures included the investigated 75 *T. pyogenes* isolates, *T. pyogenes* CVUAS 0222, the reference strains of genera *Trueperella* and *Arcanobacterium* (*T. pyogenes* DSM 20630^T, *T. pyogenes* DSM 20594, *T. abortisuis* DSM 19515^T, *T. bernardiae* DSM 9152^T, *T. bialowiezensis* DSM 17162^T, *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. phocisimile* DSM 26142^T, *A. pluranimalium* DSM 18483^T and *Arcanobacterium canis* DSM 25104^T). The cultures included also *Actinomyces hyovaginalis* CVUAS 4295, the reference strains *Actinomyces bovis* DSM 43014^T, *Actinomyces weissii* DSM 24894^T and *Actinomyces canis* DSM 15536^T. Furthermore, ß-hemolytic *S. aureus, Rhodococcus equi* and *S. agalactiae* were used as indicator strains.The *T. pyogenes* composed of 57 isolates from bovine mastitis, an unusual gelatinase negative *T. pyogenes* which was also isolated from bovine mastitis, 14 *T. pyogenes* which were recovered from bovine cervical swabs and three *T. pyogenes* which were isolated from three grey slender lorises (*Loris lydekkerianus nordicus*). The 57 *T. pyogenes* isolates from bovine clinical mastitis were isolated from cow milk of dairy farms across Hesse (n=54) and North Rhine-Westphalia (n=3) during routine microbiological diagnostics. The *T. pyogenes* isolates were kindly provided by Dr. M. Zschöck, Dr. T. Eisenberg and Dr. K. Schlez (Landesbetrieb Hessisches Landeslabor (LHL), Schubertstraße 60, 35392 Gießen, Germany). The 57 *T. pyogenes* were collected in a period of 3 years from 2009 to 2012. The isolates 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. 2. For two of the *T. pyogenes* isolates (*T. pyogenes* 1265/79 and *T. pyogenes* 944/81) no further information about the milk condition was available. The geographical distribution and the sampling locations of the *T. pyogenes* isolates from Hesse and North Rhine-Westphalia involved in this study are shown in Fig. 5.

Tab. 2: Additional information of the 57 *T. pyogenes* isolates from bovine mastitis.

	Isolates designation	Sample submission	Location	Farm	Cow	Infected quarter	Flakes / Cell count (×1000/ml)	Presence of *T. pyogenes*/* Additionally isolated microorganisms
1	*T. pyogenes* 1220/112	18.09.2009	1	E	GR	HL	Flakes	+++/-
2	*T. pyogenes* 1221/4	18.09.2009	2	S	IN	HL	Flakes	+++/-
3	*T. pyogenes* 1265/79	01.10.2009	3	S	780	HL	n.d.	+++/-
4	*T. pyogenes* 1366/28	21.10.2009	4	V	SU	HL	Flakes	+++/-
5	*T. pyogenes* 1383/3	23.10.2009	5	S	66280	FL	Flakes	+++/-
6	*T. pyogenes* 219/27	18.02.2010	6	H	172	FL	Flakes	+++/-
7	*T. pyogenes* 239/30	24.02.2010	7	M	1510	HR	Flakes	+++/-
8	*T. pyogenes* 254/208	24.02.2010	8	B	29	HL	1294	++/-
9	*T. pyogenes* 857/23	24.06.2010	9	S	89	FL	Flakes	+++/-
10	*T. pyogenes* 944/81	12.07.2010	9	S	511	FR	n.d.	+++/-
11	*T. pyogenes* 983/277	16.07.2010	10	H	293	FR	Flakes	+++/-
12	*T. pyogenes* 1022/30	22.07.2010	11	Z	GE	HR	Flakes	+++/-
13	*T. pyogenes* 1059/7	29.07.2010	12	T	342	FL	Flakes	+++/Esculin-positive streptococci +
14	*T. pyogenes* 1240/384	26.08.2010	13	F	95944	HL	14963	+++/-
15	*T. pyogenes* 1247/96	26.08.2010	14	W	LI	HL	Flakes	+++/*E. coli* ++
16	*T. pyogenes* 1512/21	14.10.2010	15	W	21	HR	Flakes	+++/-
17	*T. pyogenes* 1800/9	20.12.2010	16	V	137	FR	18313	++/-
18	*T. pyogenes* 10/46	06.01.2011	17	H	337	HR	22698	+++/-
19	*T. pyogenes* 30/4	10.01.2011	18	D	51	HL	Flakes	+++/-
20	*T. pyogenes* 36/108	11.01.2011	19	W	474	HL	Flakes	+++/-
21	*T. pyogenes* 56/178	14.01.2011	20	S	FL	HR	Flakes	+++/-
22	*T. pyogenes* 58/4	14.01.2011	21	M	61284	HL	Flakes	+++/-
23	*T. pyogenes* 59/11	14.01.2011	15	W	52	FL	1197	+/Aerobic bacilli +
24	*T. pyogenes* 79/15	18.01.2011	22	Z	21479	FL	Flakes	+++/*Streptococcus dysgalactiae* +
25	*T. pyogenes* 185/2	03.02.2011	23	G	HE	HR	Flakes	+/-
26	*T. pyogenes* 220/1	11.02.2011	21	M	83	FR	Flakes	+/*Corynebacterium* sp. +, Coagulase-negative staphylococci +
27	*T. pyogenes* 313/63	02.03.2011	24	S	AN	FL	11754	+++/-
28	*T. pyogenes* 350/1	09.03.2011	25	F	46	FR	Flakes	+/-
29	*T. pyogenes* 523/8	14.04.2011	26	K	66288	HL	Flakes	+++/-
30	*T. pyogenes* 543/31	19.04.2011	27	O	TR	FL	6005	++/-
31	*T. pyogenes* 625/2	09.05.2011	28	O	486	FR	Pus	+/-
32	*T. pyogenes* 640/1	11.05.2011	29	K	416	FR	Flakes	++/-
33	*T. pyogenes* 843/2	15.06.2011	16	S	111	HR	Flakes	+++/-
34	*T. pyogenes* 868/215	20.06.2011	30	K	479	FL	552	+++/Coagulase-negative staphylococci ++
35	*T. pyogenes* 887/77	21.06.2011	31	R	520	FR	17475	++/-
36	*T. pyogenes* 899/2	22.06.2011	32	L	16	HR	2009	+/Aerobic bacilli +, Coagulase-negative staphylococci +
37	*T. pyogenes* 898/95	24.06.2011	33	K	34825	FL	21284	++/-

Continued on the next page

Tab. 2 (Continued)

	Isolates designation	Sample submission	Location	Farm	Cow	Infected quarter	Flakes / Cell count (×1000/ml)	Presence of *T. pyogenes*/* Additionally isolated microorganisms
38	*T. pyogenes* 1056/5	20.07.2011	34	C	59	FR	3702	++/-
39	*T. pyogenes* 1065/41	21.07.2011	35	A	94120	FR	17964	+/-
40	*T. pyogenes* 1065/47	21.07.2011	35	A	29041	FL	12457	++/-
41	*T. pyogenes* 1183/105	12.08.2011	36	Q	134	FR	Flakes	+++/-
42	*T. pyogenes* 1234/1	22.08.2011	37	B	1-4	FR	Flakes	+++/-
43	*T. pyogenes* 1256/2	24.08.2011	38	M	J	HR	Flakes	++/-
44	*T. pyogenes* 1268/20	25.08.2011	39	V	976	HL	20433	++/-
45	*T. pyogenes* 1295/3	01.09.2011	40	W	0662	FL	18525	+++/-
46	*T. pyogenes* 1584/22	24.10.2011	41	O	62932	HR	Flakes	+++/-
47	*T. pyogenes* 1668/18	02.11.2011	42	S	52	HR	1413	+++/-
48	*T. pyogenes* 1749/204	17.11.2011	43	T	42	HL	1540	++/-
49	*T. pyogenes* 1815/17	29.11.2011	44	O	2614	FR	6865	+++/-
50	*T. pyogenes* 1881/14	09.12.2011	45	S	B	HR	Flakes	++/-
51	*T. pyogenes* 199/135	30.01.2012	46	H	IR	FL	1935	+/-
52	*T. pyogenes* 534/20	30.01.2012	47	Z	387	HL	1092	+/-
53	*T. pyogenes* 336/1	23.02.2012	48	W	5134	FR	Flocks	+++/-
54	*T. pyogenes* 336/2	23.02.2012	48	W	5134	HL	Flocks	+++/-
55	*T. pyogenes* 336/4	23.02.2012	48	W	5134	FL	Flocks	+++/-
56	*T. pyogenes* 467/67	19.03.2012	49	E	P	HL	Flocks	+++/Coagulase-negative staphylococci +, *S. dysgalactiae* ++
57	*T. pyogenes* 506/74	23.03.2012	31	L	525	HR	7939	++/-

FR = forward right, FL = forward left, HR = hind right, HL = hind left

* + = low, + + = moderate, +++ = high rate of isolation; n.d. = no data available

One additionally investigated *T. pyogenes* isolated from bovine mastitis, showed unusual behavior. The unusual proteinase negative *T. pyogenes* 754B was kindly obtained from Dr. R. Huber-Schlenstedt (Tiergesundheitsdienst Bayern e.V., Fachabteilung Eutergesundheitsdienst und Milchhygiene, Senator-Gerauer-Str. 23, 85586 Poing, Germany), Dr. R. Weiß and Dr. E. Prenger-Berninghoff (Institut für Hygiene und Infektionskrankheiten der Tiere, Justus-Liebig-Universität Gießen, Frankfurterstraße 85-89, 35392 Gießen, Germany).

The 14 *T. pyogenes* isolated from cervical swabs of bovines with endometritis (n=8) and from cervical swabs of apparently healthy cows (n=6) were kindly obtained from Prof. Dr. J. Aurich (Klinische Abteilung für Geburtshilfe, Gynäkologie und Andrologie) and from Dr. J. Spergser (Institut für Mikrobiologie), both from Veterinärmedizinische Universität Wien, Austria. The isolates designation, the origin and additional information regarding the clinical findings of the animals are summarized in Tab. 3.

Tab. 3: Additional information of the *T. pyogenes* isolated from bovine endometritis (1-8) and from cervical swabs of apparently healthy cows (9-14).

	Isolates designation	Specimen	Endometritis
1	*T. pyogenes* Wi10	Cervix	Yes*
2	*T. pyogenes* Wi24B	Cervix	Yes
3	*T. pyogenes* Wi2770	Cervix	Yes
4	*T. pyogenes* Wi25B	Cervix	Yes
5	*T. pyogenes* Wi23B	Cervix	Yes
6	*T. pyogenes* Wi1263	Cervix	Yes
7	*T. pyogenes* Wi371	Cervix	Yes
8	*T. pyogenes* Wi30A	Cervix	Yes
9	*T. pyogenes* Wi4	Cervix	No
10	*T. pyogenes* Wi844	Cervix	No
11	*T. pyogenes* Wi1361	Cervix	No
12	*T. pyogenes* Wi593	Cervix	No
13	*T. pyogenes* Wi17	Cervix	No
14	*T. pyogenes* Wi12	Cervix	No

* Information given by Prof. Dr. J. Aurich (Petit et al., 2009)

Fig. 5: The geographical distribution and the sampling locations of the *T. pyogenes* isolates from Hesse and North Rhine-Westphalia states.

The three *T. pyogenes* isolates of grey slender lorises origin were isolated from three grey slender lorises, which died in a period of two years at Frankfurt Zoo. The three grey slender lorises, originated from a European Association of Zoo and Aquaria (EAZA) breeding program, died in 2011 and 2012 and were kindly provided by Dr. T. Eisenberg (Landesbetrieb Hessisches Landeslabor (LHL), Schubertstraße 60, 35392 Gießen, Germany). Further data about the three *T. pyogenes* is shown in Tab. 4.

Tab. 4: Additional information of the *T. pyogenes* isolates from three grey slender lorises.

	Isolate designation	Sample submission	Specimen	Presence of *T. pyogenes*/* Additionally isolated microorganisms
1	*T. pyogenes* 11-7-D-03394	30.09.2011	Facial abscess	+++/ Coagulase-negative staphylococci +
2	*T. pyogenes* 121008157	25.05.2012	Nasal swab	+++/*Pseudomonas aeruginosa* +, *Pasteurella* sp. +++, *Enterococcus* sp. +++, Coliform bacteria +
3	*T. pyogenes* 121018522	11.12.2012	Nasal swab	+++/*Pseudomonas aeruginosa* +

* + = low, +++ = high rate of isolation

3.2 Cultivation on sheep and rabbit blood agar

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

Blood Agar Base, Cat. No. 1.10886.	40 g
Defibrinated blood	50 ml
Distilled water	up to 1000 ml

pH: 6.8 ± 0.2 at 25 °C.

The medium was prepared according to the manufacturer's specifications. The Blood Agar Base was suspended and autoclaved for 15 min at 121 °C, then cooled to 45-50 °C and 5-8% defibrinated blood were added and mixed. Sheep and rabbit blood agar were processed aseptically at Institut für Hygiene und Infektionskrankheiten der Tiere, Justus-Liebig-Universität Gießen, Frankfurterstraße 85-89, 35392 Gießen, Germany. 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).

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

3.3 Preservation of the bacteria

The bacteria were cultivated on sheep blood agar (3.2). Using sterile cotton swabs (Böttger, Bodenmais, Germany) the freshly grown bacteria were collected and transferred into a sterile microtube 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 Hemolytic properties and CAMP-like hemolytic reactions

The bacterial cultures were cultivated on sheep or rabbit blood agar (3.2) to determine the colony morphology and hemolysis. The evaluation was made after microaerobic incubation for 48-72 h at 37 °C in a candle jar. To read the hemolytic reaction on a blood agar plate, the plate must be held up to a light source and observed with the light coming from behind (transmitted light). The reference strains and field isolates in this study were also investigated for synergistic or CAMP-like and antagonistic or reverse hemolytic reactions. The CAMP-reaction, named for Christie, Atkins and Munch Petersen (1944) who elaborated that test to differentiate group B streptococci. The CAMP-reaction principle is based on β-lysin produced by β-hemolysin producing *S. aureus* which has a synergistic hemolytic effect with the CAMP factor produced by both β-hemolytic and non-hemolytic *Streptococcus agalactiae* (group B) (Fraser, 1964). The determination of CAMP-like and antagonistic hemolytic reactions was performed on sheep blood agar (3.2) using ß-hemolytic *S. aureus, S. agalactiae*, *Rhodococcus equi* as indicator strains and the reference strains *A. haemolyticum* DSM 20595^T, *T. bonasi* DSM 17163^T, *T. pyogenes* DSM 20630^T and *T. pyogenes* DSM 20594 as control strains. The indicator strains 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 supplied from the strain collection of Institut für Pharmakologie und Toxikologie, Justus-Liebig-Universität Gießen. The incubation of the inoculated plates were incubated for 48 h at 37°C under microaerobic conditions. (3.2). The positive result of the CAMP reaction is indicated by an "arrowhead"-shaped enhanced zone of staphylococcal β-hemolysis (Lányi, 1988) or as a half-moon forming zone of complete hemolysis in close proximity of the indicator strains. The antagonistic or reverse CAMP reaction is an arc-shaped zone of inhibition of staphylococcal ß-

hemolysin in the presence of exotoxins of *A. haemolyticum* and *A. phocae*. A reverse CAMP reaction is a characteristic property of *A. haemolyticum* and *A. phocae* (Fraser, 1964; Johnson et al., 2003).

3.4.2 Biochemical characterization using a commercial test system

The API Coryne system (Biomerieux, Nürtingen, Germany) consists of 20 microtubes containing dehydrated substrates for the detection of 11 enzymatic activities (nitrate reduction, pyrazinamidase, pyrrolidonyl arylamidase, alkaline phosphatase, β-glucuronidase, β-galactosidase, α-glucosidase, N-acetyl-β-glucosaminidase, esculin hydrolysis, urease, gelatin hydrolysis and catalase) and 8 carbohydrate fermentation tests (D-glucose, D-ribose, D-xylose, D-mannitol, D-maltose, D-lactose, D-saccharose and glycogen). The tray of the incubation box was wetted with 5 ml of sterile distilled water to provide a moist atmosphere, which prevents drying of the strip. Using a freshly subcultured bacterium to form a saline suspension with a minimum turbidity of McFarland 6-standard (Biomerieux) a plastic tray holding 20 mini-test tubes was inoculated (as per manufacturer's directions). Some tubes were completely filled with the suspension medium and other tubes (urea hydrolysis and carbohydrate fermentations), were overlaid with mineral oil in which an anaerobic reaction is carried out. After incubation for 48 h at 37 °C under microaerobic conditions, one drop of Nit 1 and Nit 2 reagent (Biomerieux) has been added to the nitrate microtube, one drop of PYZ reagent (Biomerieux) to pyrazinamidase and one drop of each Zym A and Zym B reagent (Biomerieux) to the pyrrolidonyl-arylamidase, alkaline phosphatase, β-glucuronidase, β-galactosidase, α-glucosidase and N-acetyl-β-glucosaminidase microtubes. The color reactions were read according to reading table. The catalase activity was estimated by adding one drop of hydrogen peroxide (3%) to the ESC or GEL test.

3.4.3 Detection of enzyme activity with Rosco Diatabs and 4-methylumbelliferyl-conjugated substrates

A bacterial suspension of the tested isolates of McFarland 4-standard were prepared in 0.25 ml sterilized 0.9% sodium chloride (Roth, Karlsruhe, Germany) in 1.5 ml Eppendorf tubes (Sarstedt). A diagnostic test tablet Diatabs™ (Rosco Diagnostica A/S, Taastrup, Denmark) was added to the bacterial suspension for detection of the enzymes. The tube was sealed and incubated for 4 h or up to 18-24 h at 37 °C were incubated for 48 h at 37°C under

microaerobic conditions. These tests were performed according to the instructions provided by the manufacturer (Rosco Diagnostica A/S), for the detection of the enzymes α-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase and α-mannosidase. A yellow color indicated a positive reaction and a colorless or very slight yellow was registered as negative reaction. For the detection of α-galactosidase *A. phocae* DSM 10002^T was used as positive control and *T. pyogenes* DSM 20630^T as negative control. For the detection of β-glucuronidase *T. pyogenes* DSM 20594 was used as positive control and *A. phocae* DSM 10002^T as negative control. For the detection of α-glucosidase *T. pyogenes* DSM 20630^T was used as positive control and *T. bonasi* DSM 17163^T as negative control. For the detection of β-glucosidase *A. pluranimalium* DSM 13483^T was used as positive control and *T. pyogenes* DSM 20630^T as negative control. For the α-mannosidase test *A. haemolyticum* DSM 20595^T was used as positive control and *T. bernardiae* DSM 9152^T as negative control.

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) were purchased from Sigma-Aldrich, Steinheim, Germany. They were used according to the information given by Maddocks and Greenan (1975), Slifkin and Gil (1983) and Bravo-Torres et al., (2003). The bacterial enzyme detection is based on the generation of fluorescence when free 4-methylumbelliferone, which is the fluorogenic substrate, is released by enzymatic hydrolysis of the non-fluorescent 4-methylumbelliferyl-conjugated substrates. According to Maddocks and Greenan (1975) and Slifkin and Gil (1983), the 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 in 0.2 ml of dimethyl sulfoxide (Roth). The final volume of each solution was filled up to 10 ml with 0.2 mol/l sodium acetate buffer pH 5.2. In order to investigate the presence of the bacterial enzymes, a loop of the freshly cultivated bacterial colonies (3.2) was spread onto a filter paper (Macherey-Nagel, Düren, Germany), followed by adding 20 µl of the 4-methylumbelliferyl-conjugated substrates to the bacterial smear and incubated at 37 °C for 1 h. The determination of fluorescence was made using an ultraviolet light (360 nm) in a dark room after adding 20 µl of NaOH (0.1 mol/l) to the inoculated substrate to enhance the fluorescence intensity of 4-

methylumbelliferone. For β-glucuronidase and β-galactosidase *T. pyogenes* DSM 20630^T was used as positive control, *A. phocae* DSM 10002^T and *T. bonasi* DSM 17163^T, respectively as negative control. For α-glucosidase and N-acetyl-β-glucosaminidase *T. pyogenes* DSM 20594 was used as positive control, *T. bonasi* DSM 17163^T and *A. phocae* DSM 10002^T, respectively as negative control.

3.4.4 Detection of the catalase activity

The catalase test is essential for differentiating catalase-positive from catalase-negative bacteria. Meanwhile it is a primarily useful tool for differentiation between genera and a valuable tool in speciation of certain Gram-positives (Cullimore, 2010). The catalase enzyme decomposes of hydrogen peroxide (H_2O_2) into water and oxygen ($2H_2O_2$ + Catalase $\rightarrow$ $2H_2O + O_2$) associated with formation of bubbles. There are many applications and method variations of the catalase test. In this study, it is carried out using a slide (drop) method by suspending some bacterial colonies in one to two drops of 3% H_2O_2 on a microscopic slide. The positive reaction was seen as bubble formation while negative reaction appeared in a uniform turbidity of the solution without bubble formation (Prescott et al., 2002; Leboffe and Pierce, 2012). *A. pluranimalium* DSM 13483^T was used as positive control, while *T. bernardiae* DSM 9152^T served as negative control.

3.4.5 Growth on Loeffler medium

Loeffler medium was used for the determination of proteolytic activities of microorganisms (Hartwigk and Marcus, 1962). The Loeffler medium was prepared in small glass Petri dishes by adding of bovine serum (PAA Laboratories GmbH) to standard I nutrient broth (containing additional 6 g glucose per liter) in the ration 7:3.

The basic ingredients of the medium were as follows:

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.

The peptone content of the bovine serum provides the medium with amino acids and other complex nitrogenous substances, which is necessary for bacterial growth. The bovine serum is

coagulated during the sterilization process and supplies the bacteria with protein source during metabolism allowing the determination of proteolytic activities. The Loeffler medium was inoculated with a swab of the microorganism directly over the surface followed by incubation for 48 h at 37°C under microaerobic conditions. A positive reaction is indicated by the formation of a groove in the solidified serum beneath the inoculation line (Hartwigk and Marcus, 1962). *T. pyogenes* DSM 20630^T was used as positive control, while *T. bernardiae* DSM 9152^T served as negative control.

3.4.6 Casein hydrolysis

Caseinase is an exoenzyme that is produced by some bacteria in order to degrade casein. Casein is a large protein that is responsible for the white color of milk. If an organism can produce casein, there will be a zone of clearing around the bacterial growth on the milk 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.

A loop of freshly cultivated bacterial colonies was inoculated on the plate in straight line, followed by an incubation of the inoculated casein hydrolysis test agar for 48 h at 37°C under microaerobic conditions (3.2). After incubation, 10% trichloroacetic acid (Sigma Aldrich) was poured on the medium in order to precipitate the casein forming a turbid area. A positive result is indicated by the formation of a clear area around the growth of the culture resulting from the breakdown of casein by the organism due to the production of caseinase. *T. pyogenes* DSM 20630^T was used as positive control, while *A. phocae* DSM 10002^T served as negative control.

3.4.7 Detection of the DNase activity

In 1957, Weckman and Catlin showed a correlation between increased DNase activity of *S. aureus* and a positive coagulase activity. The DNase is an exoenzyme that catalyzes the hydrolysis of DNA into small fragments (oligonucleotides) or single nucleotides. Jeffries et al. (1957) reported about a rapid agar method for showing DNase activity of microorganisms

using a semi-synthetic medium containing nucleic acid. The detection of the 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.

A loop of freshly cultivated bacterial colonies was inoculated on the plate in straight line. The incubation of the inoculated DNase test agar plates were incubated for 48 h at 37°C C under microaerobic conditions (3.2), followed by flooding the surface of the medium with 1 N HCl. A positive result was indicated by a clear zone of the medium surrounding the inoculated microorganism resulting from the degradation of the DNA. The size of this clear zone has a reversibly proportional relation with the amount of DNase produced. A negative result was indicated by the formation of a cloudy precipitate due to the absence of DNase activity. The acid reacts with the intact nucleic acid. *T. pyogenes* DSM 20630^{T} was used as positive control, while *T. bonasi* DSM 17163^{T} served as negative control.

3.4.8 Detection of amylase activity

Amylase is an enzyme which breaks down the insoluble starch into soluble end products such as glucose or maltose, which are absorbed into bacteria. Amylase plays a role in extracellular digestion of bacteria and fungi. The freshly cultivated bacterial colonies were cultivated on Mueller-Hinton-agar containing starch.

The Mueller-Hinton-agar composition was 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.

A fresh culture of the bacteria to be tested was used as an inoculation source. The inoculated starch agar plates were incubated for 48 h at 37 °C under microaerobic conditions. After incubation the plate was flooded with Gram's iodine. A positive reaction was indicated by a

clear area around the growth of the culture due to the hydrolysis of starch by amylase. A negative reaction was indicated by forming a dark blue colored complex. *T. pyogenes* DSM 20630^T was used as positive control, while *T. bonasi* DSM 17163^T served as negative control.

3.4.9 Detection of gelatinase activity

Gelatinases are proteolytic enzymes secreted extracellularly by some bacteria which hydrolyze or liquefy gelatin. Gelatinase could be determined by using a nutrient gelatin medium which contains a peptic digest of animal tissue (peptone), beef extract and gelatin.
Gelatinase enzyme activity was determined with two methods:

A- Nutrient gelatin stab method was composed as follows:

Peptone	5.0 g/liter
Beef extract	3.0 g/liter
Gelatin	120.0 g/liter

Final pH: 6.8 ± 0.2 at 25°C.

According to Zimbro et al. (2009), 48 -hour-old test bacteria were inoculated into tubes containing nutrient gelatin. Both the inoculated tube and an uninoculated control tube were subsequently incubated for 48 h at 37°C under microaerobic conditions. After the incubation period, the tubes were immersed in an ice bath for 15 to 30 minutes. Hydrolyzed gelatin will appear as a liquid medium even after exposure to cold temperature, while the uninoculated control medium will remain solid (Zimbro et al., 2009).

B- Nutrient gelatin plate method was composed as follows:

Yeast extract	2.0 g/liter
Peptone	5.0 g/liter
Sodium chloride	5.0 g/liter
Gelatin	30.0 g/liter
Agar	15.0g/liter

Final pH: 7.4 ± 0.2.

A second test to detect gelatin hydrolysis was the nutrient gelatin plate method. Using this method, a heavy inoculum of a 48-hour-old test bacterium was inoculated on nutrient gelatin plates. The inoculated plates were incubated for 48 h at 37°C under microaerobic conditions. A positive result was indicated by turbid zones around gelatinase-positive colonies, while

negative results were indicated by clear zones (Smith and Goodner, 1958). *T. pyogenes* DSM 20630^T was used as positive control, while *T. bonasi* DSM 17163^T served as negative control.

3.4.10 Cross reaction with streptococcal serogroup G specific antiserum

A serological identification of *T. pyogenes* was mentioned by Lämmler and Blobel (1988). The authors reported that they could identify *T. pyogenes* by using soft agar media containing streptococcal serogroup G specific antisera. The test to determine cross reactions was performed with Streptococcal Grouping Kit (Oxoid, Wesel, Germany). For this 2 to 5 colonies from freshly cultivated bacteria were emulsified in 100 µl of the extraction enzyme provided by the kit (Oxoid) in an Eppendorf tube and incubated for 5 min at 37 ºC. The tube was shaken vigorously and incubated for another 5 min. The agglutination step was performed by gently mixing 20 µl of the enzymatic extract with 20 µl of the latex reagent coated with group G streptococcal antisera to room temperature on a microscopic slide. A positive result was indicated when agglutination occurred after 30 to 60 s. *T. pyogenes* DSM 20594 was used as positive control while *T. bonasi* DSM 17163^T 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

To analyse the molecular pattern of the bacteria, a protein extraction was done using the Bruker ethanol formic acid preparation procedure (Bruker Daltonik, Bremen, Germany). Few colonies of freshly cultivated bacteria were transferred using a disposable loop to a 2.0 ml Eppendorf tube and mixed with 300 µl of HPLC grade water (Roth) to suspend the bacterial cells. HPLC grade absolute ethanol (900 µl, Roth) was subsequently added to the solution and the bacterial isolates were stored at -18^o C until processing. The bacterial isolates were centrifuged for 2 min (Biofuge A, Heraeus, Rabenau, Germany), the supernatant was removed followed by another centrifugation step. The residual fluid was removed by pipetting and the pellet was resuspended in 30 µl 70% formic acid. The same volume of pure acetonitrile (30 µl) was added and mixed. That was followed by centrifugation at 13,000 rpm for 2 min. The supernatant was transferred to a polished steel MALDI target plate (Bruker Daltonik) on 2 spots to increase the data reliability of each bacterial sample. This was allowed to dry at room

temperature. Finally, 1 µl of matrix (10 mg/ml α-cyano-4-hydroxy-cinnamic acid in 50% acetonitrile/2.5% trifluoroacetic acid) was deposited onto the dried sample and also allowed to dry at room temperature.

3.5.2 MALDI-TOF MS measurements

All the samples were analyzed using microflex LT MALDI-TOF mass spectrometer (Bruker Daltonik). Before the sample measurements and data analysis, the instrument should be optimized in order to identify the best combination of parameters that would give visible and intense spectra signals with less noise. The used 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. Peptide profile spectra were determined within the mass range of 2 to 20 kDa. The bacterial test standard (BTS, Daltonik), containing a typical extract of *E. coli DH5alpha*, which is spiked with two additional proteins was used for instrument calibration. The overall mass range covered by the bacterial test standard (BTS, Daltonik) was 3.6 to 17 kDa. The MALDI Biotyper version 3.1 software package from Bruker Daltonik that was developed for bacterial identification processed the database reference spectra which are main spectra (MSP; generated here using at least six raw spectra) including information on the average mass and the average intensity of the selected peaks as well as the frequency of the peaks in multiple measurements for each sample. The flexControl 3.0 software (Bruker Daltonik) contributes in acquiring the MALDI-TOF MS spectra. The raw spectra quality is checked using the software flexAnalysis 3.0 (Bruker Daltonik) visually. Finally, the reference spectra were created with the MALDI Biotyper 3.1 software package that includes the manufacturer's reference database and to the new acquired MSPs from 12 reference strains representing ten species of genera *Arcanobacterium*, *Trueperella* and *Actinomyces* namely, *T. pyogenes* DSM 20630^T, *T. pyogenes* DSM 20594, *T. abortisuis* DSM 19515^T, *T. bernardiae* DSM 9152^T, *T. bialowiezensis* DSM 17162^T, *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 (Hijazin, 2012a).

This system calculates the log score value, or similarity score, by considering the matching proportion of the test spectra with the database reference spectra. It also considers the consistency of peak intensities among sample and reference spectra. The masses obtained from MALDI spectra are compared to a reference library for identification in what is called

the fingerprint approach. The MALDI Biotyper software checked the suitability of the created main spectra for Biotyper-based species identification by cross-matching them to the entire database. The logarithmized score values range from 0 to 3 were 0 is no homology and 3 is absolute identity. Identification was carried out using the Biotyper 3.1 software tool, following the manufacturer's recommendation on identification based on the calculated log score values. Values of ≥ 2.0 to 3.0 represent probable and highly probable (≥ 2.3) species level matching, while scores of ≥ 1.7 to 1.999 represent probable genus level matching. The data analyses were kindly performed by Dr. Markus Timke and Dr. Markus Kostrzewa (Bruker Daltonik).

3.6 Identification of the bacteria by Fourier Transform Infrared Spectroscopy (FT-IR spectroscopy)

For FT-IR spectroscopy all isolates were cultivated on sheep blood agar for 48 h (+/- 0.5 h) at 37°C in 6-10 replicates under microaerobic conditions (GasPak^{TM} EZ Campy Container System; Becton, Dickinson and Company, Heidelberg, Germany). Harvesting bacterial biomass and preparation of bacterial films on zinc selenide (ZnSe) plates were performed as described previously (Kuhm et al., 2009), using an aliquot of 25 µl in a sample zone of a 96 well format ZnSe-plate. Every isolate for the database was measured at least six times using a TENSOR 27 FT-IR spectrometer supplemented with a HTS-XT module (Bruker Optik GmbH, Ettlingen, Germany) in transmission mode from 500 to 4000 cm^{-1} with the coupled software (OPUS 6.5). The data set for the isolates used for the construction of the differentiation method was divided into two equal parts, as described by Kuhm et al. (2009). The first one, called creation set, was used to create the method. The second one was used to verify the created method to gain the recovery rates (internal recovery set). An internal validation was performed with this internal recovery set. The 57 well described *T. pyogenes* isolates from bovine mastitis were used for external validation. Results were given as probability for repeated determination, based on the results of the respective internal and external recovery sets. In order to expand the application of FT-IR spectroscopy for mastitis diagnostics on *T. pyogenes*, a hierarchically structured method based on reference isolates from genus *Trueperella* comprising all type-strains, was created. Additionally, a taxonomically close reference isolates were cultivated on the same conditions inorder to be used for comparative purposes. This taxonomically close relatives reference isolates included

Arcanobacterium haemolyticum DSM 20595^T, *Arcanobacterium canis* DSM 25104^T, *Arcanobacterium hippocoleae* DSM 15539^T, *Arcanobacterium phocae* DSM 10002^T, *Arcanobacterium phocae* DSM 10003, *Arcanobacterium phocisimile* DSM 26142, *Arcanobacterium pluranimalium* DSM 18483^T, *Actinomyces bovis* DSM 43014^T, *Actinomyces hyovaginalis* CVUAS 4295, *Actinomyces weissii* DSM 24894^T and *Actinomyces canis* DSM 15536^T. In this first step, the isolates were divided into three classes, representing the genus, which were used as a preliminary filter. *Trueperella* isolates were differentiated in a second step down to the species level, distinguishing all recently described members of this genus. For *T. pyogenes* the creation of the FT-IR method succeeded by using three selected isolates (*T. pyogenes* DSM 20594, *T. pyogenes* DSM 20630^T and *T. pyogenes* CVUAS 0222). Therewith an adequate segregation of this species from the other species of genus *Trueperella* and the used taxonomically close members of *Arcanobacterium* and *Actinomyces* was achieved. The infrared spectra of the creation set were used in development of the differentiation methods with NeuroDeveloper software (Synthon GmbH, Heidelberg, Germany), which is based on an artificial neural network strategy (ANN) (Udelhoven et al., 2003). The second derivatives of the vector-normalized, five-point smoothed spectra of the creation set in the wave number ranges from 2800-3000 cm^{-1} and 500 to 1800 cm^{-1} were used for data analysis in covar mode with a significance of 95%. Four-fifth randomly assorted spectra of the creation set were used as the "training set" of the developer module. With the remaining one-fifth of the spectra, put in the "validation set", the internal method optimization of wavelength combinations was done (Udelhoven et al., 2000). In this way a hierarchical classification scheme is build, consisting of a top level dividing the three genera (*Actinomyces, Arcanobacterium* and *Trueperella*) and a subsequent classification level, differentiating the five *Trueperella*-species. Single infrared spectra of all *Trueperella* isolates were compared by cluster analysis as described (Contzen et al., 2011). For this collation, the second derivatives of vector normalized spectra in the wave number ranges of 500-1200 cm^{-1} and 2800-3000 cm^{-1} were used for calculation with Ward's algorithm (OPUS 4.2) (Ward, 1963). The dendrogram obtained depicts the arrangement of isolates according to their spectral differences. The FT-IR spectroscopy procedure and data analyses were kindly performed by Dr. Jörg Rau (Chemisches und Veterinäruntersuchungsamt Stuttgart, Fellbach, Germany).

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

3.7.1 DNA extraction

The genomic DNA from the investigated bacteria was isolated using DNeasy tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's specifications. A single colony of each investigated isolate was cultivated and incubated for 48 h on sheep blood agar under microaerobic conditions (3.2). A few freshly subcultured bacterial colonies were 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 Aldrich), followed by an incubation for one hour at 37 °C. Afterwards, 25 µl proteinase K (Qiagen) and 200 µl of lysis buffer AL (Qiagen) were added and incubated for 2 h at 56 °C. Subsequently 200 µl ethanol (Roth) was added to the incubated mixture and mixed thoroughly. The reaction mixture was then transferred to DNeasy silica membrane filter (Qiagen) and centrifuged for 1 min at 6,000 rpm. The eluent was discharged, followed by the addition of 500 µl buffer AW1 (Qiagen) and centrifuged for 1 min at 6,000 rpm. The eluent was discharged again. This step was repeated once again using 500 µl buffer AW2 (Qiagen). After the second washing step, a short centrifugation at 13,000 rpm for 30 sec removed completely the washing solution from the silica filter. The silica filter was subsequently transferred to another clean 2 ml Eppendorf tube, 200 µl elution buffer AE (Qiagen) was added to the filter and incubated for 5 min at room temperature. Then the Eppendorf tube containing the silica filter was centrifuged for 2 min at 6,000 rpm. The resulting DNA eluate was cooled and used as template in the PCR reaction or stored at -20 °C.

3.7.2 Polymerase chain reaction

The amplification of DNA fragments by polymerase chain reaction (PCR) was performed by use of a master mix containing heat stable DNA polymerase, deoxyribonucleoside triphosphates (dNTP); a set of primers that were complimentary to the targeted DNA and the DNA template. The master mix was as follows:

Aqua bidest.	19.9 µl
10X Taq Buffer with KCl[a]	3.0 µl
MgCl2 (25 mmol/l, Applied Biosystems, Darmstadt, Germany)	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, Thermo Fisher Scientific, Darmstadt, Germany)	0.2 µl

[a](100 mmol/l Tris-HCl, 500 mmol/l KCl, pH 8.8)

Alternatively, another master mix was used. This master mix was as follows:

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, Thermo Fisher Scientific)	0.2 µl

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

The reaction mix was run in a volume of 30 µl containing 27.5 µl of the master mix which was dispensed in 0.2 ml sterile PCR reaction tubes and mixed with 2.5 µl of the prepared DNA (3.6.1). Afterward, a standard PCR was performed using the samples at 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).

3.7.3 Agarose gel electrophoresis

The expected size of the PCR products was confirmed by electrophoresis in a 1.5% agarose gel which was used to separate DNA samples. The gel composition was 1.5% w/v ultra-pure agarose in TBE buffer (4.0 mmol/l Tris, 1 mmol/l EDTA, 1.14 mol/l glacial acetic acid, pH 8.0). The PCR product (8 µl) 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 - 1,000 bp or 100 - 1,0000 bp-ladders, Fermentas) as molecular size standard. The electrophoresis was carried out at 100 mA for approximately 1 h.

3.7.4 Staining and documentation

After electrophoresis, the gel was stained for approximately 30 min with an ethidium bromide solution (5µg/ml, Sigma Aldrich) or GelRed™ Nucleic Acid Gel Stain 10,000X stock reagent (Biotium, Hayward, USA). 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. GelRed™ is a sensitive, stable and environmentally safe fluorescent nucleic acid dye designed to replace the highly toxic ethidium bromide (EB) for staining dsDNA, ssDNA or RNA in agarose gels or polyacrylamide gels. GelRed™ is far more sensitive than EB without requiring a distaining step. 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.

3.7.5 PCR amplicon purification

The purification procedure is based on the use of a silica membrane that binds the DNA in high-salt buffer followed by elution of the DNA with a low-salt buffer or water. The procedure removes primers, nucleotides, enzymes, mineral oil, salts, agarose and other impurities from DNA samples. The purification of amplicons was performed using the HiYield Gel/PCR DNA Extraction Kit (SLG, Gauting, Germany) according to the manufacturer's instructions. Up to 100 µl of the PCR amplicon was transferred to a 1.5 microcentrifuge tube. 5 volumes of DF Buffer was added to 1 volume of the sample and mixed by vortex. The DF Column (containing the silica membrane that binds the DNA) was placed in a 2 ml collection tube and the sample mixture was added into the DF Column, followed by centrifugation at 14,000 -16,000 rpm for 30 seconds. The filtrate was discarded, the DF Column was placed back in the 2 ml collection tube. Then 600 µl of washing buffer (100 ml absolute ethanol was added to wash buffer prior to initial use) was added into the center of the DF Column, incubated for 1 min at room temperature and centrifuged at 14,000 - 16,000 rpm for 30 seconds. The eluent was discarded and the DF column was placed back in the 2 ml collection tube and centrifuged for 3 minutes at 14,000-16,000 rpm to dry the column matrix. The dried DF column was transferred to a new 1.5 ml microcentrifuge tube and 20-50 µl of elution buffer or TE was added into the center of the column matrix and incubated for at least 2 minutes to ensure that the elution buffer was completely absorbed. This was followed by centrifuging for 2 minutes at 14,000-16,000 rpm to elute the purified DNA. The DNA-

containing supernatant was then collected by pipetting and could be used as template for PCR reaction or was stored at -20 °C.

3.7.6 DNA preparation from agarose gels and sequencing

The kit to extrude DNA rapidly from the agarose gel matrix was HiYield Gel/PCR DNA Extraction Kit (3.7.5). Approximately 30 microliters of the PCR Product was electrophoresed through a 1.5% agarose gel and stained using ethidium bromide or GelRed™ Nucleic Acid Gel Stain. After trimming away excess agarose, the gel slice (≤ 300 mg) containing relevant DNA fragments was transferred into an Eppendorf tube. DF Buffer 500 µl was added to the sample, mixed by vortex and incubated at 55-60ºC for 10-15 minutes (every 2-3 minutes the tube is inverted) to ensure that the gel slice has been completely dissolved. The dissolved sample mixture was cooled to room temperature. The DF Column was subsequently placed in a 2 ml collection tube. Subsequently 800 µl of the sample mixture was transferred to the DF column, followed by centrifuging at 14,000-16,000 rpm for 30 seconds. The eluent was discarded and the DF column was placed back to the 2 ml collection tube. When the sample mixture was more than 800 µl the first step was repeated. The washing buffer containing ethanol (600 µl) was added to the DF column and incubated for 1 minute. Then, the DF column was centrifuged at 14,000 -16,000 rpm for 30 seconds and the filtrate discarded. This step was repeated once again to ensure that the residual ethanol was completely removed. The DF column was placed back in the 2 ml collection tube and centrifuged at 14.000 -16.000 rpm for 3 minutes to dry the column matrix. The dried DF column was transferred to a new 1.5 ml microcentrifuge tube and 20-50 µl of elution buffer or TE was added into the center of the column matrix. The purified DNA was recovered by the addition of 20-50 µl of elution buffer or TE added into the center of the column matrix, mixed, incubated for at least 2 minutes and followed by centrifugation at 14,000-16,000 rpm for 2 min. The DNA-containing supernatant was then collected by pipetting and used for sequencing purposes or stored at -20 °C.

The gel-purified PCR products (3.7.5 and 3.7.6) were sequenced by SEQLAB Sequence Laboratories (Göttingen, Germany). Sequence analysis and comparison was performed using the National Center for Biotechnology Information data base (NCBI: http://www.ncbi.nlm.nih.gov/). Sequencing data were checked and edited using computer programs Finch TV version 1.4.0, DNASTAR Lasergene version 7.0.0. The nucleotide sequences, amino acid sequences and evolutionary relationship analyses were conducted with

MEGA version MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0 at the Institut für Pharmakologie und Toxikologie, Justus-Liebig-Universität Gießen.

3.7.7 Amplification and sequencing of the 16S rRNA gene

The 16S rRNA gene was amplified using universal primers 16S rDNA UNI-L with the sequence 5`-AGA GTT TGA TCA TGG CTC AG-3' and 16S rDNA UNI-R with the sequence 5`-GTG TGA CGG GCG GTG TGT AC-3' as described by Hassan et al. (2009). The PCR product of the 16S rRNA gene was amplified with an expected size of 1,403 bp. The PCR reaction was performed by preparation of the master mix mentioned in 3.7.2. 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

The PCR product of the 16S rRNA gene was sequenced using the oligonucleotide primer consisted of 16S rDNA-533F with the sequence 5'-GTG CCA GCM GCC GCG GTA A-3' and 16S rDNA-907R with the sequence 5'-CCG TCA ATT CMT TTG AGT TT-3' as described by Weisburg et al. (1991), Henckel et al. (1999) and Muyzer et al. (1995).

3.7.8 Amplification and sequencing of gene *gap*

Gene *gap* was amplified using the oligonucleotide primers Gap-F with the sequence 5'-TCG AAG TTG TTG CAG TTA ACG A-3' and Gap-R with the sequence 5'-CCA TTC GTT GTC GTA CCA AG-3' as described by Sammra et al. (2014a). The PCR product of gene *gap* was amplified with an expected size of 830 bp. The PCR reaction was performed by preparation of the master mix mentioned in 3.7.2. The PCR thermal cycler program was carried out as follows:

1 cycle	94 °C	3	min	Initial denaturation
30 cycle	94 °C	30	sec	Denaturation
	50 °C	40	sec	Primer annealing
	72 °C	60	sec	Extension

1 cycle	72 °C	5	min	Final extension

3.7.9 PCR-mediated identification by amplification of species specific parts of the bacterial genome

3.7.9.1 Amplification of *T. pyogenes* ISR

PCR amplification of the ISR of *T. pyogenes* specific regions was carried out using the oligonucleotide primers Apy-ISR-F and Apy-ISR-r as described by Ülbegi (2010). The sequences were originally obtained from the ISR sequence of *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 with the sequence 5'-AAG CAG GCC CAC GCG CAG G-3'. The PCR reaction mixture was described in 3.7.2. The following PCR conditions were used:

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

3.7.9.2 Amplification of *T. pyogenes* superoxide dismutase A encoding gene *sodA*

PCR amplification of the specific regions of gene *sodA* of *T. pyogenes* was carried out using the oligonucleotide primer Apy-sodA-F with the sequence 5´-CGA GCT CGC CGA CGC TAT TGC T-3' and Apy-sodA-R with the sequence 5´ GAG CAT GAG AAT CGG GTA AGT GCC A-3' as described by Hijazin et al. (2011) and Hijazin (2012a). The PCR reaction mixture was described in 3.7.2. The following PCR conditions were used:

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

3.7.10 PCR-mediated amplification of *T. pyogenes* putative virulence factor encoding genes

3.7.10.1 Amplification of *T. pyogenes* pyolysin encoding gene *plo*

PCR amplification of gene *plo* of *T. pyogenes* encoding pyolysin was carried out using the oligonucleotide primer plo-F and plo-R as described by Ülbegi (2010). The oligonucleotide primer plo-F had the sequence 5'-CGA TCC CTC TGG TGT ACT TGC-3' and plo-R had the sequence 5'-GCT TGA CAA AAA TCT GGC GTC C-3'. The PCR reaction mixture was described in 3.7.2. The following PCR conditions were used:

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

3.7.10.2 Amplification of *T. pyogenes* collagen-binding protein encoding gene *cbpA*

PCR amplification of gene *cbpA* of *T. pyogenes* was carried out using the oligonucleotide primer cbp-F with the sequences 5'-CTT GAA ATC GAA CTT AAG GCT GG-3' and cbp-R with the sequence 5'-ATC GCC AGT CAC CTT AGA CG-3' as described by Ülbegi (2010). The PCR reaction mixture was described in 3.7.2. The following PCR conditions were used:

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

3.7.10.3 Amplification *T. pyogenes* neuraminidase encoding genes *nanH* and *nanP*

PCR amplification of genes *nanH* and *nanP* of *T. pyogenes* were carried out using the oligonucleotide primer sequences described by Silva et al. (2008). For *nanH* the oligonucleotide primer nanH-F had the sequence 5'-CGC TAG TGC TGT AGC GTT GTT

AAG T-3' and nanH-R the sequence 5'-CCG AGG AGT TTT GAC TGA CTT TGT-3'. For *nanP* the oligonucleotide primer nanP-F had the sequence 5'-TTG AGC GTA CGC AGC TCT TC-3'and nanP-R the sequence 5'-CCA CGA AAT CGG CCT TAT TG-3'. The PCR reaction mixture was described in 3.7.2. The following PCR conditions were used:

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

3.7.10.4 Amplification of *T. pyogenes* fimbriae encoding genes *fimA, fimC* and *fimE*

PCR amplification of genes *fimA*, *fimC* and *fimE* of *T. pyogenes* encoding fimbriae were carried out using the oligonucleotide primer sequences as described by Silva et al. (2008). The fimbriae encoding gene *fimA* was amplified using oligonucleotide primer fimA-F with the sequence 5'-CAC TAC GCT CAC CAT TCA CAA G-3' and fimA-R with the sequence 5'-GCT GTA ATC CGC TTT GTC TGT G-3'. The fimbriae encoding gene *fimC* was amplified with the oligonucleotide primer fimC-F with the sequence 5'-TGT CGA AGG TGA CGT TCT TCG-3' and fimC-R with the sequence 5'-CAA GGT CAC CGA GAC TGC TGG-3'. The fimbriae encoding gene *fimE* was amplified with the oligonucleotide primer fimE-F with the sequence 5'-GCC CAG GAC CGA GAG CGA GGG C-3' and fimE-R with the sequence 5´-GCC TTC CAC AAA TAA CAG CAA CC -3'. The PCR reaction mixture was described in 3.7.2. The following PCR conditions were used:

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 (*fimA* =57 °C, *fimC* = 60 °C and *fimE* = 55 °C)

3.7.10.5 Amplification of *T. pyogenes* tetracycline resistance encoding gene *tet(W)*

PCR amplification of the *T. pyogenes* tetracycline resistance gene *tet(W)* was carried out using the oligonucleotide primer sequence described by Billington and Jost (2006). The

tetracycline resistance gene *tet(W)* was amplified with the oligonucleotide primer tet(W)-F with the sequence 5´-GAC AAC GAG AAC GGA CAC TAT G-3´ and tet(W)-R the with sequence 5´-CGC AAT AGC CAG CAA TGA ACG C-3´. The PCR reaction mixture was described in 3.7.2. The following PCR conditions were used:

1 cycle	94 °C	10	min	Initial denaturation
35 cycle	94 °C	60	sec	Denaturation
	55°C	60	sec	Primer annealing
	72 °C	120	sec	Extension
1 cycle	72 °C	7	min	Final extension

3.8 Identification and classification of bacteria using DNA fingerprinting methods

3.8.1 Genomic fingerprinting using (rep)-PCRs and RAPD-PCR

Genomic fingerprinting of the *T. pyogenes* isolates was performed using four genomic DNA fingerprint methods. This included three repetitive element primed (rep)-PCRs (ERIC-PCR, BOX-PCR and $(GTG)_5$-PCR) and random amplification polymorphic DNA (RAPD-PCR) analysis. The dendrogram and the cluster analysis of genomic fingerprint pattern was performed in GelCompar II version 4.5 (Applied Maths) using the unweighted pair-group method using arithmetic average (UPGMA) clustering method based on the Pearson correlation (0.5% optimization; 1% position tolerance) and based on the matrix of F-values, which considers the presence/ absence and the intensity of DNA bands. A consensus matrix was calculated and a composite clustering was performed. The genomic fingerprinting methods were kindly performed by Dr. Stefanie P. Glaeser (Institut für Angewandte Mikrobiologie, Fachbereich Agrarwissenschaften, Ökotrophologie und Umweltmanagement, Justus-Liebig-Universität Gießen, Heinrich-Buff-Ring 26, 35392 Gießen, Germany).

3.8.1.1 Genomic fingerprinting using ERIC-PCR

PCR amplification of *T. pyogenes* Enterobacterial Repetitive Intergenic Consensus was carried out using the oligonucleotide primer sequence described by Versalovic et al. (1991). The oligonucleotide primers used were ERIC1RF with the sequence 5'-ATG TAA GCT CCT GGG GAT TCA C-3' and ERIC2 with the sequence 5'-AAG TAA GTG ACT GGG GTG

AGC-3'. The PCR reaction mixture was described in 3.7.2. The following PCR conditions were used:

1 cycle	95 °C	3	min	Initial denaturation
30 cycle	94 °C	30	sec	Denaturation
	53 °C	60	sec	Primer annealing
	70 °C	8	min	Extension
1 cycle	72 °C	16	min	Final extension

3.8.1.2 Genomic fingerprinting using BOX-PCR

PCR amplification of *T. pyogenes* BOX elements was carried out using the oligonucleotide primer sequence described by Versalovic et al. (1994). One oligonucleotide primer was tested in order to assess its usefulness in generating polymorphism, enhance the discriminatory power in subtyping, and test its abilities to differentiate between the isolates. The oligonucleotide primer used was BOXA1R with the sequence 5'-CTA CGG CAA GGC GAC GCT GAC G-3'. The PCR reaction mixture was described in 3.7.2. The following PCR conditions were used:

1 cycle	95 °C	3	min	Initial denaturation
30 cycle	94 °C	30	sec	Denaturation
	53 °C	60	sec	Primer annealing
	70 °C	8	min	Extension
1 cycle	72 °C	16	min	Final extension

3.8.1.3 Genomic fingerprinting using $(GTG)_5$-PCR

PCR amplification of *T. pyogenes* repetitive bacterial DNA elements $(GTG)_5$ was carried out using the oligonucleotide primer sequence as described by Versalovic et al. (1994). One oligonucleotide primer was tested in order to assess its usefulness in generating polymorphism, enhance the discriminatory power in subtyping, and test its abilities to differentiate between the isolates. The oligonucleotide primer used was $(GTG)_5$ with the sequence 5'-GTG GTG GTG GTG GTG-3'. The PCR reaction mixture was described in 3.7.2. The following PCR conditions were used:

1 cycle	95 °C	3	min	Initial denaturation
30 cycle	94 °C	30	sec	Denaturation

	53 °C	60	sec	Primer annealing
	70 °C	3	min	Extension
1 cycle	72 °C	16	min	Final extension

3.8.2 Genomic fingerprinting using RAPD-PCR

PCR amplification of *T. pyogenes* for Random Amplification Polymorphic DNA fingerprinting was carried out using the oligonucleotide primer sequence described by Ziemke et al. (1997). One oligonucleotide primer was tested in order to assess its usefulness in generating polymorphism, enhance the discriminatory power in subtyping, and test its abilities to differentiate between the isolates. The oligonucleotide primer used was RAPD primer B with the sequence 5'- ATC TGG CAG C -3'. The PCR reaction mixture was described in 3.7.2. The following PCR conditions were used:

1 cycle	95 °C	3	min	Initial denaturation
45 cycle	94 °C	15	sec	Denaturation
	34 °C	60	sec	Primer annealing
	70 °C	2	min	Extension
1 cycle	72 °C	10	min	Final extension

3.8.3 Genomic fingerprinting using MLSA

MLSA was performed by partial sequencing of the translation elongation factor G encoding gene *fusA*, translation elongation factor Tu encoding gene *tuf*, methionyl-tRNA synthetase encoding gene *metG* and DNA gyrase, subunit A encoding gene *gyrA*. The sequences of the oligonucleotide primers for amplification of the above mentioned four housekeeping genes were designed with the sequence data of *Actinomyces naeslundii* MG1 genome project (J. Craig Venter Institute - JCVI) and *A. haemolyticum* DSM 20595^T genome project (Yasawong et al., 2010). The target genes, the sequences of the oligonucleotide primers and the thermocycler programs are summarized in Tab. 5. MLSA included a partial sequencing of the housekeeping genes. The analyses were performed at the nucleotide and amino acid sequence level with *T. pyogenes* DSM 20630^T, *T. pyogenes* DSM 20594 and *A. haemolyticum* DSM 20595^T as controls. MLSA analysis was performed using MEGA6 (Tamura et al., 2013). Full-length gene sequences from genome sequenced *A. haemolyticum* DSM 20595^T genome project were used as reference sequences to obtain the correct open reading frame

(ORF) for translation into amino acid sequences. Alignments of nucleotide and amino acid sequences were performed with MUSCLE implemented in MEGA6 (Edgar, 2004). The phylogenetic trees in single gene base analysis of the four target genes, respectively were constructed with the Maximum-likelihood method (Stamatakis, 2006) using the Kimura-2-parameter model for nucleotide sequence (Kimura, 1980) or the JTT matrix-based method for amino acid sequences (Jones et al., 1992). Partial sequences of the four genes were concatenated with nucleotide sequences (in the following order *fusA, tuf, metG* and *gyrA*) and with amino acid sequences (in the following order FusA, Tuf, MetG and GyrA). The phylogenetic trees of the concatenated sequences were constructed using the maximum-likelihood method based on evolutionary distances calculated with the general time reversible model for nucleotide sequences (Nei and Kumar, 2000) and again with the JTT matrix-based method for amino acid sequences. A discrete gamma distribution was used to model evolutionary rate differences among sites (5 categories; +G) and a rate variation model allowed for some sites to be evolutionarily invariable (+I). Nucleotide and amino acid sequence similarities of single and concatenated genes were determined based on p-distances calculated in MEGA6.

Tab. 5: Nucleotide sequences of PCR and sequencing primers designed for DNA amplification and sequencing of four housekeeping gene fragments used for MLSA of *T. pyogenes*.

Oligonucleotide primers	Sequence	Expected size of PCR product (bp)
1. fusA-F 2. fusA-R	5'-GCT TCA TCA ACA AGA TGG AC-3' 5'-CTC GAT TG CGA CGT GG AT-3'	828
3. tuf-F 4. tuf-R	5'-GGA CGG TGA TTG GAG AAG AAT GG-3' 5'-CCA GGT TGA TTA CGC TCC AGA AGA-3'	796
5. metG-F 6. metG-R	5'-GCC GGT TTT GGT GTT CC-3' 5'-GGC CAA ATC TGG GAA TGG-3'	837
7. gyrA-F 8. gyrA-R	5'-CCA CCA GAT CGA GGT CAT C-3' 5'-TCG TCG GCA GTG AAA CGC A-3'	937

3.8.3.1 Amplification of *T. pyogenes* translation elongation factor G encoding gene *fusA*

PCR amplification of *T. pyogenes* translation elongation factor G encoding gene *fusA* was carried out using the oligonucleotide primer fusA-F and fusA-R. The PCR reaction mixture was described in 3.7.2. The following PCR conditions were used:

1 cycle	94 °C	3	min	Initial denaturation
30 cycle	94 °C	45	sec	Denaturation
	57 °C	30	sec	Primer annealing
	72 °C	90	sec	Extension
1 cycle	72 °C	7	min	Final extension

3.8.3.2 Amplification of *T. pyogenes* translation elongation factor Tu encoding gene *tuf*

PCR amplification of *T. pyogenes* translation elongation factor Tu encoding gene *tuf* was carried out using the oligonucleotide primer tuf-F and tuf-R. The PCR reaction mixture was described in 3.7.2. The following PCR conditions were used:

1 cycle	94 °C	3	min	Initial denaturation
30 cycle	94 °C	45	sec	Denaturation
	57 °C	40	sec	Primer annealing
	72 °C	60	sec	Extension
1 cycle	72 °C	7	min	Final extension

3.8.3.3 Amplification of *T. pyogenes* methionyl-tRNA synthetase encoding gene *metG*

PCR amplification of *T. pyogenes* methionyl-tRNA synthetase encoding genes metG was carried out using the oligonucleotide primer metG-F and metG-R. The PCR reaction mixture was described in 3.7.2. The following PCR conditions were used:

1 cycle	94 °C	3	min	Initial denaturation
35 cycle	94 °C	45	sec	Denaturation
	52 °C	30	sec	Primer annealing
	72 °C	90	sec	Extension

1 cycle	72 °C	10	min	Final extension

3.8.3.4 Amplification of *T. pyogenes* DNA gyrase (subunit A) encoding gene *gyrA*

PCR amplification of *T. pyogenes* DNA gyrase (subunit A) encoding gene *gyrA* was carried out using the oligonucleotide primer gyrA-F and gyrA-R. The PCR reaction mixture was described in 3.7.2. The following PCR conditions were used:

1 cycle	94 °C	3	min	Initial denaturation
35 cycle	94 °C	45	sec	Denaturation
	52 °C	30	sec	Primer annealing
	72 °C	90	sec	Extension
1 cycle	72 °C	10	min	Final extension

4 Results

4.1.1 Phenotypic and genotypic properties of *T. pyogenes* isolated from bovine mastitis

The identification and characterization was done using cultural methods, various phenotypical tests, MALDI-TOF MS and FT-IR spectroscopy and by PCR amplification of various molecular targets.

4.1.1.1 Phenotypic results

All 57 *T. pyogenes* of the present study and the reference strains *T. pyogenes* DSM 20630^T and *T. pyogenes* DSM 20594 showed a zone of hemolysis after cultivation on sheep blood agar. However, as shown in Tab. 6, no enhanced hemolysis of the *T. pyogenes* isolates could be recorded after cultivation of the bacteria on rabbit blood agar compared to sheep blood agar. The 57 *T. pyogenes* and the two *T. pyogenes* reference strains were positive for CAMP-like hemolytic reactions with β-hemolysin producing strain of *S. aureus* and *R. equi* but not with *S. agalactiae* as indicator strains. None of the isolates showed a reverse CAMP-like hemolytic reaction (Tab. 6)

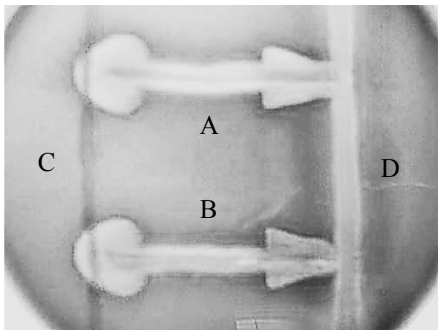

Fig. 6: A typical CAMP-like hemolytic reaction of *T. pyogenes* 467/67 (A) and *T. pyogenes* 1022/30(B) with β-hemolysin producing strain of *S. aureus* (C) and *R. equi* (D) as indicator strains.

Tab. 6: Hemolytic properties and CAMP-like hemolytic reactions of 57 *T. pyogenes* isolates and both *T. pyogenes* reference strains.

Hemolytic property		Number of *T. pyogenes* isolates			Results of *T. pyogenes* reference strains*	
		Tested	Positive	% positive	DSM 20630^T	DSM 20594
Hemolysis on	Sheep blood agar	57	57	100	+	+
	Rabbit blood agar	57	57	100	+	+
CAMP-like hemolysis with	β-hemolysin producing strain of *S. aureus*	57	57	100	+	+
	S. agalactiae	57	0	0	-	-
	R. equi	57	57	100	+	+
Reverse CAMP reaction		57	0	0	-	-

*= results obtained from Hijazin et al. (2011); + = positive; - = negative.

In addition, all 57 *T. pyogenes* isolates yielded a positive reaction for the enzymes β-D-glucuronidase, α-D-glucosidase and N-acetyl-β-D-glucosaminidase (3.4.3). Positive reactions were also recorded for serolysis on Loeffler medium (3.4.5), casein hydrolysis (3.4.6) and for the enzyme DNase (3.4.8). All 57 isolates were negative for the enzymes β-D-glucosidase (3.4.3) and catalase (3.4.4). However, the results were variable for the enzymes α-galactosidase, β-galactosidase, α-mannosidase (3.4.3) and amylase (3.4.9). α-galactosidase enzyme activities could be detected in 14% (n=8), β-galactosidase enzyme activities in 98% (n=56), α-mannosidase enzyme activities in 5% (n=3) and amylase enzyme activities in7% (n=4) of the *T. pyogenes* isolated from the milk samples of dairy cattle. The enzymatic extracts of all 57 *T. pyogenes* and the two *T. pyogenes* reference strains displayed cross reactions with streptococcal serogroup G specific antiserum (3.4.10) (Tab. 7).

Tab. 7: Phenotypical properties of the 57 *T. pyogenes* isolates of the present study and the two *T. pyogenes* reference strains.

Phenotypical properties	Number of *T. pyogenes* isolates			Results of *T. pyogenes* reference strains**	
	Tested	Positive	% positive	DSM 20630^T	DSM 20594
β-Glucuronidase (β-GUR) +[1,2]	57	57	100	+	+
α-Galactosidase (α-GAL)[1]	57	8*	14.0	-	-
β-Galactosidase (β-GAL)[2]	57	1*	1.8	+	+
α-Glucosidase (α-GLU)[1,2]	57	57	100	+	+
β-Glucosidase (β-GLU)[1]	57	0	0	-	-
N-acetyl- β-Glucosaminidase (β-NAG)[2]	57	57	100	+	+
α-Mannosidase[1]	57	3*	5.3	-	-
Catalase	57	0	0	-	-
Serolysis on Loeffler agar	57	57	100	+	+
Caseinase	57	57	100	+	+
Starch hydrolysis (amylase)	57	4	7.0	+	-
Cross reaction with streptococcal serogroup G specific antiserum	57	57	100	+	+

* all these positive isolates exhibited weak reaction. ** = results obtained from Hijazin et al. (2011). + = positive reaction; - = negative reaction. 1 = Tablets containing substrates (3.4.3); 2 = 4-methylumbelliferyl-conjugated substrates (3.4.4).

4.1.1.2 Identification by MALDI-TOF MS

All 57 *T. pyogenes* isolates of the present study and the 12 reference strains representing nine species of genera *Arcanobacterium* and *Trueperella* were subjected to MALDI-TOF MS analysis (3.5). For phylogenetic analysis, a hierarchical clustering of the mass spectra was performed for all tested bacterial isolates that were included in the present study and the reference library entries using the mean spectrum projection (MSP) dendrogram function of MALDI Biotyper, version 3.0. The spectral analysis was carried out using the MALDI Biotyper, version 3.1 species identification software and the BioTyper database from Bruker Daltonics.

All 57 isolates were identified by MALDI-TOF MS to the species level with a score of ≥ 2.0. The dendrogram generated from hierarchical cluster analysis of MALDI-TOF MS consists of

four significantly different main branches of genera *Arcanobacterium* and *Trueperella*. The branch represented by 57 *T. pyogenes* isolates and the reference strains *T. pyogenes* DSM 20630^T and *T. pyogenes* DSM 20594 was more dissimilar to the other *Arcanobacterium* and *Trueperella* species, which were included as an external reference and the cluster was also clearly separated from other reference strains. The clustering of the species within each main branch of the dendrogram showed different degrees of similarity.

This cluster clearly separated the 57 *T. pyogenes* isolates from bovine mastitis included in this study from 12 reference strains of genera *Arcanobacterium* and *Trueperella.* Routine identification based on the BioTyper workflow revealed that all of the examined mass spectra of the *T. pyogenes* isolates were assigned to the correct species with an average score value of 2.69. The minimum log (score) value was assigned between the two isolates *T. pyogenes* 219/27 and *T. pyogenes* 10/46 with a log score value of 2.44. Mainwhile, the maximum log (score) value was assigned between the two isolates *T. pyogenes* 1584 and *T. pyogenes* 1183/105 with a log score value of 2.84. These values suggest a large degree of similarity (spectral quality, number of signals, peak positions) between the spectra recorded in this study and the entries for *T. pyogenes* contained in the BioTyper database. All isolates belonging to the same species was clustered consistently in the same group. A dendrogram analysis of MALDI-TOF main spectra of the 57 *T. pyogenes* isolates from dairy cattle and 12 reference strains of genera *Arcanobacterium* and *Trueperella* is shown in Fig. 7. The 57 *T. pyogenes* isolates investigated in the present study matched against MSPs of the reference strains *T. pyogenes* DSM 20630^T and *T. pyogenes* DSM 20594 and against reference library entries. The 57 *T. pyogenes* isolates and the reference strains (*T. pyogenes* DSM 20630^T and *T. pyogenes* DSM 20594) matched with each other with log (score) values ranging from 2.23 to 2.84. The two reference strains *T. pyogenes* DSM 20630^T and *T. pyogenes* DSM 20594 had a log (score) value of 2.15 against each other. Also, the two reference strains *A. phocae* DSM 10002^T and *A. phocae* DSM 10003 matched with a log (score) value of 2.68 among each other and could be identified to species level. The evaluation of the log (score) values of the 57 *T. pyogenes* isolates among each other yielded the lowest log (score) value of 2.44 to a maximal score of 2.84. The maximum log (score) value was between the two isolates *T. pyogenes* 1584/22 and *T. pyogenes* 1183/105. Both of the two isolates were obtained from different animals and different locations with the same phenotypic properties but different genotypic properties. *T. pyogenes* 1584/22 was positive for *nanH, nanP* and negative for *tet*(*W*). Contrariwise, *T. pyogenes* 1183/105 was negative for *nanH, nanP* and positive for *tet*(*W*). This indicates that

there is no clonal relationship between the two strains. However, *T. pyogenes* 336/1, *T. pyogenes* 336/2 and *T. pyogenes* 336/4 were obtained from one animal. According to the MALDI-TOF MS fingerprint data *T. pyogenes* 336/1 and *T. pyogenes* 336/2 matched with *T. pyogenes* 336/4 with high log (score) values between 2.82 and 2.75.

In addition to that, MALDI-TOF MS successfully identified 12 reference strains representing nine species of genera *Arcanobacterium* and *Trueperella* to the species level by matching against the reference database library entries using the MALDI Biotyper 3.1 software package and the newly acquired MSPs from the 12 reference strains. All nine species were reliably differentiated with no false positive cluster formation.

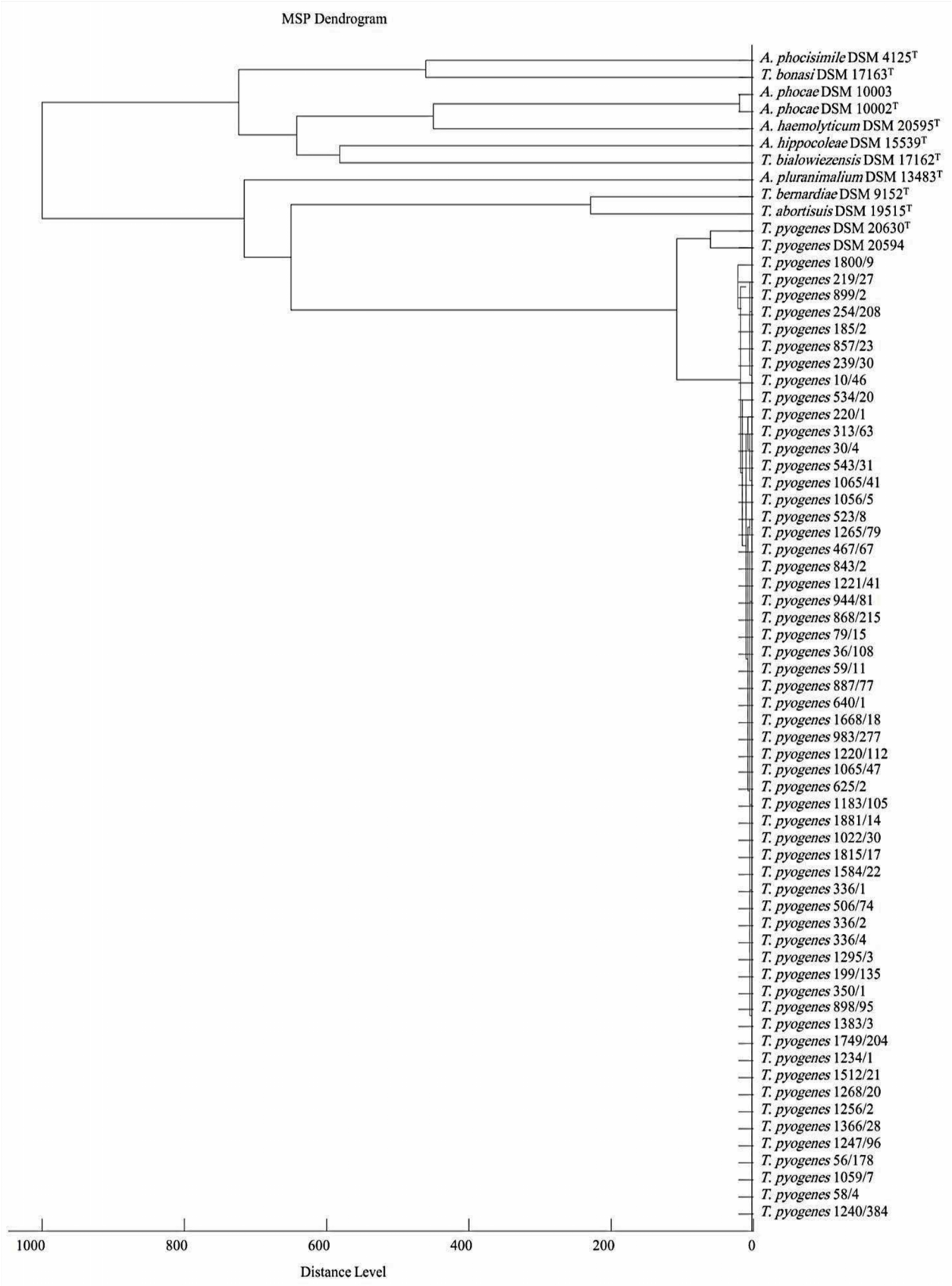

Fig. 7: A score oriented dendrogram of MALDI-TOF main spectra profiles of 57 *T. pyogenes* isolates of the present study with twelve reference strains of genera *Arcanobacterium* and *Trueperella*, also including the two *T. pyogenes* reference strains.

4.1.1.3 Identification by FT-IR spectroscopy

In order to expand the application of FT-IR spectroscopy for mastitis diagnostics on *T. pyogenes*, a hierarchically structured method based on reference isolates from genus *Trueperella* was created. Additionally, a taxonomically close relatives reference isolates from genera *Arcanobacterium* and *Actinomyces* were cultivated on the same conditions and integrated in the first level of the method. The *Trueperella* isolates comprise the type-strains of all recently described members of this genus. The spectral characteristics of five species of genus *Trueperella*, presented in this study, are based on the results of a comparative analysis of their FT-IR spectra. For *T. pyogenes* the creation of the method succeeded by using three selected isolates. An adequate segregation of this species from other species of genus *Trueperella* was achieved (Tab. 8). Additionally, this model allowed the correct classification of the external 57 *T. pyogenes* isolates obtained from bovine clinical mastitis as *T. pyogenes*, with a probability of 98.8% and no error (Tab. 8). Most noticeable is the hierarchical classification of the infrared data which yielded two distinct groups or classes. The first group clustered the *Trueperella* reference strains *T. abortisuis* DSM 19515^T, *T. bernardiae* DSM 9152^T, *T. bialowiezensis* DSM 17162^T and *T. bonasi* DSM 17163^T. The second group clustered the external 57 *T. pyogenes* isolates obtained from bovine clinical mastitis. However, *T. pyogenes* 336-1, *T. pyogenes* 336-2 and *T. pyogenes* 336-4 isolated from the same cow clustered together in a separate subgroup, which indicate epidemiological relationship (Fig. 9). Cluster analysis over the entire range of spectra and their first derivatives showed that isolates of *T. pyogenes* showed that there are differences to an extent that is observed between species. All other bacterial species comprised mostly single isolates. Therefore, a synoptic appraisal for internal validation on genus level was performed. For more than 92% of the *Arcanobacterium*, *Actinomyces* and *Trueperella* isolates were assigned to the respective genus correctly.

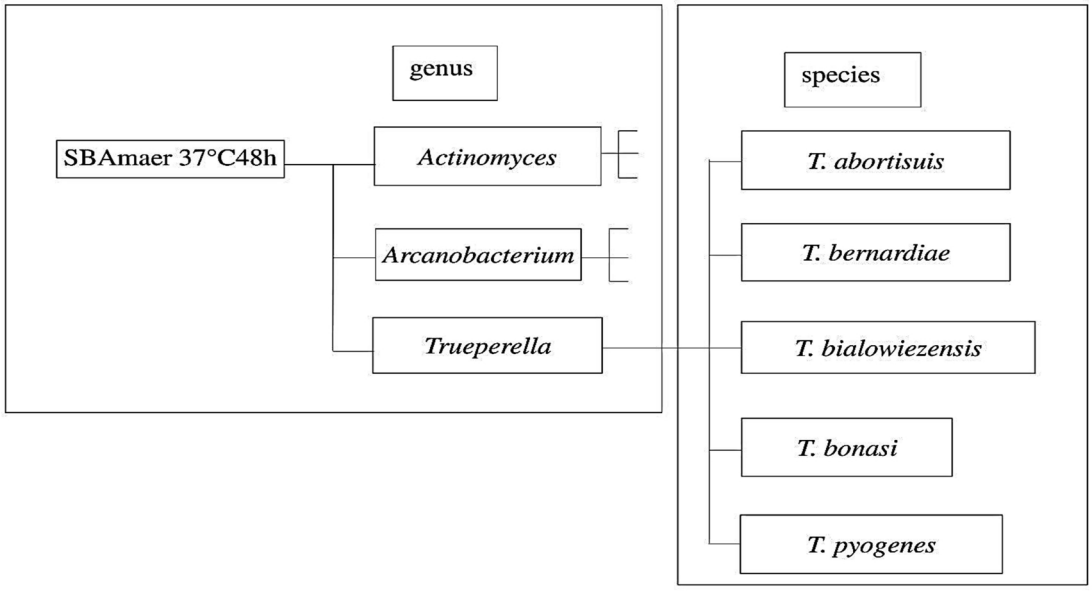

Fig. 8: Hierarchical ANN classification scheme for the differentiation of *Actinomycetales* strains at the genus level and the *Trueperella* strains at species level. The isolates were cultivated on sheep blood agar (SBA) for 48 h (+/-0.5 h) at 37°C under microaerobic conditions.

Tab. 8: Validation of genus and species classification by FT-IR, given as probability for correct identification of strains (repeated determinations).

Organism	Strains and isolates	Spectra	n_{ref}	n_{val}	Identification (%)[a]	
					Correct	Incorrect
Actinomyces (genus level)	4	36	13	23	95.8	0
Arcanobacterium (genus level)	7	87	35	52	92.6	0
Trueperella (genus level)	7	89	31	58	95.8	0
T. abortisuis	1	15	5	10	96.0	0
T. bernardiae	1	13	4	9	100	0
T. bialowiezensis	1	19	5	14	91.8	0
T. bonasi	1	12	3	9	88.9	0
T. pyogenes	3	30	14	16	97.9	0
T. pyogenes from milk-samples	57	342	0 [b]	342	98.8 [b]	0

n_{ref}, number of spectra used for reference; n_{val}, number of spectra used for validation. For internal validation, all spectra which had not been included in the reference data set were used. [a]The probability of obtaining uncertain results during repeated determinations is given by the residual to 100%. [b]For identification of *T. pyogenes*, an external validation was applied using all isolates not included in the reference.

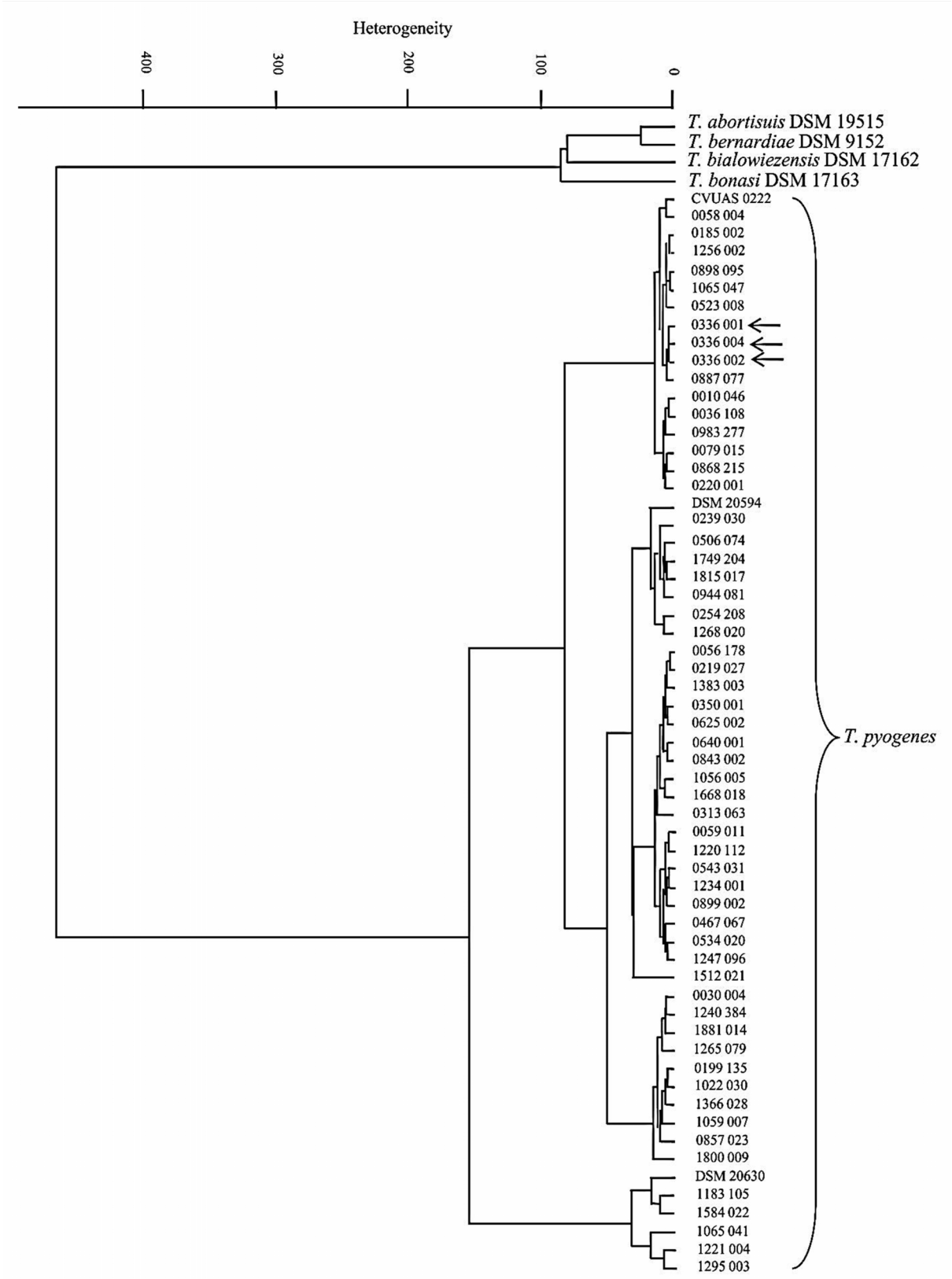

Fig. 9: Dendrogram of infrared spectra of 57 *T. pyogenes* isolates from milk samples in comparison with reference strains from the same genus. Cluster analysis was performed by using the second derivatives of the spectra in the spectral ranges of 500 to 1,200 cm^{-1} and 2,800 to 3,000 cm^{-1}. Ward's algorithm was applied. The arrows show three independent isolates from the same cow.

4.1.1.4 PCR-mediated identification and characterization

The oligonucleotide primers Apy-F and Apy-R were used for amplification of a ISR specific part of *T. pyogenes* as described in 3.7.9.1. An approximately 120 bp amplicon was amplified from all 57 investigated *T. pyogenes* isolates and for the reference strains *T. pyogenes* DSM 20630^T and *T. pyogenes* DSM 20594 (Fig. 10; Tab. 9). The oligonucleotide primers Apy-sodA-F and Apy-sodA-R were used for amplification of a *sodA* specific part of *T. pyogenes* as described in 3.7.9.2. An approximately 200 bp amplicon was amplified for the 57 investigated *T. pyogenes* isolates and for the two reference strains *T. pyogenes* DSM 20630^T and *T. pyogenes* DSM 20594 (Fig. 11; Tab. 9). No cross reactivity could be recorded with any of the control strains.

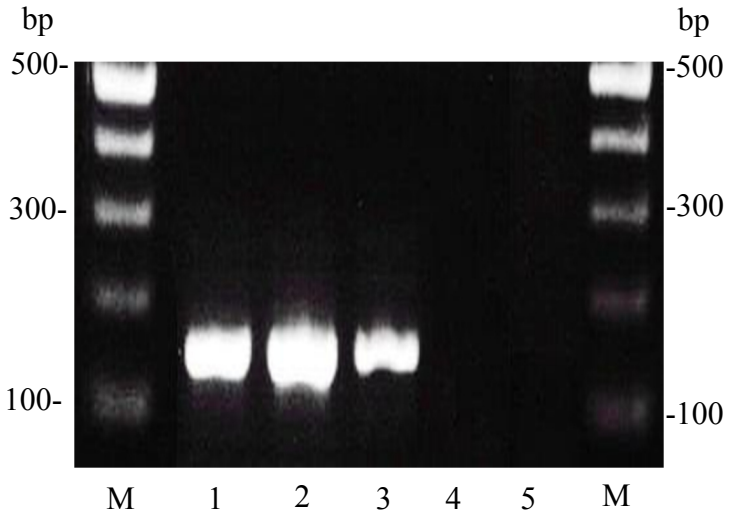

Fig. 10: A typical amplification reaction of an approximately 120 bp part of *T. pyogenes* ISR (1, 2 and 3) using the specific oligonucleotide primer pair Apy-F and Apy-R. *T. bonasi* DSM 17163^T (4) and *T. bernardiae* DSM 9152^T (5) served as negative control. M = Gene Ruler 100 bp - 1,000 bp DNA ladder (3.7.3).

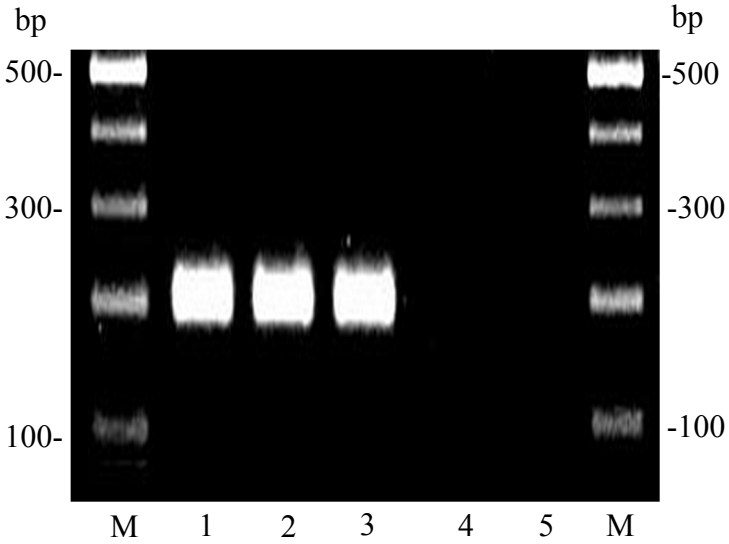

Fig. 11: A typical amplification reaction of an approximately 200 bp *T. pyogenes sodA* fragment (1, 2 and 3) using the specific oligonucleotide primer pair Apy-sodA-F and Apy-sodA-R. *T. bonasi* DSM 17163^T (4) and *T. bernardiae* DSM 9152^T (5) served as negative control. M = Gene Ruler DNA ladder (3.7.3).

The oligonucleotide primers plo-F and plo-R were used for amplification of a gene *plo* specific part of *T. pyogenes* (3.7.10.1). With the oligonucleotide primer a 704 bp amplicon was amplified from all 57 investigated *T. pyogenes* isolates and from the two reference strains *T. pyogenes* DSM 20630^T and *T. pyogenes* DSM 20594 (Fig. 12; Tab. 9). As described in 3.7.10.2 the oligonucleotide primers cbp-F and cbp-R were used for amplification of gene *cbpA* specific parts of *T. pyogenes*. With the oligonucleotide primer an approximate size of 330 bp (327 bp expected size) amplicon was amplified from a single isolate (1.8%) of the 57 investigated *T. pyogenes* isolates and for the reference strain *T. pyogenes* DSM 20630^T. However, reference strain *T. pyogenes* DSM 20594 and the remaining 56 isolates were *cbpA* negative (Fig. 13; Tab. 9).

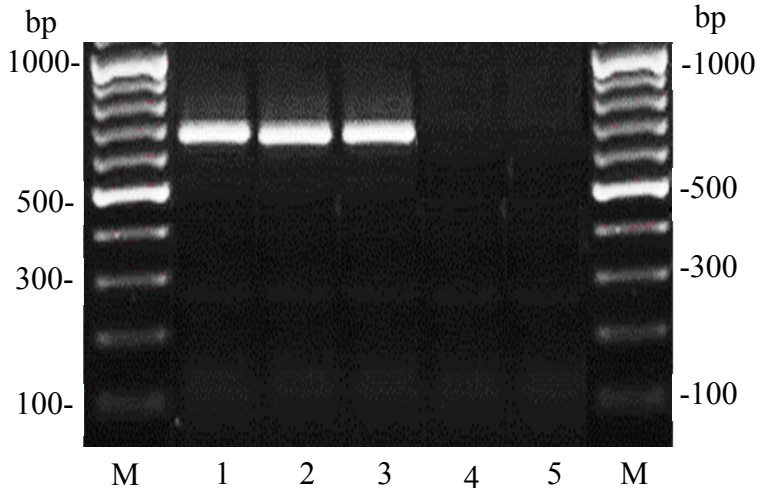

Fig. 12: A typical amplification reaction of an approximately 700 bp *T. pyogenes* gene *plo* fragment (1, 2 and 3) using the oligonucleotide primer pair plo-F and plo-R. *T. bonasi* DSM 17163^T (4) and *T. bialowiezensis* DSM 17162^T (5) served as negative control. M = Gene Ruler DNA ladder (3.7.3).

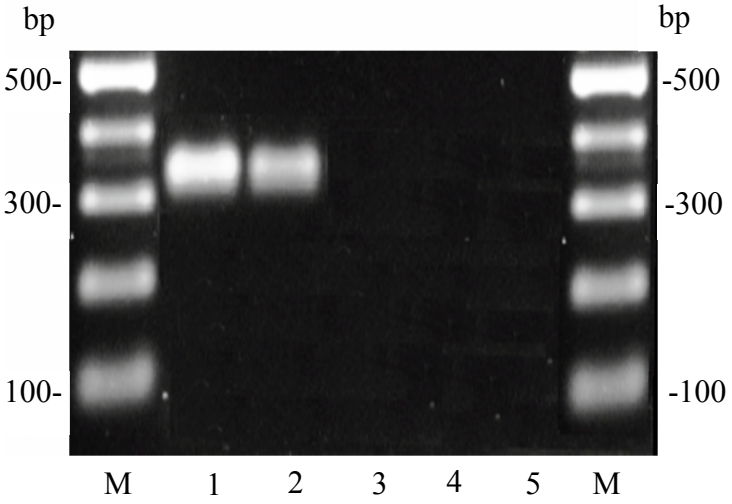

Fig. 13: A typical amplification reaction of an approximately 330 bp *T. pyogenes* gene *cbpA* fragment (1 and 2) using the specific oligonucleotide primer pair cbp-F and cbp-R. *T. pyogenes* DSM 20594 (3), *T. bialowiezensis* DSM 17162^T (4) and *T. bonasi* DSM 17163^T (5) served as negative control. M = Gene Ruler DNA ladder (3.7.3).

As described in 3.7.10.3 the oligonucleotide primer nanH-F and nanH-R were used for amplification of a gene *nanH* specific part of *T. pyogenes*. With the oligonucleotide primer an approximately 780 bp (781 bp expected size) amplicon was amplified for 39 (68%) of the 57 investigated *T. pyogenes* isolates and for both reference strains *T. pyogenes* DSM 20630^T and *T. pyogenes* DSM 20594 (Fig. 14; Tab. 9). As described in 3.7.10.3 the oligonucleotide primers nanP-F and nanP-R were used for amplification of gene *nanP*. With the oligonucleotide primers an approximately 150 bp amplicon was amplified for 48 (84%) of the 57 investigated *T. pyogenes* isolates and for both reference strains *T. pyogenes* DSM 20630^T and *T. pyogenes* DSM 20594 (Fig. 15; Tab. 9).

The neuraminidase encoding gene *nanH* could be detected in 35 *T. pyogenes* isolates possessing neuraminidase encoding gene *nanP*. Gene *nanH* was recorded in four *T. pyogenes* isolates alone without the simultaneous presence of neuraminidase encoding gene *nanP*. The neuraminidase encoding gene *nanP* was recorded in 13 *T. pyogenes* isolates alone without the presence of neuraminidase encoding gene *nanH*. Both of the neuraminidase encoding gene *nanH* and gene *nanP* were recorded negatively in five *T. pyogenes* isolates. The two *T. pyogenes* reference strains carried both genes *nanH* and *nanP*.

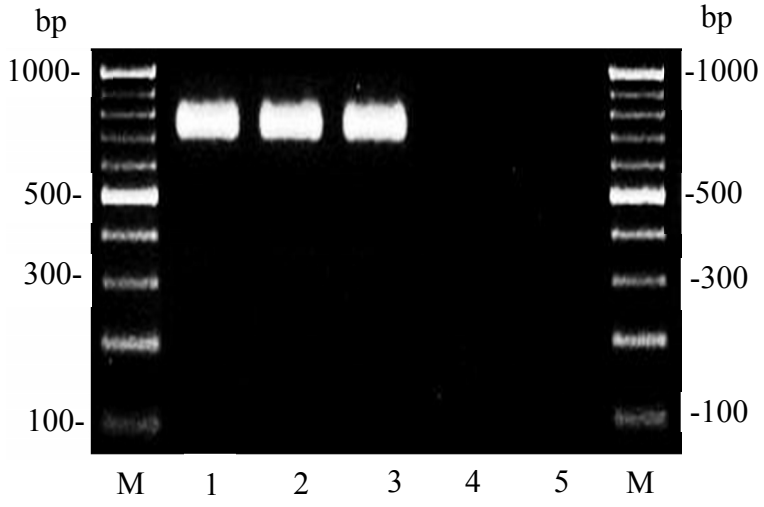

Fig. 14: A typical amplification reaction of an approximately 780 bp *T. pyogenes* gene *nanH* fragment (1, 2 and 3) using the oligonucleotide primer pair nanH-F and nanH-R *T. abortisuis* DSM 19515^T (4) and *T. bialowiezensis* DSM 17162^T (5) served as negative control. M = Gene Ruler DNA ladder (3.7.3).

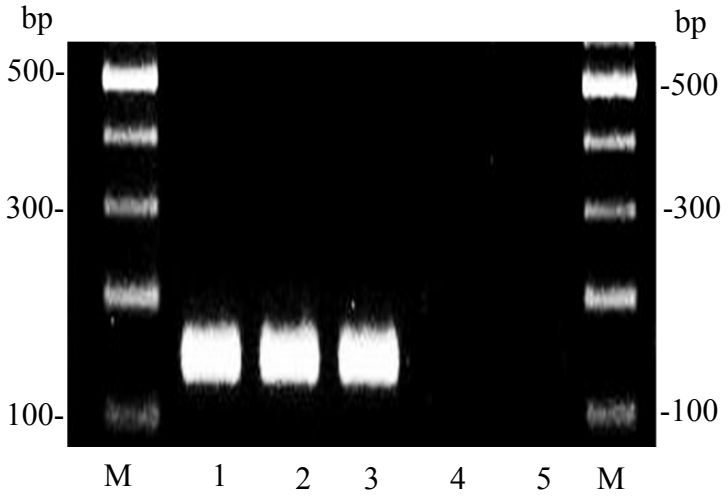

Fig. 15: A typical amplification reaction of a 150 bp *T. pyogenes* gene *nanP* fragment (1, 2 and 3) using the oligonucleotide primer pair nanP-F and nanP-R. *T. abortisuis* DSM 19515^T (4) and *T. bialowiezensis* DSM 17162^T (5) served as negative control. M = Gene Ruler DNA ladder (3.7.3).

As described in 3.7.10.4 the oligonucleotide primer fimA-F and fimA-R were used for amplification of a gene *fimA* specific part of *T. pyogenes*. An approximately 610 bp (605 bp expected size) amplicon was amplified for all 57 investigated *T. pyogenes* isolates and for the reference strain *T. pyogenes* DSM 20594 (Fig. 16; Tab. 9). As described in 3.7.10.4 the oligonucleotide primers fimC-F and fimC-R were used for amplification of a gene *fimC* specific part of *T. pyogenes*. An approximately 850 bp (843 bp expected size) amplicon was amplified for 53 (61.4%) of the 57 investigated *T. pyogenes* isolates and for the 2 reference strains *T. pyogenes* DSM 20630^T and *T. pyogenes* DSM 20594 (Fig. 17; Tab. 9). As described in 3.7.10.4 the oligonucleotide primers fimE-F and fimE-R were used for amplification of a gene *fimE* specific part of *T. pyogenes*. An approximately 780 bp (775 bp expected size) amplicon was amplified for 52 (91.2%) of the 57 investigated *T. pyogenes* isolates and for the 2 reference strains *T. pyogenes* DSM 20630^T and *T. pyogenes* DSM 20594 (Fig. 18; Tab. 9). No cross reactivity could be recorded with any of the control strains.

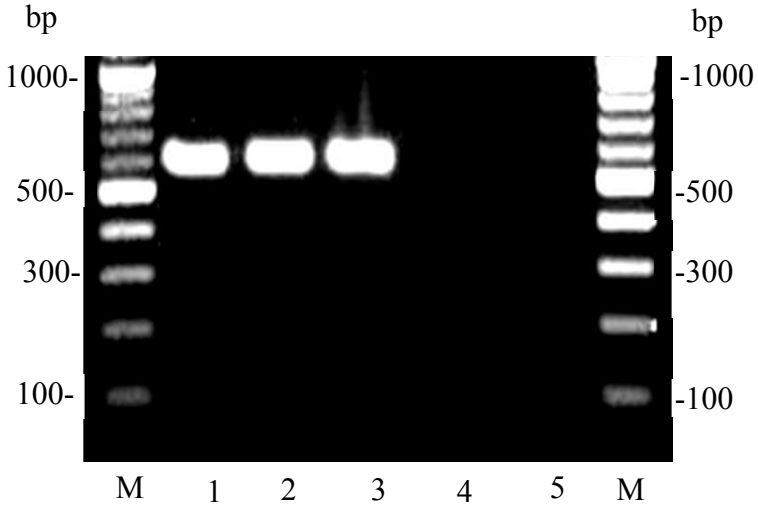

Fig. 16: A typical amplification reaction of an approximately 600 bp *T. pyogenes* gene *fimA* fragment (1, 2 and 3) using the specific oligonucleotide primer pair fimA-F and fimA-R. *T. abortisuis* DSM 19515^T (4) and *T. bialowiezensis* DSM 17162^T (5) served as negative control. M = Gene Ruler DNA ladder (3.7.3).

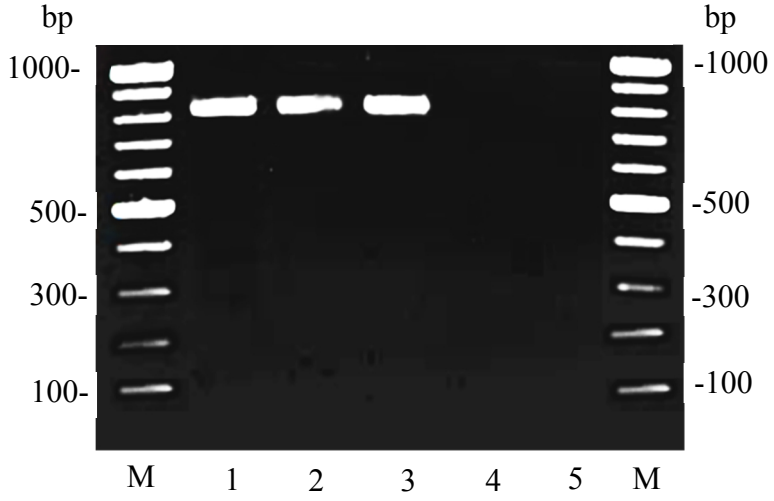

Fig. 17: A typical amplification reaction of an approximately 850 bp *T. pyogenes* gene *fimC* fragment (1, 2 and 3) using the specific oligonucleotide primer pair fimC-F and fimC-R. *T. abortisuis* DSM 19515^T (4) and *T. bialowiezensis* DSM 17162^T (5) served as negative control. M = Gene Ruler DNA ladder (3.7.3).

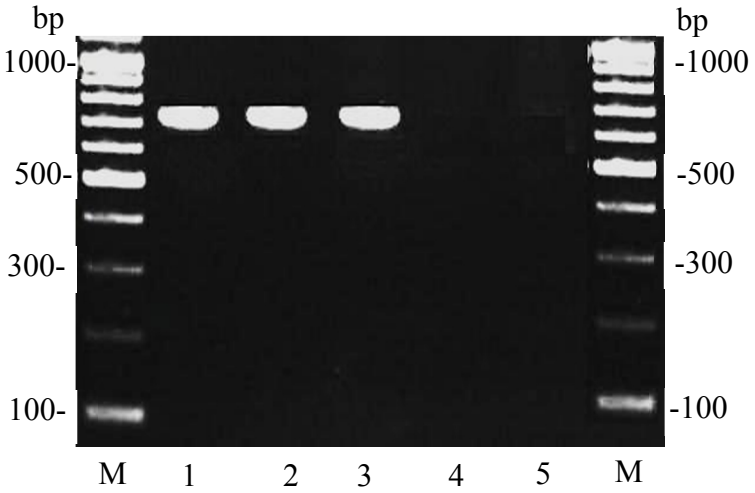

Fig. 18: A typical amplification reaction of an approximately 780 bp *T. pyogenes* gene *fimE* fragment (1, 2 and 3) using the specific oligonucleotide primer fimE-F and fimE-R.*T. abortisuis* DSM 19515^T (4) and (5) *T. bialowiezensis* DSM 17162^T (5) served as negative control. M = Gene Ruler DNA ladder (3.7.3).

As described in 3.7.10.5 the oligonucleotide primer tet(W)-F and tet(W)-R were used for amplification of gene *tet(W)* specific parts of *T. pyogenes*. An amplicon of an approximately size of 1,850 bp (1,843 bp expected size) was amplified from 22 (38.6%) of the 57 investigated *T. pyogenes* isolates and for reference strain *T. pyogenes* DSM 20630^T, *T. pyogenes* DSM 20594 was negative (Fig. 19; Tab. 9). No cross reactivity could be recorded with any of the control strains. A summary comparing the PCR amplified target genes, also including putative virulence factor encoding genes, of the 57 *T. pyogenes* isolates from bovine mastitis and the *T. pyogenes* reference strains of the present study is presented in Tab. 27.

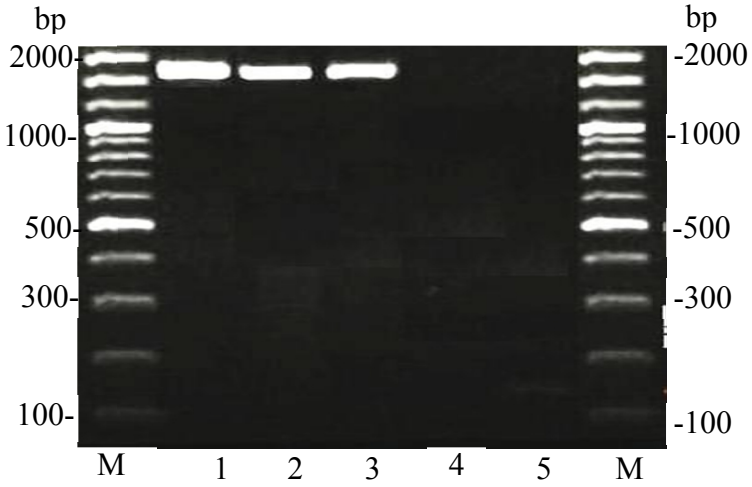

Fig. 19: A typical amplification reaction of an approximately 1,850 bp *T. pyogenes* gene *tet(W)* fragment (1, 2 and 3) using the specific oligonucleotide primer tet(W)-F and tet(W)-R. *T. pyogenes* DSM 20594 (4) and *T. bialowiezensis* DSM 17162^{T} (5) served as negative control. M = Gene Ruler DNA ladder (3.7.3).

Tab. 9: Genotypical properties of the 57 *T. pyogenes* isolates of the present study and the two *T. pyogenes* reference strains.

Genotypical properties	Number of *T. pyogenes* isolates			Results of *T. pyogenes* reference strains**	
	Tested	Positive	Positive%	DSM 20630*	DSM 20594*
T. pyogenes specific part of ISR	57	57	100	+	+
T. pyogenes specific part of gene *sod*A	57	57	100	+	+
Pyolysin encoding gene *plo*	57	57	100	+	+
Collagen-binding protein encoding gene *cbpA*	57	1	1.8	+	-
Neuraminidase H encoding gene *nanH*	57	39	68.4	+	+
Neuraminidase P encoding gene *nanP*	57	48	84.2	+	+
Fimbriae endoding gene *fimA*	57	57	100	-	+
Fimbriae endoding gene *fimC*	57	53	93	+	+
Fimbriae endoding gene *fimE*	57	52	91.2	+	+
Tetracycline resistance encoding gene *tet*(*W*)	57	22	38.6	+**	-**

The reactions are shown as follows: +; positive reaction; -; negative reaction; *= results obtained from Hijazin et al., 2011; **= results obtained from Billington et al., (2002); the numbers of positive and negative reactions are shown in parentheses.

The presence or absence of the six genes encoding virulence factors (*cbpA, nanH, nanP, fimA, fimC, fimE* and the tetracycline resistance encoding gene *tet*(*W*) diversified the *T. pyogenes* isolates from milk samples in Hesse and the reference strains *T. pyogenes* DSM 20594 and *T. pyogenes* DSM 20630^T genotypically, representing seventeen genotypes. Group A assembled 18 isolates including *T. pyogenes* 313/63, *T. pyogenes* 30/4, *T. pyogenes* 36/108, *T. pyogenes* 467/67, *T. pyogenes* 640/1, *T. pyogenes* 857/23, *T. pyogenes* 350/1, *T. pyogenes* 944/81*, T. pyogenes* 336/ 1*, T. pyogenes* 336/ 2*, T. pyogenes* 336/4, *T. pyogenes* 254/208, *T. pyogenes* 220/1, *T. pyogenes* 1668/18, *T. pyogenes* 1383/3, *T. pyogenes* 1247/96, *T. pyogenes* 1059/7 and *T. pyogenes* 10/46). Group B assembled seven isolates including *T. pyogenes* 625/2, *T. pyogenes* 239/30, *T. pyogenes* 1815/17, *T. pyogenes* 1800/9, *T. pyogenes* 1749/204, *T. pyogenes* 1366/28 and *T. pyogenes* 1295/3. Group C assembled nine isolates including *T. pyogenes* 59/11, *T. pyogenes* 58/4, *T. pyogenes* 534/20, *T. pyogenes* 523/8, *T. pyogenes* 506/74, *T. pyogenes* 199/135*, T. pyogenes* 1584/22, *T. pyogenes* 1265/79 and *T. pyogenes* 1240/384. The reference strain *T. pyogenes* DSM 20594 is also assembled to this group. Group D assembled 4 isolates including *T. pyogenes* 1256/2, *T. pyogenes* 1022/30, *T. pyogenes* 1881/14 and *T. pyogenes* 56/178). Group E assembled three isolates including *T. pyogenes* 1221/4, *T. pyogenes* 1220/112 and *T. pyogenes* 219/27. Group F assembled three isolates including *T. pyogenes* 1268/20, *T. pyogenes* 1056/5 and *T. pyogenes* 843/2. Group G assembled two isolates including *T. pyogenes* 1183/105 and *T. pyogenes* 1065/47. Group H assembled two isolates including *T. pyogenes* 887/77 and *T. pyogenes* 185/2. Group I assembled two isolates including *T. pyogenes* 543/31 and *T. pyogenes* 898/95. Group J assembled *T. pyogenes* 983/277. Group K assembled *T. pyogenes* 899/2. Group L assembled *T. pyogenes* 1512/21. Group M assembled *T. pyogenes* 868/215. Group N assembled *T. pyogenes* 1065/41. Group O assembled *T. pyogenes* 79/15. Group P assembled *T. pyogenes* 1234/1. Group Q assembled the reference strain *T. pyogenes* DSM 20630^T. The genotypic profiles of *T. pyogenes* isolated from mastitic milk of dairy cows (57 isolates) and the reference strains *T. pyogenes* DSM 20594 and *T. pyogenes* DSM 20630^T are presented in Tab. 10.

Tab. 10: Genotype profiles of *T. pyogenes* isolated from mastitic milk of dairy cows (57 isolates) and the reference strains *T. pyogenes* DSM 20594 and *T. pyogenes* DSM 20630^T.

Group	Genotype							Number of positive isolates and strains, respectivly	%
A	*nanH*	*nanP*	*fimA*	*fimC*	*fimE*	*tet(W)*		18	30.5
B	*nanP*	*fimA*	*fimC*	*fimE*	*tet*(*W*)			7	11.9
C	*nanH*	*nanP*	*fimA*	*fimC*	*fimE*			10*	16.9
D	*nanP*	*fimA*	*fimC*	*fimE*				4	6.8
E	*nanH*	*nanP*	*fimA*	*fimC*	*tet*(*W*)			3	5.1
F	*nanH*	*fimA*	*fimC*	*tet*(*W*)				3	5.1
G	*fimA*	*fimC*	*fimE*	*tet*(*W*)				2	3.4
H	*fimA*	*fimC*	*fimE*					2	3.4
I	*nanH*	*nanP*	*fimA*	*fimE*	*tet*(*W*)			2	3.4
J	*cbpA*	*nanH*	*nanP*	*fimA*	*fimC*	*fimE*	*tet*(*W*)	1	1.7
K	*nanP*	*fimA*	*fimE*					1	1.7
L	*nanH*	*nanP*	*fimA*	*fimE*				1	1.7
M	*nanP*	*fimA*	*fimC*	*fimE*				1	1.7
N	*nanH*	*nanP*	*fimA*	*fimC*				1	1.7
O	*nanH*	*fimA*	*fimC*	*fimE*				1	1.7
P	*fimA*	*fimC*						1	1.7
Q	*cbpA*	*nanH*	*nanP*	*fimC*	*fimE*	*tet*(*W*)		1**	1.7

*including *T. pyogenes* DSM 20594 **including *T. pyogenes* DSM 20630^T

4.1.1.5 Characterization of *T. pyogenes* by genomic fingerprinting using MLSA

The MLSA studies were performed with a selection of 14 of the 57 *T. pyogenes* isolates and the reference strains *T. pyogenes* DSM 20594, *T. pyogenes* DSM 20630^T and *A. haemolyticum* DSM 20595^T.

The *T. pyogenes* isolates were selected because they were isolated from the same or from different geographical locations or from the same or from different cows. The 14 *T. pyogenes* were isolated from 7 locations, 9 farms, and 12 cows. A detailed information about the origin of the 14 *T. pyogenes* isolates could be seen in Tab. 11. In this study, MLSA was employed by using partial sequences of the housekeeping genes *fusA, tuf, metG* and *gyrA* encoding the proteins FusA, Tuf, MetG and GyrA. The targeted amplified DNA fragments were double sequenced on both strands and the sequences were deposited in the GenBank

(National Center for Biotechnology Information). The GenBank accession numbers of locus sequences obtained in this study are provided in Tab. 12. The phylogenetic analysis was based on the combined utilization of the *fusA-tuf-metG-gyrA* partial gene sequences and the encoded amino acid sequences of FusA, Tuf, MetG and GyrA.

Tab. 11: Origin of the 14 *T. pyogenes* isolated from bovine milk used in the MLSA study.

	T. pyogenes isolates	Location	Farm	Cow	Genotype group
1	1220/112	1	E	GR	E
2	1512/21	15	W	21	L
3	1800/9	16	V	137	B
4	58/4	21	M	61284	C
5	59/11	15	W	52	C
6	220/1	21	M	83	A
7	843/2	16	S	111	F
8	887/77	31	R	520	H
9	1065/41	35	A	94120	N
10	1065/47	35	A	29041	G
11	336/1	48	W	5134	A
12	336/2	48	W	5134	A
13	336/4	48	W	5134	A
14	506/74	31	L	525	C

Tab. 12: GenBank accession numbers of locus sequences of the 14 *T. pyogenes* isolated from bovine milk used in this study and the two *T. pyogenes* reference strains *T. pyogenes* DSM 20630^T and *T. pyogenes* DSM 20594.

	T. pyogenes isolates and strains	*fusA*	*tuf*	*metG*	*gyrA*
1	336/1	KJ605917	KJ609091	KJ609080	KJ609066
2	336/2	KJ605918	KJ609092	KJ609081	KJ609067
3	336/4	KJ605919	KJ609093	KJ609082	KJ609068
4	1512/21	KJ605916	KJ609101	KJ609077	KJ609063
5	59/11	KJ605922	KJ609089	KJ609085	KJ609071
6	58/4	KJ605921	KJ609096	KJ609084	KJ609070
7	220/1	KJ605923	KJ609090	KJ609079	KJ609065
8	1065/41	KJ605926	KJ609099	KJ609074	-ve
9	1065/47	KJ605927	KJ609100	KJ609075	KJ609061
10	1800/9	KJ605920	KJ609088	KJ609078	KJ609064
11	843/2	KJ605924	KJ609094	KJ609086	KJ609072
12	887/77	KJ605925	KJ609095	KJ609087	KJ609073
13	506/74	KJ605928	KJ609098	KJ609083	KJ609069
14	1220/112	KJ605915	KJ609097	KJ609076	KJ609062
15	DSM 20630^T	KJ605911	HG941716	HG941708	HG941704
16	DSM 20594	KJ605910	HG941715	HG941707	HG941703

As described in 3.8.2.1 the oligonucleotide primers fusA-F and fusA-R were used for amplification of the translation elongation factor G encoding gene *fusA* of *T. pyogenes*. An approximately 828 bp amplicon was amplified from all investigated *T. pyogenes* isolates and for the reference strains *T. pyogenes* DSM 20594, *T. pyogenes* DSM 20630^T and *A. haemolyticum* DSM 20595^T. The cluster analyses of the translation elongation factor G encoding gene *fusA* had succeeded to subdivide the 14 investigated *T. pyogenes* isolates and the reference strains (*T. pyogenes* DSM 20594, *T. pyogenes* DSM 20630^T and *A. haemolyticum* DSM 20595^T). The phylogenetic tree built with the *fusA* nucleotide sequence using 730 nucleotide positions split the *T. pyogenes* isolates in the present study into three groups. The first group (GI) comprised 15 isolates and with four subgroups. The first subgroup clustering *T. pyogenes* 336-1, *T. pyogenes* 336-2 and *T. pyogenes* 336-4 (GI.I). The first subgroup also included the well defined subcluster of the isolates *T. pyogenes* 506-74, *T. pyogenes* 1800-9, and *T. pyogenes* 200-1. This previous subcluster also assembled reference strain *T. pyogenes* DSM 20630^T. The second subgroup (GI.II) clustered six *T. pyogenes* isolates. The second subgroup (GI.II) included the isolates *T. pyogenes* 1220-112,

T. pyogenes 843-2 and *T. pyogenes* 887-77 with bootstrap supports of 76%. The third subgroup (GI.III) is clustering the isolate *T. pyogenes* 1065-41. The fourth subgroup is clustering *T. pyogenes* 58-4 and reference strain *T. pyogenes* DSM 20594. The second group (GII) comprised two isolates *T. pyogenes* 1512-21 and *T. pyogenes* 59-11. However, *T. pyogenes* 1065-47 is representing an out-group (GIII) (Fig. 20A). The phylogenetic tree built with the FusA amino acid sequences of a total of 243 amino acid positions split the isolates in the present study into two groups, with final bootstrap supports for each group of 68% and 100%, respectively. The first group (GI) comprised 12 isolates and is clustering *T. pyogenes* 1065-47, *T. pyogenes* 506-74, *T. pyogenes* 1065-41, *T. pyogenes* 200-1, *T. pyogenes* 59-11, *T. pyogenes* 58-4, *T. pyogenes* 1800-9, *T. pyogenes* 336-4, *T. pyogenes* 336-2, *T. pyogenes* 336-1, *T. pyogenes* 1512-21 and the reference strain *T. pyogenes* DSM 20594. The isolates *T. pyogenes* 1220-112, *T. pyogenes* 843-2 and *T. pyogenes* 887-77 were clustered separately within the first group. The second group (GII) is clustering *T. pyogenes* DSM 20630^T (Fig. 20B). The reference strain *A. haemolyticum* DSM 20595^T was assembled and clustered separately out of the group.

Fig. 20A

0.05

Fig. 20B

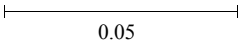

Fig. 20: Maximum-likelihood tree based on *fusA* nucleotide sequences of a total of 730 nucleotide positions (A) and FusA amino acid sequences of a total 243 amino acid positions (B) of the investigated target gene and protein of the 14 *T. pyogenes* isolates of mastitis origin, *T. pyogenes* DSM 20594, *T. pyogenes* DSM 20630^T and *A. haemolyticum* DSM 20595^T.

As described in 3.8.2.2 the oligonucleotide primers tuf-F and tuf-R were used for amplification of translation elongation factor Tu encoding gene *tuf* of *T. pyogenes*. An approximately 800 bp amplicon was amplified from all investigated *T. pyogenes* isolates and for the reference strains (*T. pyogenes* DSM 20594, *T. pyogenes* DSM 20630^T and *A. haemolyticum* DSM 20595^T). The cluster analyses of the translation elongation factor Tu encoding gene *tuf* succeeded to subdivide the 14 investigated *T. pyogenes* isolates and the reference strains *T. pyogenes* DSM 20594, *T. pyogenes* DSM 20630^T and *A. haemolyticum* DSM 20595^T. The phylogenetic tree built with the *tuf* nucleotide sequence of a total of 769 nucleotide positions split the isolates of the present study in two groups. The first group (GI) comprised 15 isolates with final bootstrap supports of 89% and is splitted in two subgroups. The first subgroup is clustering *T. pyogenes* 200-1, *T. pyogenes* 1065-47 and *T. pyogenes* 1800-9. The second subgroup is clustering 12 isolates including *T. pyogenes* 1065-41, *T. pyogenes* 1220-112, *T. pyogenes* 1512-21, *T. pyogenes* 336-1, *T. pyogenes* 336-2, *T. pyogenes* 336-4, *T. pyogenes* 506-74, *T. pyogenes* 58-4, *T. pyogenes* 59-11, *T. pyogenes* 843-2, *T. pyogenes* 887-77 and reference strain *T. pyogenes* DSM 20630^T. The second

group (GII) comprised only the reference strain *T. pyogenes* DSM 20594 (Fig. 21A). The phylogenetic tree built with the Tuf amino acid sequences of a total of 256 amino acid positions showed a 100% similarity and failed to split the isolates of the present study (except *T. pyogenes* 58-4 and *T. pyogenes* 200-1) into groups (Fig. 21B) The reference strain *A. haemolyticum* DSM 20595^T was assembled and clustered separately out of the group.

Fig. 21A

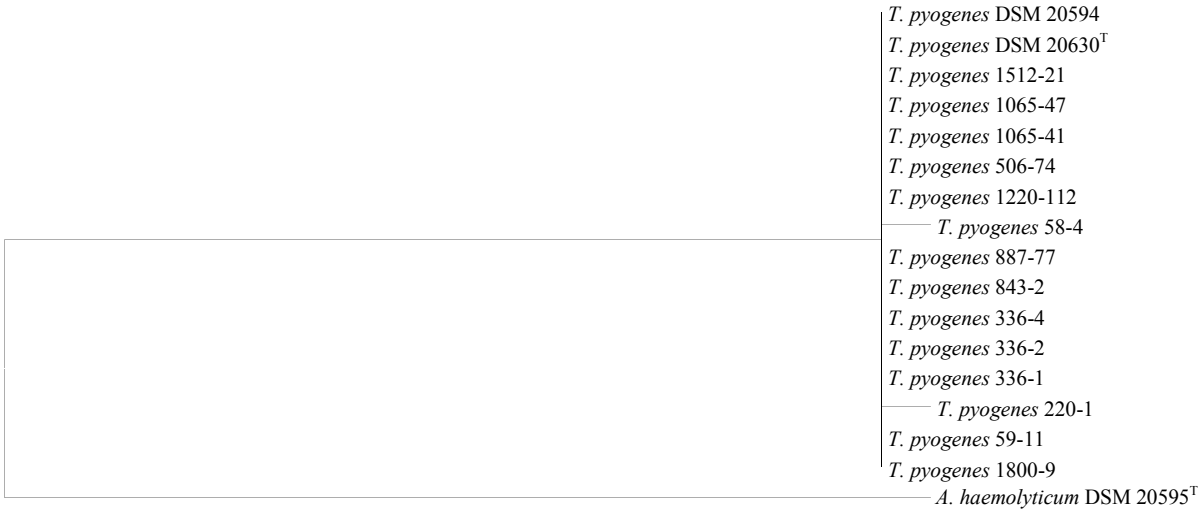

Fig. 21B

Fig. 21: Maximum-likelihood tree based on *tuf* nucleotide sequences of a total of 769 nucleotide positions (A) and Tuf amino acid sequences of a total of 256 amino acid positions (B) of the investigated target gene and protein of the 14 *T. pyogenes* isolates of bovine origin, *T. pyogenes* DSM 20594, *T. pyogenes* DSM 20630^T and *A. haemolyticum* DSM 20595^T.

As described in 3.8.2.3 the oligonucleotide primer metG-F and metG-R were used for amplification of the methionyl-tRNA synthetase encoding gene *metG* of *T. pyogenes*. An approximately 840 bp amplicon was amplified from all 14 investigated *T. pyogenes* isolates and for the reference strains *T. pyogenes* DSM 20594, *T. pyogenes* DSM 20630^T and *A. haemolyticum* DSM 20595^T. The cluster analysis of the methionyl-tRNA synthetase encoding gene *metG* succeeded to subdivide the 14 investigated *T. pyogenes* isolates and the reference strains (*T. pyogenes* DSM 20594, *T. pyogenes* DSM 20630^T and *A. haemolyticum* DSM 20595^T). The phylogenetic tree built with the *metG* nucleotide sequence of a total of 808 nucleotide positions split the isolates of the present study into two groups, with final bootstrap supports for each group of 87 and 100%, respectively. The first group (GI) comprised ten isolates and is splitted into five subgroups. The first subgroup (GI.I) is clustering *T. pyogenes* 1065-47, *T. pyogenes* 1800-9 and *T. pyogenes* 506-74, with final bootstrap supports of 93%. The second subgroup (GI.II) is clustering *T. pyogenes* 59-11 and the reference strain *T. pyogenes* DSM 20630^T with final bootstrap supports of 95%. The third subgroup (GI.III) is clustering *T. pyogenes* 1065-41, *T. pyogenes* 200-1 with final bootstrap supports of 98%. The fourth subgroup (GI.IV) is clustering *T. pyogenes* 1512-21. The fifth subgroup (GI.V) is clustering *T. pyogenes* 843-2, *T. pyogenes* 336-1, *T. pyogenes* 336-2, *T. pyogenes* 336-4 and reference strain *T. pyogenes* DSM 20594 with final bootstrap supports of 94%.The second group (G-II) comprised three isolates clustering *T. pyogenes* 1220-112, *T. pyogenes* 58-4, *T. pyogenes* 887-77 with final bootstrap supports of 100% (Fig. 22A).

The phylogenetic tree built with the MetG amino acid sequences of a total 269 amino acid positions split the isolates of the present study into in three groups. The first group (G-I) comprised five isolates and clustered *T. pyogenes* 843-2, *T. pyogenes* 336-1, *T. pyogenes* 336-2, *T. pyogenes* 336-4 and the reference strain *T. pyogenes* DSM 20594. The second group (G-II) comprised five isolates and clustered *T. pyogenes* 1220-112, *T. pyogenes* 1512-21, *T. pyogenes* 58-4 and *T. pyogenes* 887-77. The third group (G-III) comprised seven isolates clustering *T. pyogenes* 1065-47, *T. pyogenes* 1800-9, *T. pyogenes* 506-74, *T. pyogenes* 200-1, *T. pyogenes* 1065-41, 59-11 and the reference strain *T. pyogenes* DSM 20630^T (Fig. 22B). The reference strain *A. haemolyticum* DSM 20595^T was assembled and clustered separately out of the group.

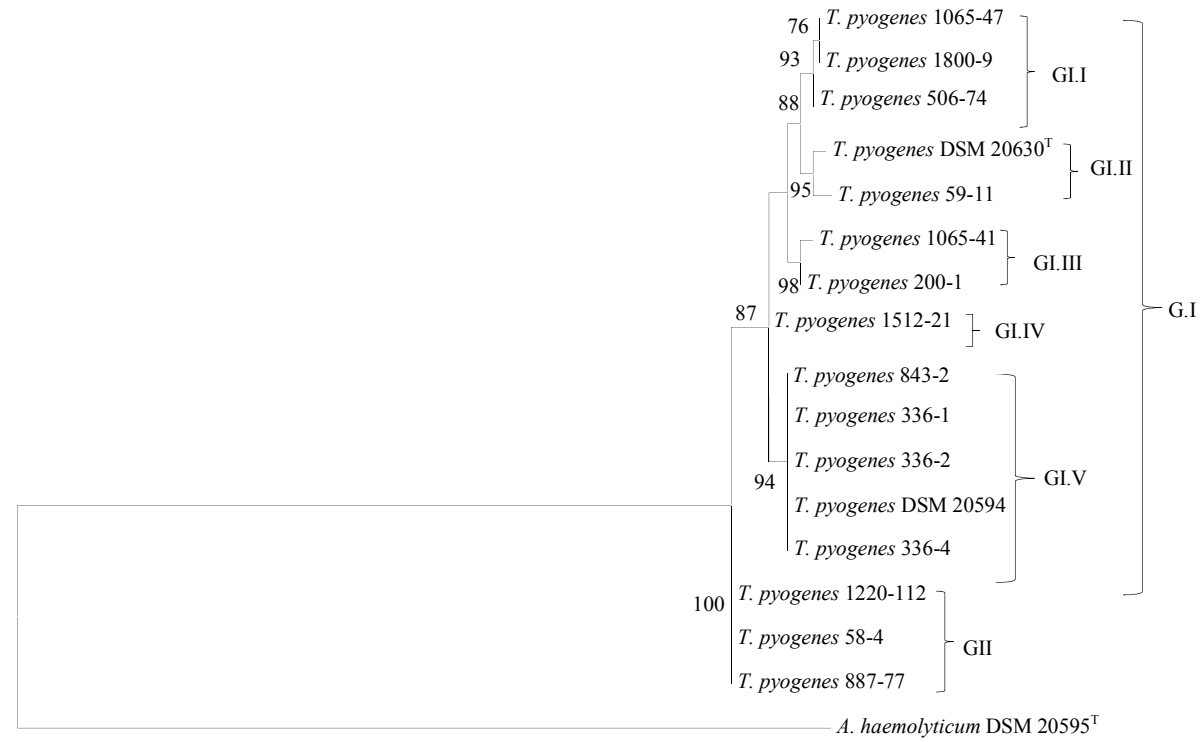

Fig. 22A

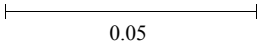

Fig. 22B

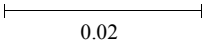

Fig. 22: Maximum-likelihood tree based on *metG* nucleotide sequences of a total of 808 nucleotide positions (A) and MetG amino acid sequences of a total of 269 amino acid positions (B) of the investigated target gene and protein of the 14 *T. pyogenes* of bovine origin, *T. pyogenes* DSM 20594, *T. pyogenes* DSM 20630^{T} and *A. haemolyticum* DSM 20595^{T}.

As described in 3.8.2.4 the oligonucleotide primers GyrA-F and GyrA-R were used for amplification of DNA gyrase (subunit A) encoding gene *gyrA* of *T. pyogenes*. An approximately 940 bp amplicon was amplified from the investigated *T. pyogenes* isolates and for the reference strains *T. pyogenes* DSM 20594, *T. pyogenes* DSM 20630^T and *A. haemolyticum* DSM 20595^T. However, *T. pyogenes* DNA gyrase (subunit A) encoding gene *gyrA* could not be amplified from isolate *T. pyogenes* 1065-41. The cluster analyses of the DNA gyrase (subunit A) encoding gene *gyrA* succeeded to subdivide the 13 investigated *T. pyogenes* isolates and the reference strains (*T. pyogenes* DSM 20594, *T. pyogenes* DSM 20630^T and *A. haemolyticum* DSM 20595^T). The phylogenetic tree built with the *gyrA* nucleotide sequence of a total of 870 nucleotide positions split the isolates of the present study into in three groups. The first group (GI) comprised 12 isolates with two subgroups. The first subgroup (GI.I) comprised 7 isolates clustering *T. pyogenes* 58-4, *T. pyogenes* 59-11, *T. pyogenes* 200-1, *T. pyogenes* 1512-21, *T. pyogenes* 1800-9, *T. pyogenes* 1065-47 and *T. pyogenes* 1220-112. The second subgroup (GI.II) comprised five isolates clustering *T. pyogenes* 506-74 and reference strain *T. pyogenes* DSM 20594. The second subgroup is also clustering *T. pyogenes* 336-1, *T. pyogenes* 336-2 and *T. pyogenes* 336-4 which formed a distinct cluster at the nucleotide sequence level with bootstrap supports of a nucleotide similarity of 100%. The second group (GII) comprised two isolates clustering *T. pyogenes* 843-2 and *T. pyogenes* 887-77 with bootstrap supports of 78%. The third group (GIII) clustered reference strain *T. pyogenes* DSM 20630^T alone (Fig. 23A).

The phylogenetic tree built with the GyrA amino acid sequences of a total of 290 amino acid positions split the isolates in the present study into two groups, with final bootstrap supports for each group of 93% and 100%, respectively. The first group (GI) comprised 14 isolates and was clustering *T. pyogenes* 1065-47, *T. pyogenes* 506-74, *T. pyogenes* 200-1, *T. pyogenes* 59-11, *T. pyogenes* 58-4, *T. pyogenes* 1800-9, *T. pyogenes* 336-4, *T. pyogenes* 336-2, *T. pyogenes* 336-1, *T. pyogenes* 1512-21, *T. pyogenes*1220-112 and *T. pyogenes* 887-77 and reference strains *T. pyogenes* DSM 20630^T and *T. pyogenes* DSM 20594. The second group (GII) comprised *T. pyogenes*1800-9 (Fig. 23B). The reference strain *A. haemolyticum* DSM 20595^T was assembled and clustered separately out of the group.

Fig: 23 A

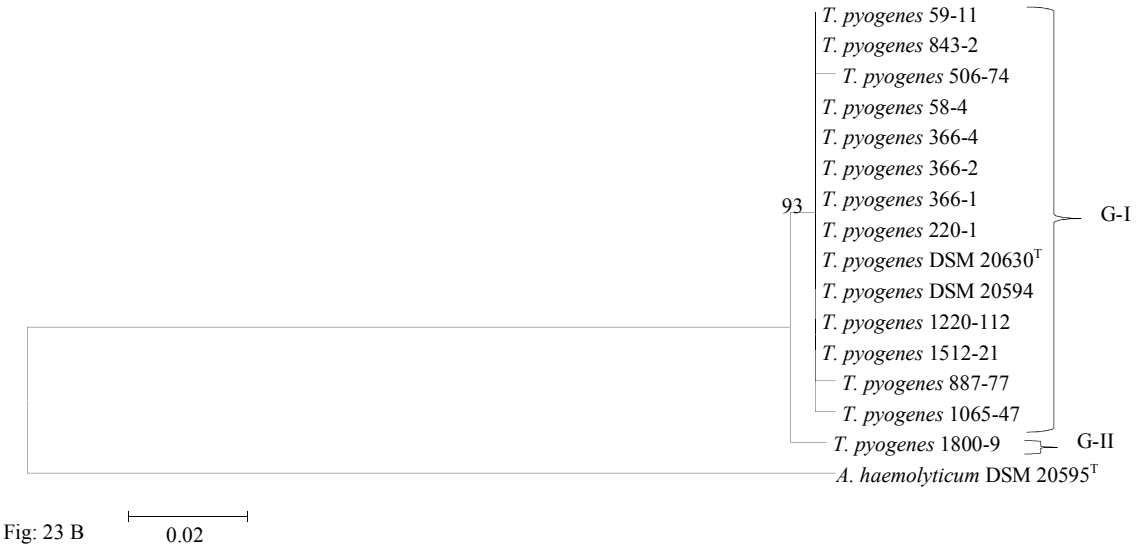

Fig: 23 B

Fig. 23: Maximum-likelihood tree based on *gyrA* nucleotide sequences of a total of 870 nucleotide positions (A) and GyrA amino acid sequences of a total of 290 amino acid positions (B) of the investigated target gene and protein of the 13 *T. pyogenes* isolates of bovine origin, *T. pyogenes* DSM 20594, *T. pyogenes* DSM 20630^{T} and *A. haemolyticum* DSM 20595^{T}.

All four partial nucleotide sequences and amino acid sequences of the housekeeping genes of the *T. pyogenes* isolates and the reference strains *T. pyogenes* DSM 20594, *T. pyogenes* DSM 20630^T and *A. haemolyticum* DSM 20595^T (*T. pyogenes* 1065-41 was not included) with a total nucleotide sequence of 3,177 bp and a total amino acid sequence of 1,058 sites were concatenated in the following order: *fusA-tuf-metG-gyrA* and FusA-Tuf-MetG-GyrA. The cluster analysis of the phylogenetic trees of concatenated sequences had succeeded to subdivide the 13 investigated *T. pyogenes* isolates and the reference strains (*T. pyogenes* DSM 20594, *T. pyogenes* DSM 20630^T and *A. haemolyticum* DSM 20595^T). The concatenated tree built with the nucleotide sequence with 3,177 bp clustered the investigated strains and isolates in five groups. The first group (GI) comprised five isolates with two subgroups. The first subgroup (GI.I) subclustering the three isolates *T. pyogenes* 336-1, *T. pyogenes* 336-2 and *T. pyogenes* 336-4 which formed a distinct cluster at the nucleotide sequence level with 100% similarity and bootstrap supports of 97%. The second subgroup (GI.II) subclustering *T. pyogenes* 843-2 and the reference strain *T. pyogenes* DSM 20594. The second group (GII) clustering *T. pyogenes* 506-74 and the reference strain *T. pyogenes* DSM 20630^T. The third group (GIII) was clustering five isolates and within the third subgroup two subclusters were well defined. The first subcluster assembled with the isolate *T. pyogenes* 1512-21 and *T. pyogenes* 59-11. The second subcluster assembled with the isolate *T. pyogenes* 1800-9 and *T. pyogenes* 1065-47 together with *T. pyogenes* 200-1. The fourth group (GIV) assembled the isolate *T. pyogenes* 58-4 together with *T. pyogenes* 1220-112. The fifth group (GV) assembled the isolate *T. pyogenes* 887-77 (Fig. 24A). The average GC content of the concatenated sequences of the four housekeeping genes ranged from 61.2 mol% to 61.5 mol% within the *T. pyogenes* species and 54.8 mol% for the reference strain *A. haemolyticum* DSM 20595^T (Tab. 13). The analysis of intra genomic GC content of the investigated *T. pyogenes* isolates showed a low GC content difference indicating a homogeneity within the *T. pyogenes* species. A pairwise comparison of MLSA among the *T. pyogenes* species revealed a sequence distance between 0% and 0.25% (Tab. 14).

The concatenated tree built with the amino acid sequence with 1,058 sites clustered the investigated isolates and represented them in four groups. The first group (GI) comprised five isolates and is clustering *T. pyogenes* 336-1, *T. pyogenes* 336-2, *T. pyogenes* 336-4, *T. pyogenes* 843-2 and the reference strain *T. pyogenes* DSM 20594. The second group (GII) comprised four isolates containing two subgroup, the subgroup (GII.I) assembled *T.*

pyogenes 1220-112 and *T. pyogenes* 887-77, while the subgroup (GII.II) assembled *T. pyogenes* 1512-21 and *T. pyogenes* 58-4. The third group (GIII) is clustering *T. pyogenes* 59-11, *T. pyogenes* 200-1, *T. pyogenes* 506-74 and *T. pyogenes* 1065-47 and the reference strain *T. pyogenes* DSM 20630^T. The fourth group (GIV) was clustering *T. pyogenes* 1800-9 (Fig. 24B). The percentage similarity of the amino acids composition among *T. pyogenes* and *A. haemolyticum* DSM 20595^T was ranging from 80.4% to 80.7%, while within the species *T. pyogenes* was ranging from 99.4% to 100% (Tab. 15).

The analysis of the amino acid composition of the concatenated FusA-Tuf-MetG-GyrA of the 13 *T. pyogenes* isolates of bovine origin, *T. pyogenes* DSM 20594 and *T. pyogenes* DSM 20630^T revealed 20 different amino acids. The investigated *T. pyogenes* shared identical compositions for six amino acids. These similarities were considered for cysteine (Cys) with 0.85%, glycine (Gly) with 7.57%, arginine (Arg) with 6.24%, serine (Ser) with 4.25%, tryptophan (Trp) with 1.04% and tyrosine (Tyr) with 3.02%. Variations in the amino acids composition were observed for 14 amino acids including, alanine (Ala) between 8.32% and 8.41%, aspartic acid (Asp) between 8.32% and 8.41%, glutamic acid (Glu) between 8.13% and 8.41%, phenylalanine (Phe) between 2.36% and 2.46%, histidine (His) between 1.98% and 2.08%, isoleucine (Ile) between 6.52% and 6.43%, lysine (Lys) between 4.82% and 4.91%, leucine (Leu) between 9.17% and 9.26%, methionine (Met) between 2.27% and 2.36%, asparagine (Asn) between 3.21% and 3.50%, proline (Pro) between 4.54% and 4.63%, glutamine (Gln) between 3.31% and 3.40%, threonine (Thr) between 5.2% and 5.39% and valine (Val) between 8.13% and 8.22%. Reference strain *A. haemolyticum* DSM 20595^T showed the same 20 amino acid content like the species *T. pyogenes,* one amino acids was identical in composition with the species *T. pyogenes* (Trp) and rest 19 amino acid were different (Ala, Cys, Asp, Phe, Gly, His, Ile, Lys, Leu, Met, Asn, Pro, Gln, Arg, Ser, Thr, Glu, Val and Tyr).

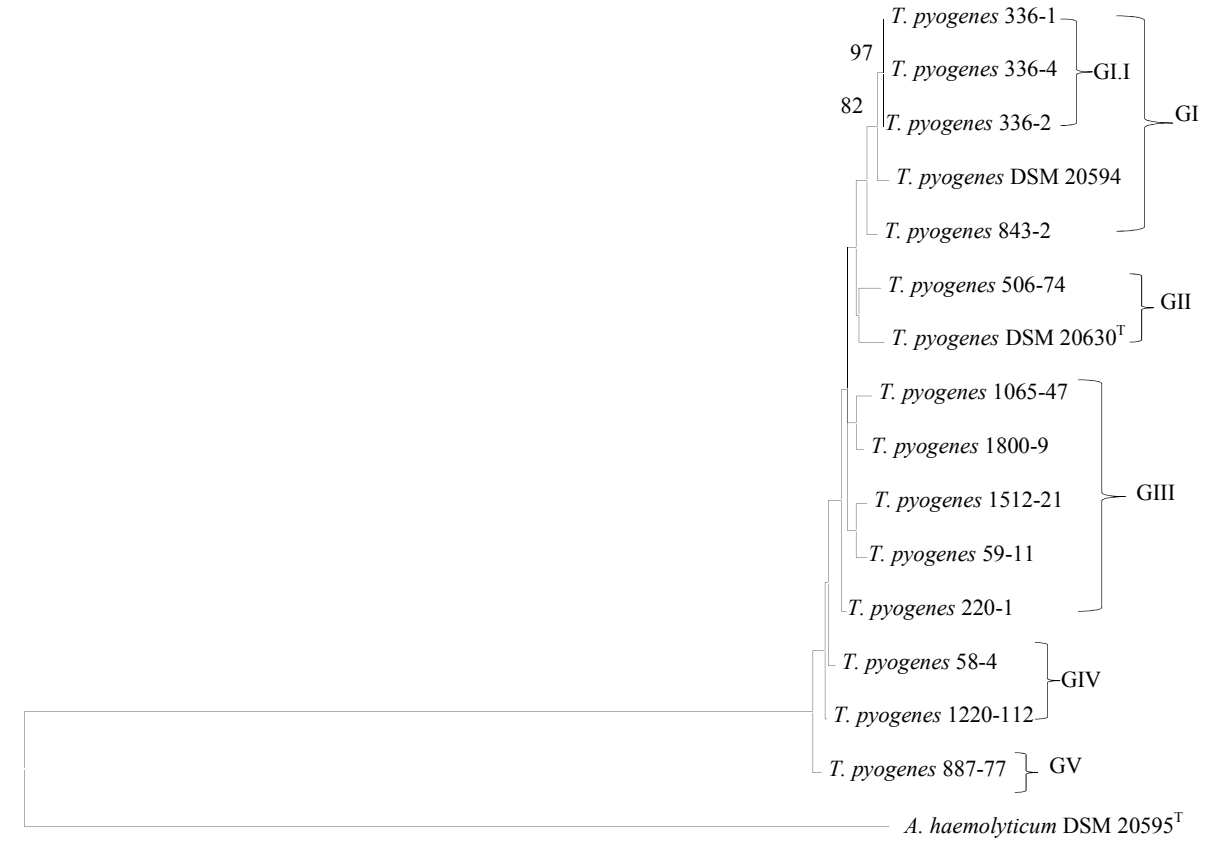

Fig. 24A

Fig. 24B

Fig. 24: Phylogenetic analysis based on concatenated partial *fusA-tuf-metG-gyrA* nucleotide sequences of a total of 3,177 nucleotide positions (A) and FusA-Tuf-MetG-GyrA amino acid sequences of a of total 1058 amino acid positions (B) of the investigated target gene and protein of the 13 *T. pyogenes* isolates of bovine origin, *T. pyogenes* DSM 20594, *T. pyogenes* DSM 20630^T and *A. haemolyticum* DSM 20595^T.

Tab. 13: The average percent of GC (guanine-cytosine) content of the concatenated sequences for the 4 locus sequences of the 13 *T. pyogenes* isolates of bovine origin, *T. pyogenes* DSM 20630^T, *T. pyogenes* DSM 20594, and *A. haemolyticum* DSM 20595^T.

	Isolates or strains	G mol%	C mol%	G+C mol%
1	*T. pyogenes* 336/1	29.8	31.4	61.2
2	*T. pyogenes* 336/2	29.8	31.4	61.2
3	*T. pyogenes* 336/4	29.8	31.4	61.2
4	*T. pyogenes* 1512/21	29.9	31.6	61.5
5	*T. pyogenes* 59/11	29.8	31.5	61.3
6	*T. pyogenes* 58/4	29.8	31.5	61.3
7	*T. pyogenes* 220/1	29.9	31.5	61.4
8	*T. pyogenes* 1065/47	29.8	31.6	61.4
9	*T. pyogenes* 1800/9	29.8	31.6	61.4
10	*T. pyogenes* 843/2	29.8	31.5	61.3
11	*T. pyogenes* 887/77	29.7	31.5	61.2
12	*T. pyogenes* 506/74	29.9	31.5	61.4
13	*T. pyogenes* 1220/112	29.8	31.5	61.3
14	*T. pyogenes* DSM 20630^T	29.9	31.4	61.3
15	*T. pyogenes* DSM 20594	29.8	31.4	61.2
16	*A. haemolyticum* DSM 20595^T	28	26.8	54.8

Tab. 14: Average pairwise distances calculated using Kimura two-parameter model of the concatenated sequences for the 4 locus sequences of the 13 *T. pyogenes* isolates of bovine origin, *T. pyogenes* DSM 20630^T, *T. pyogenes* DSM 20594, and *A. haemolyticum* DSM 20595^T.

	Isolates	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	*T. pyogenes* 336/1																
2	*T. pyogenes* 336/2	0.000															
3	*T. pyogenes* 336/4	0.000	0.000														
4	*T. pyogenes* 1512/21	0.006	0.006	0.006													
5	*T. pyogenes* 59/11	0.008	0.008	0.008	0.004												
6	*T. pyogenes* 58/4	0.008	0.008	0.008	0.004	0.008											
7	*T. pyogenes* 220/1	0.007	0.007	0.007	0.005	0.006	0.005										
8	*T. pyogenes* 1065/47	0.009	0.009	0.009	0.005	0.005	0.008	0.006									
9	*T. pyogenes* 1800/9	0.008	0.008	0.008	0.007	0.006	0.007	0.004	0.004								
10	*T. pyogenes* 843/2	0.005	0.005	0.005	0.006	0.009	0.008	0.007	0.008	0.008							
11	*T. pyogenes* 887/77	0.009	0.009	0.009	0.008	0.012	0.006	0.008	0.011	0.010	0.004						
12	*T. pyogenes* 506/74	0.008	0.008	0.008	0.009	0.009	0.010	0.008	0.009	0.007	0.008	0.009					
13	*T. pyogenes* 1220/112	0.007	0.007	0.007	0.005	0.008	0.002	0.004	0.007	0.006	0.005	0.004	0.010				
14	*T. pyogenes* DSM 20630^T	0.009	0.009	0.009	0.010	0.008	0.011	0.008	0.010	0.009	0.008	0.010	0.009	0.010			
15	*T. pyogenes* DSM 20594	0.003	0.003	0.003	0.008	0.011	0.009	0.009	0.011	0.011	0.005	0.009	0.008	0.009	0.010		
16	*A. haemolyticum* DSM 20595^T	0.250	0.250	0.250	0.248	0.249	0.247	0.248	0.250	0.250	0.250	0.247	0.249	0.247	0.250	0.249	

Tab. 15: Percentage of amino acids composition similarity between the proteins of the investigated genes of the 13 *T. pyogenes* isolates of bovine origin, *T. pyogenes* DSM 20630^{T}, *T. pyogenes* DSM 20594 and *A. haemolyticum* DSM 20595^{T}.

	Isolates	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	*T. pyogenes* 336/1																
2	*T. pyogenes* 336/2	100															
3	*T. pyogenes* 336/4	100	100														
4	*T. pyogenes* 1512/21	99.9	99.9	99.9													
5	*T. pyogenes* 59/11	99.8	99.8	99.8	99.9												
6	*T. pyogenes* 58/4	99.8	99.8	99.8	99.9	99.8											
7	*T. pyogenes* 220/1	99.7	99.7	99.7	99.8	99.9	99.7										
8	*T. pyogenes* 1065/47	99.7	99.7	99.7	99.8	99.9	99.7	99.8									
9	*T. pyogenes* 1800/9	99.6	99.6	99.6	99.7	99.8	99.6	99.7	99.8								
10	*T. pyogenes* 843/2	99.9	99.9	99.9	99.8	99.7	99.7	99.6	99.6	99.5							
11	*T. pyogenes* 887/77	99.7	99.7	99.7	99.8	99.7	99.7	99.6	99.6	99.5	99.8						
12	*T. pyogenes* 506/74	99.7	99.7	99.7	99.8	99.9	99.7	99.8	99.8	99.7	99.6	99.6					
13	*T. pyogenes* 1220/112	99.8	99.8	99.8	99.9	99.8	99.8	99.7	99.7	99.6	99.9	99.9	99.7				
14	*T. pyogenes* DSM 20630^{T}	99.4	99.4	99.4	99.5	99.6	99.4	99.5	99.5	99.4	99.3	99.3	99.5	99.4			
15	*T. pyogenes* DSM 20594	100.0	100.0	100.0	99.9	99.8	99.8	99.7	99.7	99.6	99.9	99.7	99.7	99.8	99.4		
16	*A. haemolyticum* DSM 20595^{T}	80.6	80.6	80.6	80.6	80.7	80.5	80.6	80.7	80.8	80.5	80.4	80.6	80.5	80.6	80.6	

Tab. 16: Content (mol%) of the amino acids composition in the concatenated protein sequences derived from partially sequenced *fusA, tuf, metG* and *gyrA* genes of the 13 *T. pyogenes* isolates of bovine origin, *T. pyogenes* DSM 20630^T, *T. pyogenes* DSM 20594 and *A. haemolyticum* DSM 20595^T.

	Isolates	Ala	Cys	Asp	Glu	Phe	Gly	His	Ile	Lys	Leu	Met	Asn	Pro	Gln	Arg	Ser	Thr	Val	Trp	Tyr
1	*T. pyogenes* 336/1	8.41	0.85	8.32	8.32	2.36	7.56	1.98	6.52	4.82	9.17	2.27	3.50	4.63	3.31	6.24	4.25	5.29	8.13	1.04	3.02
2	*T. pyogenes* 336/2	8.41	0.85	8.32	8.32	2.36	7.56	1.98	6.52	4.82	9.17	2.27	3.50	4.63	3.31	6.24	4.25	5.29	8.13	1.04	3.02
3	*T. pyogenes* 336/4	8.41	0.85	8.32	8.32	2.36	7.56	1.98	6.52	4.82	9.17	2.27	3.50	4.63	3.31	6.24	4.25	5.29	8.13	1.04	3.02
4	*T. pyogenes* 1512/21	8.41	0.85	8.32	8.32	2.36	7.56	1.98	6.43	4.82	9.17	2.36	3.50	4.63	3.31	6.24	4.25	5.29	8.13	1.04	3.02
5	*T. pyogenes* 59/11	8.41	0.85	8.41	8.32	2.36	7.56	1.98	6.43	4.82	9.17	2.36	3.40	4.63	3.31	6.24	4.25	5.29	8.13	1.04	3.02
6	*T. pyogenes* 58/4	8.41	0.85	8.32	8.32	2.46	7.56	1.98	6.43	4.82	9.17	2.36	3.50	4.63	3.31	6.24	4.25	5.20	8.13	1.04	3.02
7	*T. pyogenes* 220/1	8.41	0.85	8.41	8.22	2.36	7.56	1.98	6.43	4.82	9.17	2.36	3.40	4.63	3.31	6.33	4.25	5.29	8.13	1.04	3.02
8	*T. pyogenes* 1065/47	8.41	0.85	8.32	8.32	2.36	7.56	2.08	6.43	4.82	9.17	2.36	3.40	4.63	3.31	6.24	4.25	5.29	8.13	1.04	3.02
9	*T. pyogenes* 1800/9	8.41	0.85	8.32	8.22	2.36	7.56	1.98	6.43	4.82	9.17	2.36	3.40	4.63	3.40	6.24	4.25	5.29	8.22	1.04	3.02
10	*T. pyogenes* 843/2	8.41	0.85	8.41	8.22	2.36	7.56	1.98	6.52	4.82	9.17	2.27	3.50	4.63	3.31	6.24	4.25	5.29	8.13	1.04	3.02
11	*T. pyogenes* 887/77	8.41	0.85	8.41	8.13	2.36	7.56	1.98	6.43	4.91	9.17	2.36	3.50	4.63	3.31	6.24	4.25	5.29	8.13	1.04	3.02
12	*T. pyogenes* 506/74	8.32	0.85	8.41	8.32	2.36	7.56	1.98	6.43	4.82	9.17	2.36	3.40	4.63	3.31	6.24	4.25	5.39	8.13	1.04	3.02
13	*T. pyogenes* 1220/112	8.41	0.85	8.41	8.22	2.36	7.56	1.98	6.43	4.82	9.17	2.36	3.50	4.63	3.31	6.24	4.25	5.29	8.13	1.04	3.02
14	*T. pyogenes* DSM 20630^T	8.41	0.85	8.32	8.41	2.36	7.56	1.98	6.43	4.82	9.26	2.36	3.21	4.54	3.31	6.24	4.35	5.39	8.13	1.04	3.02
15	*T. pyogenes* DSM 20594	8.41	0.85	8.32	8.32	2.36	7.56	1.98	6.52	4.82	9.17	2.27	3.50	4.63	3.31	6.24	4.25	5.29	8.13	1.04	3.02
16	*A. haemolyticum* DSM 20595^T	8.70	0.66	9.36	8.32	2.65	7.47	2.17	5.77	5.01	9.45	2.65	2.74	4.44	3.12	6.43	3.78	5.29	8.32	1.04	2.65

alanine (Ala), cysteine (Cys), aspartic Acid (Asp), glutamic Acid (Glu), phenylalanine (Phe), glycine (Gly), histidine (His), isoleucine (Ile), lysine (Lys), leucine (Leu), methionine (Met), asparagine (Asn), proline (Pro), glutamine (Gln), arginine (Arg), serine (Ser), threonine (Thr), valine (Val), tryptophan (Trp), tyrosine (Tyr).

4.1.2 Phenotypic and genotypic properties of the gelatinase negative *T. pyogenes* 754B isolated from bovine mastitis

T. pyogenes 754B investigated in the present study was identified phenotypically by determination of haemolysis and CAMP-like haemolytic reactions, by using the API Coryne test system and various other phenotypical tests. *T. pyogenes* 754B showed hemolytic properties and CAMP-like hemolytic properties typical for the species *T. pyogenes*. As shown in Tab. 17 no enhanced hemolysis of *T. pyogenes* 754B could be recorded after cultivation on rabbit blood agar compared to sheep blood agar. *T. pyogenes* 754B showed a positive reaction for the enzymes β-D-glucuronidase, β-galactosidase, α-D-glucosidase, N-acetyl-β-D-glucosaminidase, amylase, pyrrolidonyl arylamidase, alkaline phosphatase, degradation of D-glucose, D-ribose, D-maltose and D-lactose D-saccharose and glycogen. The isolate also displayed a cross reactions with streptococcal serogroup G specific antiserum. Negative reactions were observed for the enzyme activites of α-galactosidase, β-glucosidase, α-mannosidase and catalase. Negative reactions were observed for nitrate reduction, pyrazinamidase, esculin hydrolysis, urease, gelatinase, catalase and degradation of D-mannitol (3.4.2). However, it was of interest that *T. pyogenes* 754B was negative in gelatinase enzyme activity. This could be determined with three independent test systems using nutrient gelatin stab method (3.4.7) and nutrient gelatin plate method (3.4.7) and API Coryne test system (3.4.2). Moreover, *T. pyogenes* 754B showed no caseinase enzyme activity (3.4.6) and did not show any serolysis of Loeffler medium (3.4.5) (Tab. 17). The MALDI-TOF MS identified the isolate 754B to the species level of *T. pyogenes* matching against reference library entries with log (score) values of 2.36 with reference strain *T. pyogenes* DSM 20594 and 2.07 with *T. pyogenes* DSM 20630^T (Fig. 25). Furthermore, isolate 754B was genotypically identified as *T. pyogenes* by sequencing the 16S rRNA gene (3.7.7) and the gene *gap* (3.7.8). The oligonucleotide primers 16SUNI-L and 16SUNI-R and the oligonucleotide primers 16S rDNA-533F and 16S rDNA-907R were used for amplification and sequencing of 5'- end the 16S rRNA gene respectively with an approximate size of 1,400 nt (1,403 nt expected size) (3.7.7). The nucleotide sequences were deposited to the GenBank and the accession number of the 16S rRNA gene of *T. pyogenes* 754B was HF947290. Sequencing the 16S rRNA gene of *T. pyogenes* 754B yielded a complete sequence identity with 16S rRNA gene of reference strain *T. pyogenes* DSM 20630^T with a sequence homology

of 100%. Comparing the sequenced fragment of the 16S rRNA gene of *T. pyogenes* 754B to the sequence of the 16S rRNA gene of other four species of genera *Trueperella* revealed a sequence homology between 91.8% and 98.7%. A dendrogram analysis of the 16S rRNA gene sequences of *T. pyogenes* 754B together with five reference strains of genera *Trueperella* is shown in Fig. 26.

The oligonucleotide primers Gap-F and Gap-R were used for partial amplification of gene *gap* of *T. pyogenes* 754B with an expected size of 830 bp. The nucleotide sequence data was deposited to the GenBank and the accession number of gene *gap* of *T. pyogenes* 754B is HF947289. Sequencing of gene *gap* of *T. pyogenes* 754B yielded a complete sequence identity with a sequence homology of 100% with reference strain *T. pyogenes* DSM 20594 (HF930768) and 99.4% with *T. pyogenes* DSM 20630^T. Comparing the sequence homology of gene *gap* of *T. pyogenes* 754B to the sequence of gene *gap* of three species of genera *Trueperella* revealed a sequence homology of 81.1% with *T. bernardiae* DSM 9152^T, 77.3% with *T. bonasi* DSM 17163^T and 88.6% with *T. abortisuis* DSM 19515^T. A dendrogram analysis of gene *gap* of *T. pyogenes* 754B isolate together with five reference strains of genera *Trueperella* is shown in Fig. 27.

T. pyogenes 754B was further identified by amplification of species specific regions of *T. pyogenes* ISR and *T. pyogenes sodA*. Amplification of the known and putative virulence factor encoding genes revealed that *T. pyogenes* 754B carried gene *plo*, *fimA*, *fimC*, *fimE* tetracycline resistance encoding gene *tet*(*W)* but not gene *cbpA*, the neuraminidases encoding gene *nanH* and the neuraminidases encoding gene *nanP* (Tab. 17). The *T. pyogenes* 754B was identical to group G as determined among the mastitis isolates depicted in Tab. 10.

A summary of the genotypic properties of *T. pyogenes* 754B and other *T. pyogenes* isolates investigated in the present study is presented in Tab. 27.

Tab. 17: Phenotypical and genotypical properties of *Trueperella pyogenes* 754B of bovine origin and two *T. pyogenes* reference strains.

Phenotypical properties	*T. pyogenes* 754B	*T. pyogenes* DSM 20630^{T}*	*T. pyogenes* DSM 20594*
Hemolysis on sheep blood agar	+	+	+
Hemolysis on rabbit blood agar	+	+	+
CAMP-like reaction with:			
Staphylococcus aureus β-hemolysin	+	+	+
Streptococcus agalactiae	-	-	-
Rhodococcus equi	+	+	+
Reverse CAMP reaction	-	-	-
Nitrate reduction[1]	-	-	-
Pyrazinamidase[1]	-	-	-
Pyrrolidonyl arylamidase[1]	+	+	+
Alkaline phosphatase[1]	+	-	-
β-Glucuronidase (β-GUR)[1,2,3]	+	+	+
α-Galactosidase (α-GAL)[2]	-	-	-
β-Galactosidase (β-GAL)[1,3]	+	+	+
α-Glucosidase (α-GLU)[1,2,3]	+	+	+
β-Glucosidase (β-GLU)[2]	-	-	-
N-acetyl- β-Glucosaminidase (β-NAG)[1,3]	+	+	+
Esculin (β-Glucosidase)[1]	-	-	-
Urease[1]	-	-	-
Gelatinase[1,4]	-	+	+
Fermentation of:			
D-Glucose[1]	+	+	+
D-Ribose[1]	+	+	+
D-Xylose[1]	+	+	+
D-Mannitol[1]	-	-	-
D-Maltose[1]	+	+	+
D-Lactose[1]	+	+	+
D-Saccharose[1]	(+)	+	+
Glycogen[1]	+	+	-
α-Mannosidase[2]	-	-	-
Catalase	-	-	-
Serolysis on Loeffler agar	-	+	+
Caseinase	-	+	+
DNase	+	+	+
Starch hydrolysis (amylase)	+	+	-
Cross reaction with streptococcal serogroup G specific antiserum	+	+	+
Genotypical properties			
T. pyogenes 16S rRNA sequence	+	+	+
T. pyogenes gene *gap* sequence	+	+	+
T. pyogenes specific part of ISR	+	+	+
T. pyogenes specific part of gene *sodA*	+	+	+

Continued on the next page

Tab. 17 (Continued)

Genes encoding virulence factors	*T. pyogenes* 754B	*T. pyogenes* DSM 20630^T*	*T. pyogenes* DSM 20594*
Pyolysin encoding gene *plo*	+	+	+
Collagen-binding protein encoding gene *cbpA*	-	+	-
Neuraminidase H encoding gene *nanH*	-	+	+
Neuraminidase P encoding gene *nanP*	-	+	+
Fimbriae endoding gene *fimA*	+	-	+
Fimbriae endoding gene *fimC*	+	+	+
Fimbriae endoding gene *fimE*	+	+	+
Tetracycline resistance encoding gene *tet*(*W*)	+	+**	-**

*= results obtained from Hijazin et al. (2011); **= results obtained from Billington et al., (2002); +, positive reaction; (+) weak positive reaction -, negative reaction. 1 = Api-Coryne test system (Biomerieux, Nürtingen, Germany); 2 = tablets containing substrates (Rosco Diagnostica A/S, Taastrup, Denmark); 3 = 4-methylumbelliferyl conjugated substrates (Sigma, Steinheim, Germany); 4 = nutrient gelatin stab method and nutrient gelatin plate method

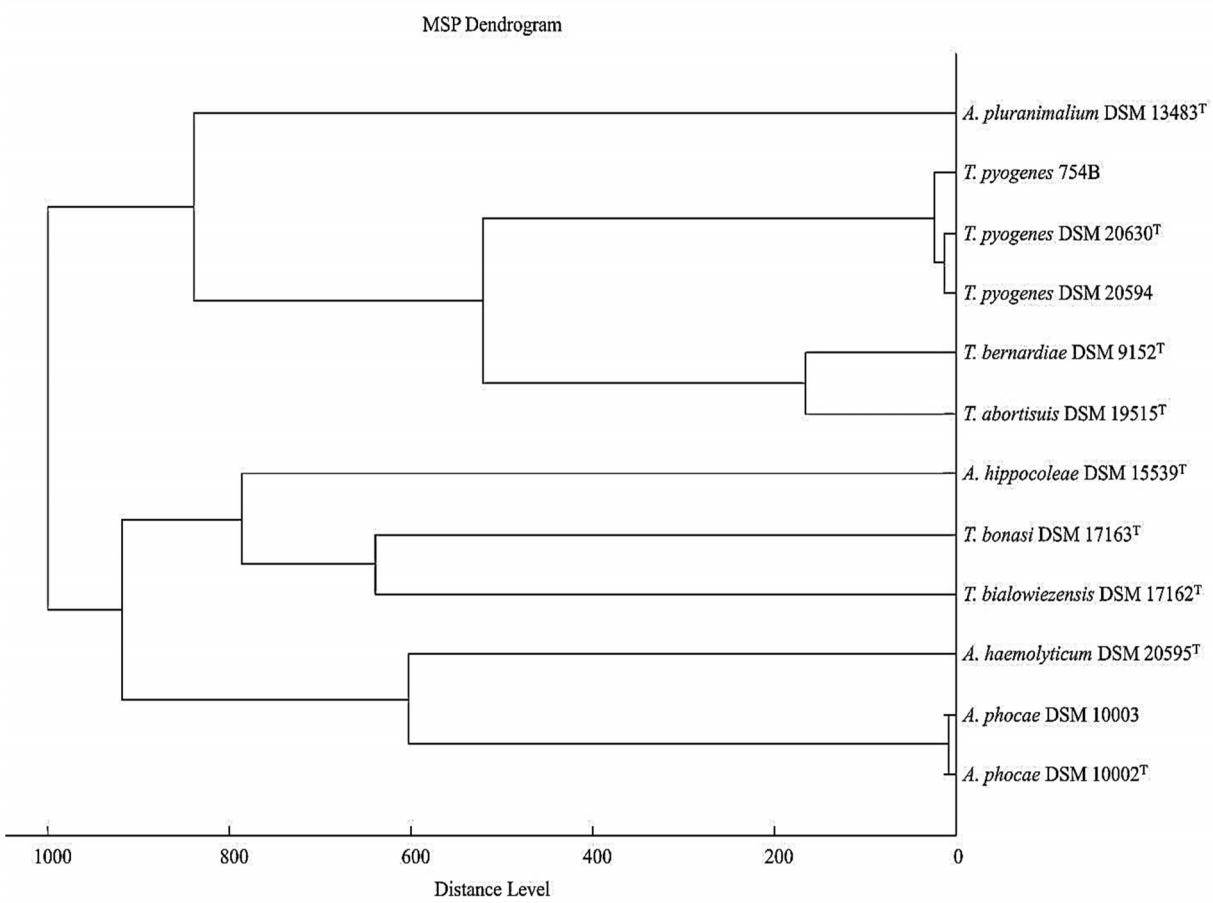

Fig. 25: A score oriented dendrogram of MALDI-TOF main spectra profile of *T. pyogenes* 754B of the present study with eleven reference strains of genera *Arcanobacterium* and *Trueperella*, also including two *T. pyogenes* reference strains.

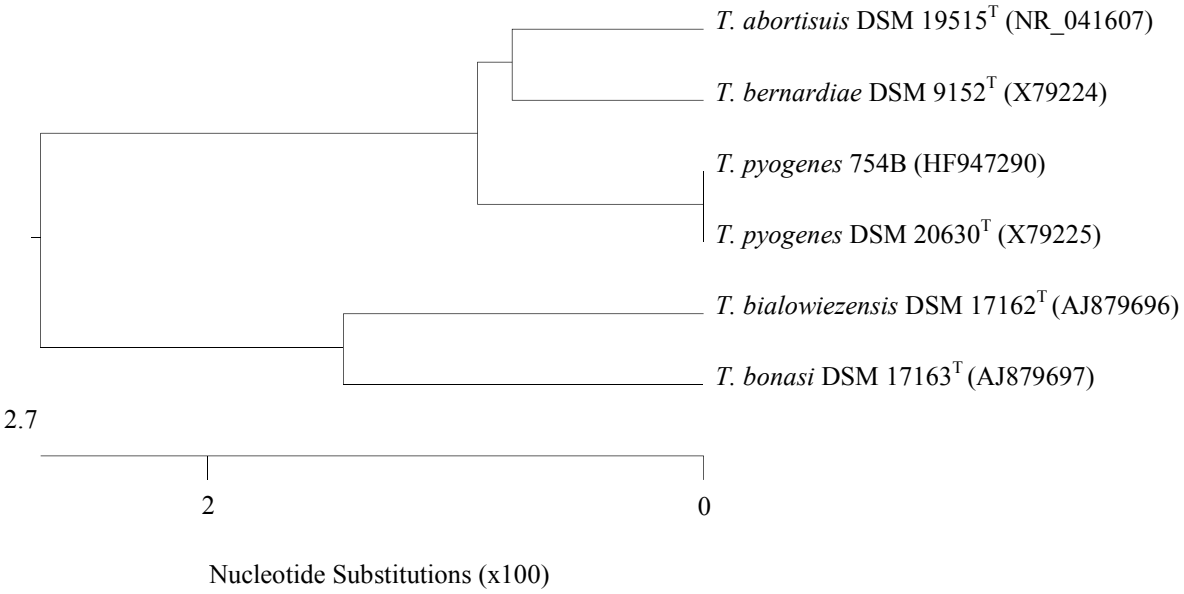

Fig. 26: Phylogenetic analysis based on 16S rRNA gene nucleotide sequences of the investigated *T. pyogenes* 754B with the reference strains *T. pyogenes* DSM 20630^T, *T. abortisuis* DSM 19515^T, *T. bernardiae* DSM 9152^T, *T. bialowiezensis* DSM 17162^T and *T. bonasi* DSM 17163^T.

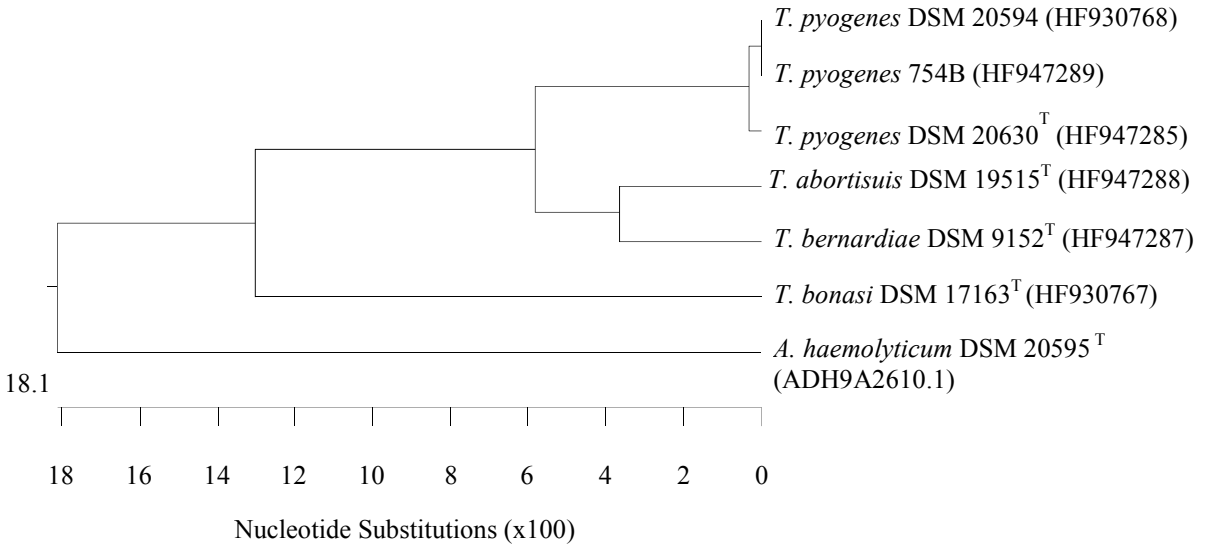

Fig. 27: Phylogenetic analysis based on the glyceraldehyde-3-phosphate dehydrogenase encoding gene *gap* nucleotide sequences of the investigated *T. pyogenes* 754B with the reference strains *T. pyogenes* DSM 20594, *T. pyogenes* DSM 20630^T, *T. abortisuis* DSM 19515^T, *T. bernardiae* DSM 9152^T, *T. bonasi* DSM 17163^T and *A. haemolyticum* DSM 20595^T.

4.1.3 Phenotypic and genotypic properties of *T. pyogenes* isolated from bovine genital tract

4.1.3.1 Phenotypic results

The *T. pyogenes* isolates recovered from bovine genital tract and investigated in the present study were tested on blood agar plates for hemolytic properties and CAMP-like hemolytic reactions. The 14 *T. pyogenes* showed a zone of hemolysis after cultivation on sheep blood agar with no enhanced hemolysis on rabbit blood agar compared to sheep blood agar and displayed CAMP-like hemolytic reactions with *R. equi and S. aureus* β-hemolysin as indicator strains with no reverse CAMP-like reaction. The 14 *T. pyogenes* isolates were positive for the enzymes β-D-glucuronidase, α-D-glucosidase, β-galactosidase and N-acetyl-β-D-glucosaminidase. Moreover, positive reactions were recorded for serolysis on Loeffler medium, casein hydrolysis and for the enzyme DNase. The enzymatic extracts of all 14 *T. pyogenes* showed cross reactions with streptococcal serogroup G specific antiserum. The results for the 14 *T. pyogenes* isolates were variable for the enzyme α-galactosidase in which one isolate (7%) showed a weak positive reaction and for amylase enzyme activity in which 4 (29%) isolates showed a positive reaction. The 14 *T. pyogenes* were negative for the enzymes β-D-glucosidase, α-mannosidase and catalase (Tab. 18).

Tab. 18: Phenotypical properties of 14 *T. pyogenes* isolated from bovine genital tract and two *T. pyogenes* reference strains.

Phenotypical properties	*T. pyogenes*			
	from endometritic cows (n=8)	from apparently healthy cows (n=6)	DSM 20630^{T}*	DSM 20594*
Hemolysis on sheep blood agar	+	+	+	+
Hemolysis on rabbit blood agar	+	+	+	+
CAMP-like reaction with:				
Staphylococcus aureus β-hemolysin	+	+	+	+
Streptococcus agalactiae	-	-	-	-
Rhodococcus equi	+	+	+	+
Reverse CAMP reaction	-	-	-	-
β-Glucuronidase (β-GUR)[1,2]	+	+	+	+
α-Galactosidase (α-GAL)[1]	-	-(5),(+)(1)	-	-
β-Galactosidase (β-GAL)[2]	+	+	+	+
α-Glucosidase (α-GLU)[1,2]	+	+	+	+
β-Glucosidase (β-GLU)[1]	-	-	-	-
N-acetyl- β-Glucosaminidase (β-NAG)[2]	+	+	+	+
α-Mannosidase[1]	-	-	-	-
Catalase	-	-	-	-
Serolysis on Loeffler agar	+	+	+	+
Caseinase	+	+	+	+
DNase	+	+	+	+
Starch hydrolysis (amylase)	-(6),+(2)	-(4),+(2)	+	-
Cross reaction with streptococcal serogroup G specific antiserum	+	+	+	+

The reactions are shown as follows: +, positive; (+), weak positive; -, negative; 1 = Tablets containing substrates (3.4.3); 2 = 4-methylumbelliferyl-conjugated substrates (3.4.4). *= results obtained from Hijazin et al., 2011; the numbers of positive and negative reactions are shown in parentheses.

4.1.3.2 Identification by MALDI-TOF MS

The MALDI-TOF MS analysis allowed an identification of all 14 *T. pyogenes* isolates from cervical swabs associated with bovine endometritis (n=8) and from cervical swabs of apparently healthy cows (n=6) to the species level of *T. pyogenes*. The log (score) values of the 14 *T. pyogenes* isolates against the two reference strains *T. pyogenes* DSM 20630^T and *T. pyogenes* DSM 20594 ranged from 1.9 to 2.44. The log (score) values of the 14 *T. pyogenes* isolates against reference strain *T. pyogenes* DSM 20630^T ranged from 1.9 to 2.21 and against reference strain *T. pyogenes* DSM 20594 from 2.13 to 2.44. A comparison of the log (score) values of the 14 isolates among each other revealed the lowest log (score) value of 2.53 of *T. pyogenes* Wi23B with *T. pyogenes* Wi2770 in which both isolates were from endometritic cows and a maximal log (score) value of 2.9 of *T. pyogenes* Wi17 with *T. pyogenes* Wi4 which were isolated from apparently healthy cows. It was not possible to identify a peak present in eight isolates from endometritis having cows which was not present in six isolates from not endometritic cows or vice versa. However, the comparison of raw spectra of the isolates from bovines with endometritis (n=8), from cervical swabs of apparently healthy cows (n=6) and 12 other reference strains of genera *Arcanobacterium* and *Trueperella* revealed high log (score) values indicating very similar mass spectra. A dendrogram analysis of MALDI-TOF main spectra of the 14 *T. pyogenes* isolates from bovines with endometritis (n=8), from cervical swabs of apparently healthy cows (n=6) and 12 reference strains of genera *Arcanobacterium* and *Trueperella* is shown in Fig. 28.

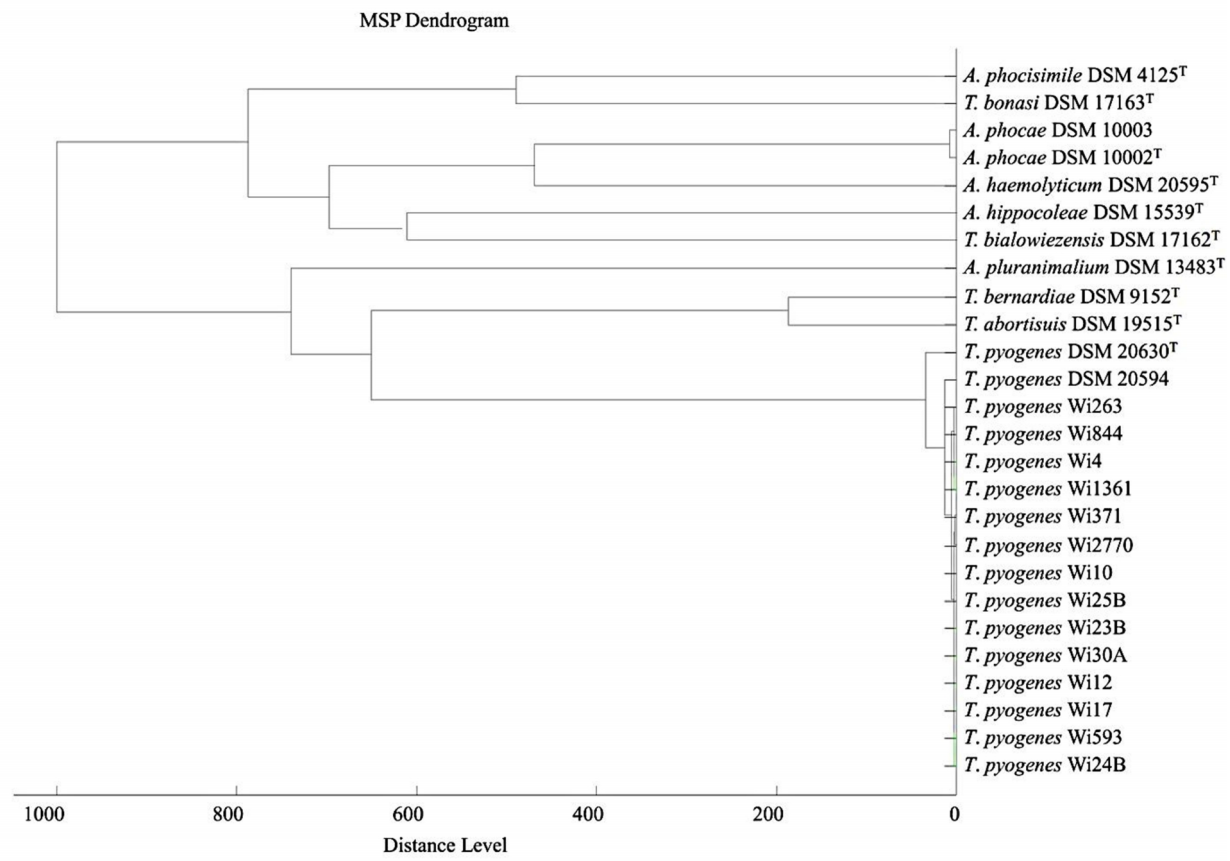

Fig. 28: A score oriented dendrogram of MALDI-TOF main spectra profiles of the 14 *T. pyogenes* isolates of the present study with twelve reference strains of genera *Arcanobacterium* and *Trueperella*, also including the two *T. pyogenes* reference strains.

4.1.3.3 PCR-mediated identification and characterization of *T. pyogenes*

The *T. pyogenes* specific parts of ISR and gene *sodA* were further examined and successfully amplified in all 14 *T. pyogenes*. In addition putative virulence factor encoding genes were screened in the 14 *T. pyogenes* isolates and showed that the 14 *T. pyogenes* isolates carried genes *plo*, *nanH*, *nanP*, *fimA*, *fimC* and *fimE*. However, gene *cbpA* was successfully amplified in only one (7%) *T. pyogenes* isolate. The tetracycline resistance encoding gene *tet*(*W)* was successfully amplified in three (37.5%) *T. pyogenes* isolated from endometritic cows and in all *T. pyogenes* from apparently healthy cows (Tab. 19).

A summary comparing the PCR mediated amplification of the various target genes also including putative virulence factor encoding genes of the 14 *T. pyogenes* isolated from bovine genital tract and the *T. pyogenes* reference strains of the present study is presented in Tab. 27.

Tab. 19: Genotypical properties of the *T. pyogenes* isolated from bovine genital tract and two *T. pyogenes* reference strains.

Genotypical properties	*T. pyogenes*			
	from endometritic cows (n=8)	from apparently healthy cows (n=6)	DSM 20630^{T}*	DSM 20594*
T. pyogenes specific part of ISR	+	+	+	+
T. pyogenes specific part of gene *soda*	+	+	+	+
Pyolysin encoding gene *plo*	+	+	+	+
Collagen-binding protein encoding gene *cbpA*	-(7),+(1)	-	+	-
Neuraminidase H encoding gene *nanH*	+	+	+	+
Neuraminidase P encoding gene *nanP*	+	+	+	+
Fimbriae endoding gene *fimA*	+	+	-	+
Fimbriae endoding gene *fimC*	+	+	+	+
Fimbriae endoding gene *fimE*	+	+	+	+
Tetracycline resistance encoding gene *tet*(*W*)	-(5),+(3)	+	+**	-**

The reactions are shown as follows: +, positive reaction; -, negative reaction; *= results obtained from Hijazin et al. (2011); **= results obtained from Billington et al., (2002) the numbers of positive and negative reactions are shown in parentheses.

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4.1.4 Phenotypic and genotypic properties of *T. pyogenes* isolated from three grey slender lorises (*Loris lydekkerianus nordicus*)

4.1.4.1 Phenotypic results

The three *T. pyogenes* isolates from three grey slender lorises produced after cultivation under microaerobic conditions in a candle jar a zone of hemolysis on sheep blood agar. No enhanced hemolysis of the *T. pyogenes* isolates could be recorded after cultivation on rabbit blood agar compared to sheep blood agar. In addition, the investigated three *T. pyogenes* isolates of the present study displayed CAMP-like hemolytic reactions with *R. equi and S. aureus* β-hemolysin but no reverse CAMP reaction. The phenotypic screening of the three *T. pyogenes* isolates showed a positive reaction for the enzymes β-glucuronidase, β-galactosidase, α-glucosidase, N-acetyl- β-glucosaminidase, pyrrolidonyl arylamidase, gelatinase, degradation of D-glucose, D-ribose, D-maltose and D-lactose and glycogen. Moreover, the investigated *T. pyogenes* isolates showed positive reaction for serolysis on Loeffler agar, casein hydrolysis, enzymes DNase and amylase and a cross reaction with streptococcal serogroup G specific antiserum. However, the three investigated *T. pyogenes* were negative for the enzymes α-galactosidase, β-glucosidase, α-mannosidase, nitrate reduction, pyrazinamidase, esculin hydrolysis, urease, catalase and degradation of D-mannitol (Tab. 20).

Tab. 20: Phenotypical properties of the three *T. pyogenes* isolated from three grey slender lorises and two *T. pyogenes* reference strains.

Phenotypical properties	*T. pyogenes* 121018522	*T. pyogenes* 121008157	*T. pyogenes* 11-07-D 03394	*T. pyogenes* DSM 20630^{T}*	*T. pyogenes* DSM 20594*
Hemolysis on sheep blood agar	+	+	+	+	+
Hemolysis on rabbit blood agar	+	+	+	+	+
CAMP-like reaction with:					
Staphylococcus aureus β-hemolysin	+	+	+	+	+
Streptococcus agalactiae	-	-	-	-	-
Rhodococcus equi	+	+	+	+	+
Reverse CAMP reaction	-	-	-	-	-

Continued on the next page

Tab. 20 (Continued)

Phenotypical properties	*T. pyogenes* 121018522	*T. pyogenes* 121008157	*T. pyogenes* 11-07-D 03394	*T. pyogenes* DSM 20630^{T}*	*T. pyogenes* DSM 20594*
Nitrate reduction[1]	-	-	-	-	-
Pyrazinamidase[1]	-	-	-	-	-
Pyrrolidonyl arylamidase[1]	+	+	+	+	+
Alkaline phosphatase[1]	+	+	+	-	-
β-Glucuronidase (β-GUR)[1,2,3]	+	+	+	+	+
α-Galactosidase (α-GAL)[2]	-	-	-	-	-
β-Galactosidase (β-GAL)[1,3]	+	+	+	+	+
α-Glucosidase (α-GLU)[1,2,3]	+	+	+	+	+
β-Glucosidase (β-GLU)	-	-	-	-	-
N-acetyl- β-Glucosaminidase (β-NAG)[1,3]	+	+	+	+	+
Esculin (β-Glucosidase)[1]	-	-	-	-	-
Urease[1]	-	-	-	-	-
Gelatinase [1,4]	-	-	-	+	+
Fermentation of:					
D-Glucose[1]	+	+	+	+	+
D-Ribose[1]	+	+	+	+	+
D-Xylose[1]	+	+	+	+	+
D-Mannitol[1]	-	-	-	-	-
D-Maltose[1]	+	+	+	+	+
D-Lactose[1]	+	+	+	+	+
Glycogen[1]	+	+	+	+	-
α-Mannosidase[2]	-	-	-	-	-
Catalase	-	-	-	-	-
Serolysis on Loeffler agar	+	+	+	+	+
Caseinase	+	+	+	+	+
DNase	+	+	+	+	+
Starch hydrolysis (amylase)	+	+	+	+	-
Cross reaction with streptococcal serogroup G specific antiserum	+	+	+	+	+

The reactions are shown as follows: +, positive; (+), weak positive; -, negative; 1 = Api-Coryne test system; 2 = tablets containing substrates; 3 = 4-methylumbelliferyl conjugated substrates; 4 = nutrient gelatin stab method and nutrient gelatin plate method; *= results obtained from Hijazin et al., 2011.

4.1.4.2 Identification by MALDI-TOF MS

MALDI-TOF MS produced useful spectra to identify the three *T. pyogenes* isolated from three grey slender lorises to the species level matching against reference library entries and the two *T. pyogenes* reference strains and differentiated them from other reference strains representing nine species of genera *Arcanobacterium* and *Trueperella.*

Matching the log (score) values of the three *T. pyogenes* isolated from three grey slender lorises against the two reference strains *T. pyogenes* DSM 20630^T and *T. pyogenes* DSM 20594 ranged with log (score) values from 2.19 to 2.55. The log (score) values of the investigated three *T. pyogenes* isolates against reference strain *T. pyogenes* DSM 20630^T ranged from 2.19 to 2.21 and against reference strain *T. pyogenes* DSM 20594 from 2.54 to 2.55. A comparison of the log (score) values of the three *T. pyogenes* isolates among each other revealed the lowest log (score) value of 2.53 between *T. pyogenes* 121008157 and *T. pyogenes* 11-07-D 03394 and the maximal log (score) value of 2.59 between *T. pyogenes* 121018522 and *T. pyogenes* 121008157. This hierarchical cluster analysis showed that the three *T. pyogenes* isolates formed a distinct cluster with the two reference strains *T. pyogenes* DSM 20630^T and *T. pyogenes* DSM 20594 and separated the species *T. pyogenes* from other species of genera *Arcanobacterium* and *Trueperella*. A dendrogram analysis of MALDI-TOF main spectra of the three investigated isolates is shown in Fig. 29.

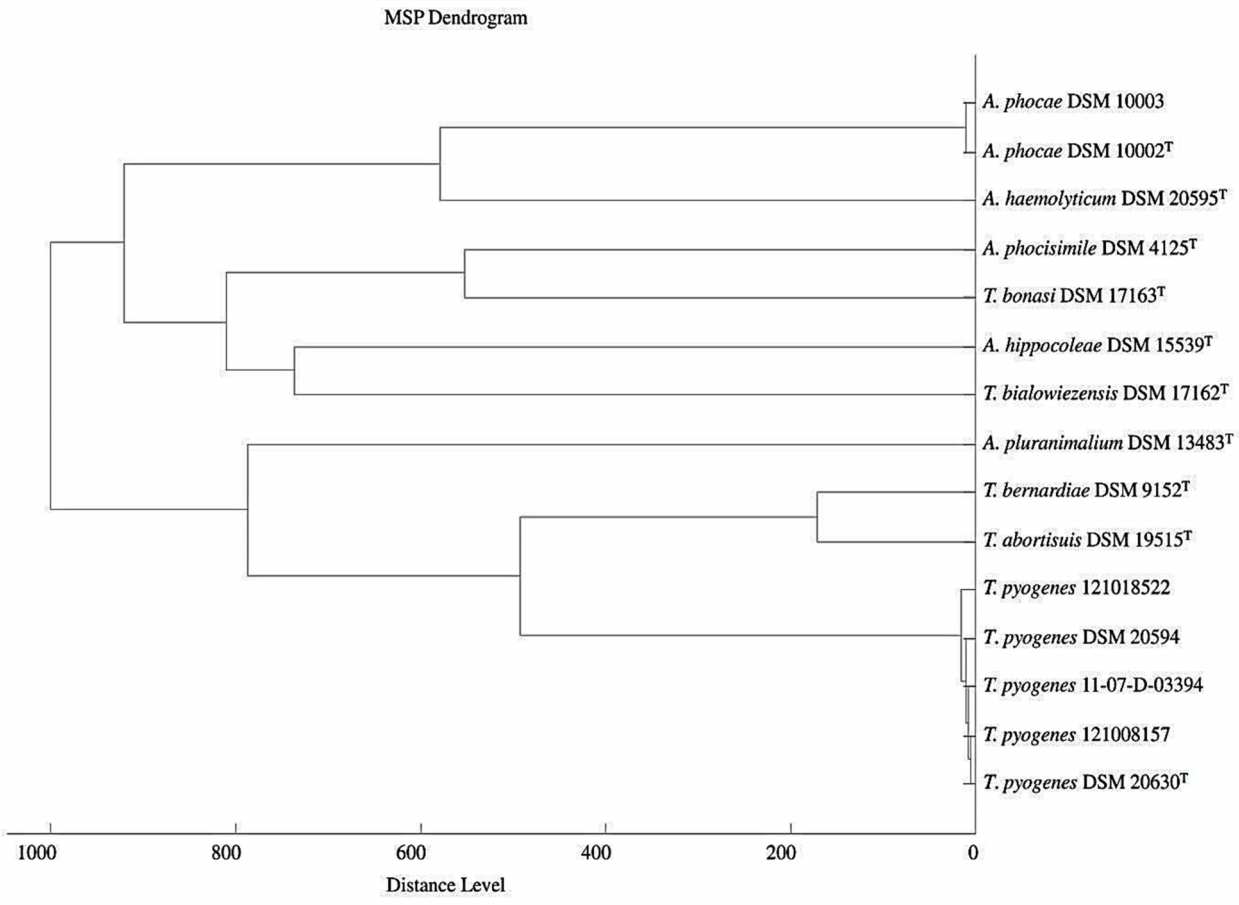

Fig. 29: A score oriented dendrogram of MALDI-TOF main spectra profiles of the three *T. pyogenes* isolated from three grey slender lorises of the present study with twelve reference strains of genera *Arcanobacterium* and *Trueperella*, also including the two *T. pyogenes* reference strains.

4.1.4.3 PCR-mediated identification and characterization

The oligonucleotide primers 16SUNI-L and 16SUNI-R used for amplification of 16S rRNA gene specific parts produced a 1,400 nt amplicon from the three investigated *T. pyogenes* isolates from grey slender lorises. The nucleotide sequence data were deposited in the GenBank and the accession numbers were HG530069, HG530070 and HG530071, respectively. For genotypic differentiation isolates 11-07-D-03394, 121008157 and 121018522 were compared with each other and with the most closely related reference strains *T. abortisuis* DSM 19515^T, *T. bernardiae* DSM 9152^T, *T. bialowiezensis* DSM 17162^T, *T. bonasi* DSM 17163^T and *A. haemolyticum* DSM 20595^T (Fig. 13). Sequencing of the 16S rRNA gene of the three investigated *T. pyogenes* isolates yielded a complete sequence identity with a sequence homology of 100% with 16S rRNA gene of reference strain *T. pyogenes* DSM 20630^T with accession number X79225. Comparing the sequence homology of the 16S rRNA gene of the three investigated *T. pyogenes* isolates from slender loris to the sequence of the 16S rRNA gene of four species of genera *Trueperella* revealed a sequence homology of 98.8% with *T. abortisuis* DSM 19515^T, 97.9% with *T. bernardiae* DSM 9152^T, 95.4% with *T. bialowiezensis* DSM 17162^T, 96% with *T. bonasi* DSM 17163^T.

A dendrogram analysis of the 16S rRNA gene for the three investigated *T. pyogenes* isolates from grey slender loris together with four reference strains of genera *Trueperella* and *A. haemolyticum* DSM 20595 ^{T} is shown in Fig. 30.

Glyceraldehyde-3-phosphate dehydrogenase encoding gene *gap* was used as additional marker. The oligonucleotide primers Gap-F and Gap-R described in 3.7.8 allowed a partial amplification of gene *gap* with size of 830 bp. The nucleotide sequence data were deposited to the GenBank. The GenBank accession numbers of gene *gap* of the three *T. pyogenes* isolates were HG530072, HG530074 and HG530073, respectively. Sequencing of gene *gap* of the three *T. pyogenes* isolates yielded a complete sequence identity with a sequence similarity of 100% with *T. pyogenes* DSM 20594 with accession number HF930768. Comparing the sequence homology of gene *gap* of the three *T. pyogenes* isolates to the sequence of the gene *gap* of six reference strains of genera *Trueperella* and *A. haemolyticum* DSM 20595^T revealed a sequence homology of 99.4% with *T. pyogenes* DSM 20630^T, 81.1% with *T. bernardiae* DSM 9152^T, 77.3% with *T. bonasi* DSM 17163^T, 88.4% with *T. abortisuis* DSM 19515^T and 73.1% with *A. haemolyticum* DSM 20595^T.

A dendrogram analysis of gene *gap* for the investigated *T. pyogenes* isolates and strains of genera *Trueperella* and *A. haemolyticum* DSM 20595 ^{T} is shown in Fig. 31.

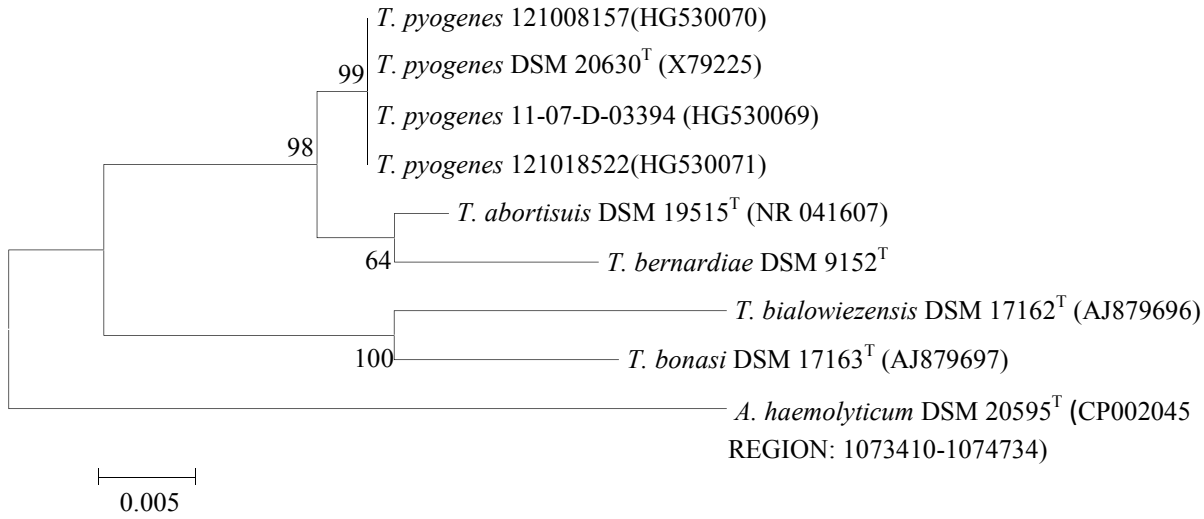

Fig. 30: Phylogenetic analysis based on 16S rRNA gene nucleotide sequences of the 3 investigated *T. pyogenes* isolates from slender loris origin and the reference strains *T. pyogenes* DSM 20630^{T}, *T. abortisuis* DSM 19515^{T}, *T. bernardiae* DSM 9152^{T}, *T. bialowiezensis* DSM 17162^{T}, *T. bonasi* DSM 17163^{T} and *A. haemolyticum* DSM 20595^{T}.

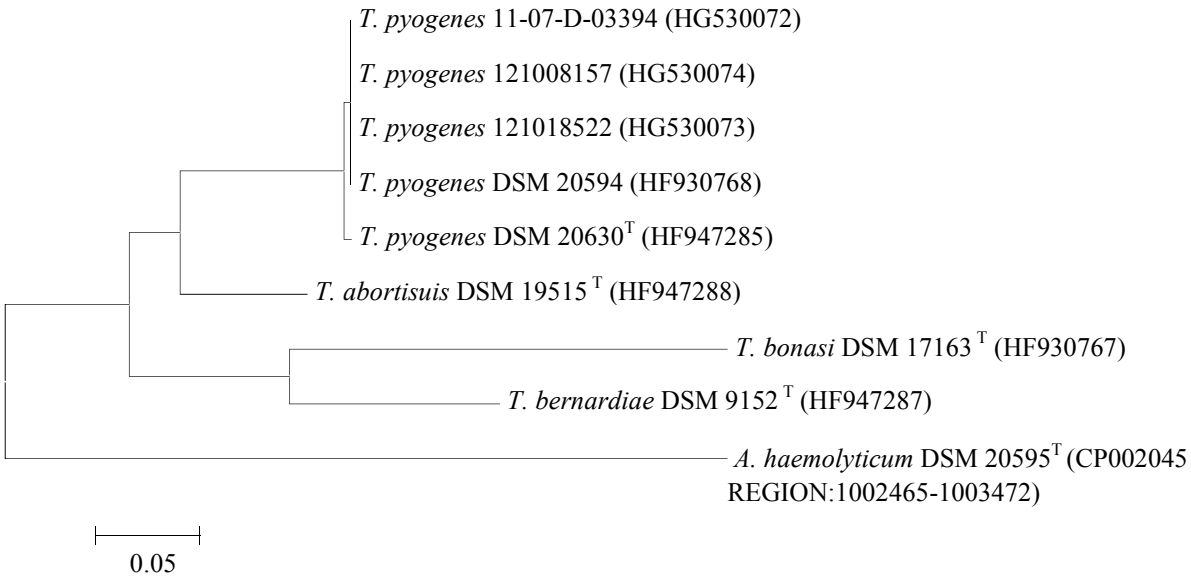

Fig. 31: Phylogenetic analysis based on the glyceraldehyde-3-phosphate dehydrogenase encoding gene *gap* nucleotide sequences of a total of 829 nucleotide positions of the 3 investigated *T. pyogenes* isolates from slender loris origin and the reference strains *T. pyogenes* DSM 20630^{T}, *T. abortisuis* DSM 19515^{T}, *T. bernardiae* DSM 9152^{T}, *T. bialowiezensis* DSM 17162^{T}, *T. bonasi* DSM 17163^{T} and *A. haemolyticum* DSM 20595^{T}.

In addition, the *T. pyogenes* specific part of ISR and gene *sodA* were amplified for all three *T. pyogenes* isolates. The three investigated *T. pyogenes* isolates from slender loris origin were employed for the validation of gene *plo*, gene *nanH*, the genes *fimA*, *fimC*, *fimE* and the tetracycline resistance encoding gene *tet*(*W)*. However, the three investigated *T. pyogenes* isolates from grey slender loris origin were negative for the presence of gene *cbpA* using the oligonucleotide primers cbp-F and cbp-R described in 3.7.10.2 and gene *nanP* using the oligonucleotide primers *nan*P-F and *nan*P-R as described in 3.7.10.3 (Tab. 21).

A summary comparing the PCR mediated amplification of the various target genes and various putative virulence factor encoding genes of the three *T. pyogenes* isolated from grey slender lorises origin and the *T. pyogenes* reference strains of the present study is presented in Tab. 27.

Tab. 21: Genotypical properties of the three *T. pyogenes* isolated from grey slender lorises origin and two *T. pyogenes* reference strains.

Genotypical properties	*T. pyogenes* 121018522	*T. pyogenes* 121008157	*T. pyogenes* 11-07-D 03394	*T. pyogenes* DSM 20630*	*T. pyogenes* DSM 20594*
T. pyogenes 16S rRNA gene sequence	+	+	+	+	+
T. pyogenes gene *gap* sequence	+	+	+	+	+
T. pyogenes specific part of ISR	+	+	+	+	+
T. pyogenes specific part of gene *soda*	+	+	+	+	+
Pyolysin encoding gene *plo*	+	+	+	+	+
Collagen-binding protein encoding gene *cbpA*	-	-	-	+	-
Neuraminidase H encoding gene *nanH*	+	+	+	+	+
Neuraminidase P encoding gene *nanP*	-	-	-	+	+
Fimbriae endoding gene *fimA*	+	+	+	-	+
Fimbriae endoding gene *fimC*	+	+	+	+	+
Fimbriae endoding gene *fimE*	+	+	+	+	+
Tetracycline resistance encoding gene *tet*(*W*)	+	+	+	+**	-**

The reactions are shown as follows: +, positive; -, negative; *= results obtained from Hijazin et al. (2011); **= results obtained from Billington et al., (2002).

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4.1.4.4 Identification and classification of bacteria using DNA fingerprinting methods

4.1.4.4.1 Genomic fingerprinting using (rep)-PCRs

4.1.4.4.1.1 Genomic fingerprinting using ERIC-PCR

The usage of ERIC1R and ERIC2 primers described in 3.8.1.1 under defined conditions led to reproducible profiles of the amplified fragments. All strains were typeable by using ERIC1R and ERIC2 -PCR and the band patterns were variable (polymorphism). The genomic patterns were obtained clearly for all strains. Some variation of band intensity could be observed. Each isolate yielded between five to 17 amplicons ranging in size from 200 bp to 2,000 bp. The analysis was repeated to ensure the reproducibility of the DNA fingerprinting profiles. The number of bands and the corresponding sizes remained constant. The intensity of the stained DNA varied for some strains. The 3 *T. pyogenes* isolates from grey slender loris origin and *T. pyogenes* reference strains *T. pyogenes* DSM 20630^T and *T. pyogenes* DSM 20594 produced 3 different profiles. The dendrogram of the five *T. pyogenes* isolates based on the similarity percentage generated two clusters. Cluster I is a major cluster and can be divided into two sub clusters consisting of three *T. pyogenes* isolates from three grey slender lorises and *T. pyogenes* DSM 20630^T. Cluster II consisted of one strain, comprising the reference strain *T. pyogenes* DSM 20594 (Fig. 32).

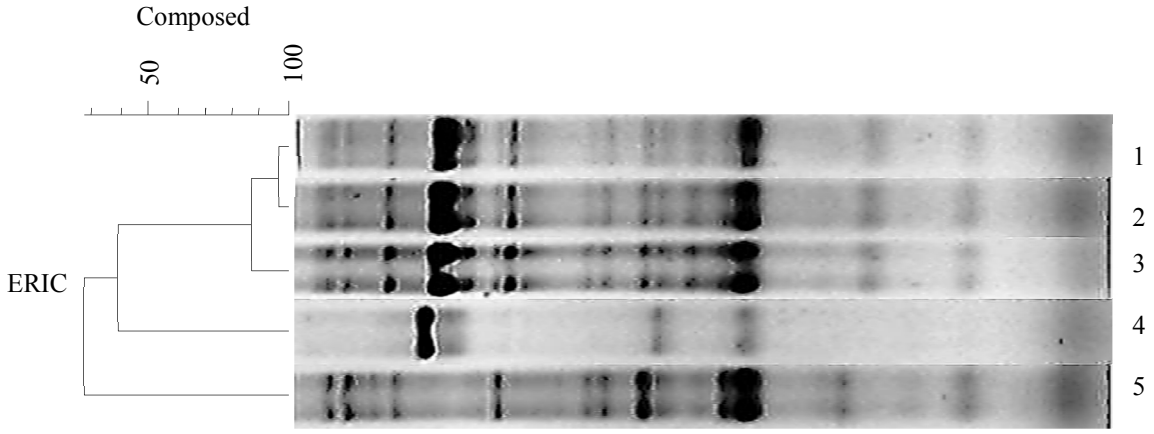


Fig. 32: Genomic fingerprint pattern of the three grey slender lorises isolates in comparison to *T. pyogenes* reference strains with ERIC-PCR. Strains: 1: *T. pyogenes* 11-07-D-03394, 2: *T. pyoge*nes 121008157 3: *T. pyogenes* 121018522, 4: *T. pyogenes* DSM 20630^T and 5: *T. pyogenes* DSM 20594.

4.1.4.4.1.2 Genomic fingerprinting using BOX PCR

The usage of primer BOXA1R under defined conditions led to reproducible profiles of the amplified fragments. All *T. pyogenes* were typeable by using BOXA1R -PCR and the band patterns were variable (polymorphism). The genomic patterns were obtained clearly for all strains. Some variation of band intensity can be observed. Each strain contained between 12 to 14 bands ranging in size from 200 bp to 2,000 bp. The analysis was repeated to ensure the reproducibility of the DNA fingerprinting profiles. The number of bands and the corresponding sizes remained constant. The intensity of the stained DNA varied for some strains. For the three *T. pyogenes* isolated from grey slender lorises and *T. pyogenes* reference strain *T. pyogenes* DSM 20630^{T} and *T. pyogenes* DSM 20594 three profiles were obtained. The dendrogram of the five *T. pyogenes* strains based on the similarity percentage generated two clusters. Cluster I is a major cluster and can be divided into two sub cluster consisting of three *T. pyogenes* isolated from the three grey slender lorises and the *T. pyogenes* DSM 20630^{T}. Cluster II consisted of one strain, the reference strain *T. pyogenes* DSM 20594 (Fig. 33).

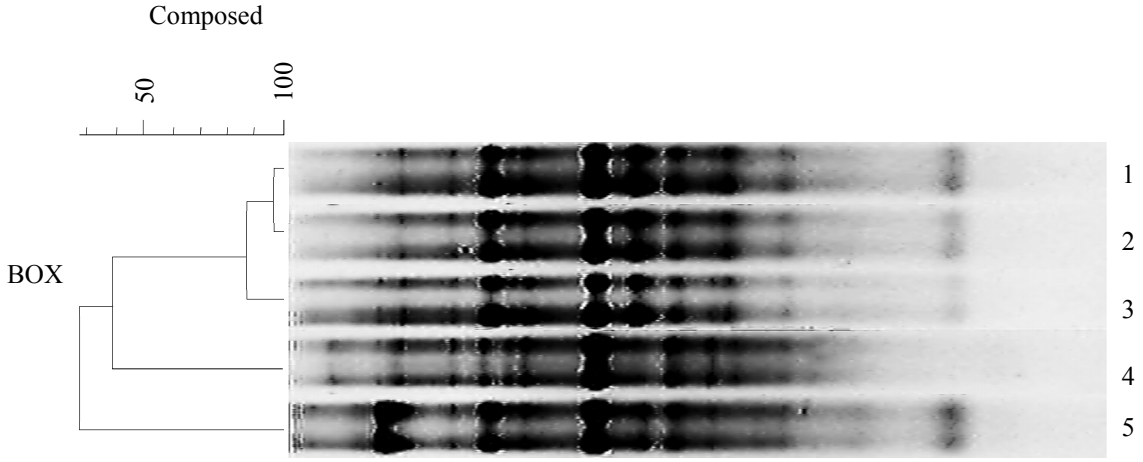


Fig. 33: Genomic fingerprint pattern of the *T. pyogenes* of the three grey slender lorises isolates in comparison to *T. pyogenes* reference strains with BOX-PCR. Strains: 1: *T. pyogenes* 11-07-D-03394, 2: *T. pyoge*nes 121008157 3: *T. pyogenes* 121018522, 4: *T. pyogenes* DSM 20630^{T} and 5: *T. pyogenes* DSM 20594.

4.1.4.4.1.3 Genomic fingerprinting using repetitive bacterial DNA elements $(GTG)_5$-PCR

The usage of the $(GTG)_5$ primers described in 3.8.1.3 under defined conditions lead to reproducible profiles of the amplified fragments. All strains were typeable by using $(GTG)_5$ primer and the band patterns were variable (polymorphism). Each strain contained between 4 to 14 bands ranging in size from 200 bp to 2,000 bp. The analysis was repeated to ensure the reproducibility of the DNA fingerprinting profiles. The number of bands and the corresponding sizes remained constant. The intensity of the stained DNA varied for some strains.

Within the three *T. pyogenes* isolates from grey slender loris and *T. pyogenes* reference strains *T. pyogenes* DSM 20630^T and *T. pyogenes* DSM 20594 three profiles were obtained. The dendrogram of the five *T. pyogenes* strains based on the similarity percentage generated two clusters. Cluster I is a major cluster and can be divided into two sub clusters consisting of three *T. pyogenes* isolated from three grey slender lorises and *T. pyogenes* DSM 20630^T. Cluster II consisted of one strain, the reference strain *T. pyogenes* DSM 20594 (Fig. 34).

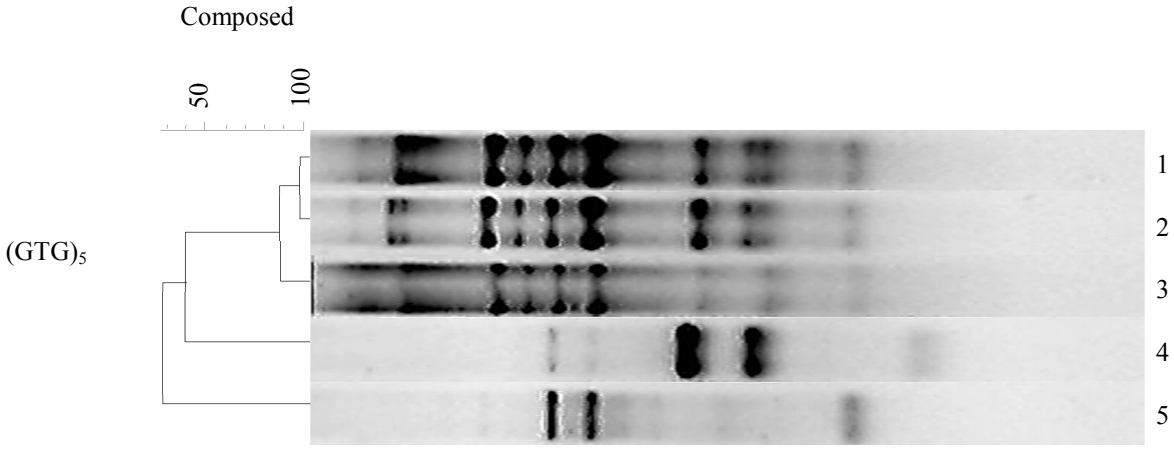


Fig. 34: Genomic fingerprint pattern of *T. pyogenes* of the three grey slender lorises isolates in comparison to *T. pyogenes* reference strains with $(GTG)_5$ primer. Strains: 1: *T. pyogenes* 11-07-D-03394, 2: *T. pyoge*nes 121008157 3: *T. pyogenes* 121018522, 4: *T. pyogenes* DSM 20630^T and 5: *T. pyogenes* DSM 20594.

4.1.4.4.2 Genomic fingerprinting using random amplification polymorphic DNA PCR (RAPD-PCR)

The usage of RAPD primer B described in 3.8.1.4 under defined conditions led to reproducible profiles of the amplified fragments. All strains were typeable by using RAPD

primer B and the band patterns were variable (polymorphism). Each strain contained between 4 to 7 bands ranging in size from 200 bp to 2000 bp. The analysis was repeated to ensure the reproducibility of the DNA fingerprinting profiles. The number of the bands and the corresponding sizes remained constant. The intensity of the stained DNA varied for some strains.

For the three *T. pyogenes* isolates from grey slender loris origin and *T. pyogenes* reference strains *T. pyogenes* DSM 20630^T and *T. pyogenes* DSM 20594 three profiles were obtained. The dendrogram of the five *T. pyogenes* strains based on the similarity percentage generated two cluster. Cluster I is a major cluster and can be divided into two sub clusters consisting of three *T. pyogenes* isolated from three grey slender lorises and *T. pyogenes* DSM 20630^T. Cluster II consisted of one strain, comprising reference strain *T. pyogenes* DSM 20594 (Fig. 35).

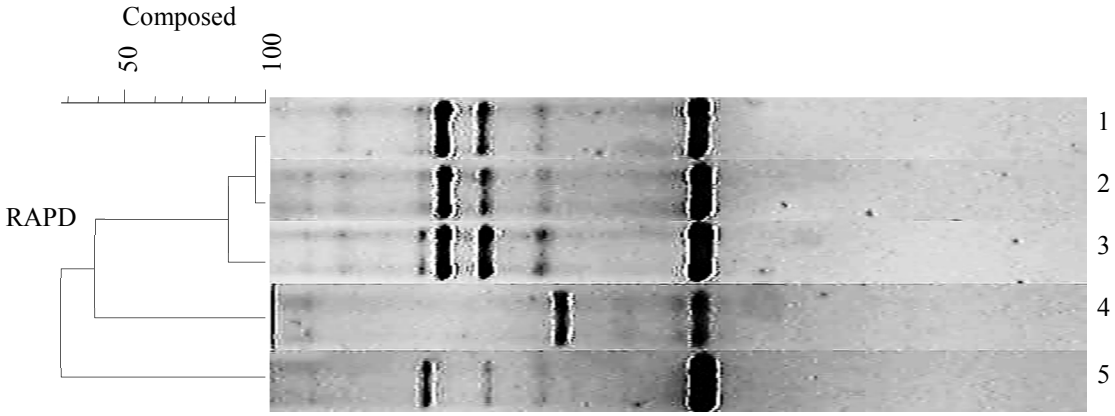


Fig. 35: Genomic fingerprint pattern of the three grey slender lorises isolates in comparison to *T. pyogenes* reference strains with RAPD primer. Strains: 1: *T. pyogenes* 11-07-D-03394, 2: *T. pyoge*nes 121008157 3: *T. pyogenes* 121018522, 4: *T. pyogenes* DSM 20630^T and 5: *T. pyogenes* DSM 20594.

4.1.4.4.2 Genomic fingerprinting using MLSA

For a detailed phylogenetic study four housekeeping protein encoding genes were included in the MLSA. The analysis is based on partial sequences of the housekeeping genes *fusA-tuf-metG-gyrA*. The sequences of the four housekeeping genes of the three *T. pyogenes* isolates of grey slender loris origin and the reference strains *T. pyogenes* DSM 20594, *T. pyogenes* DSM 20630^T and *A. haemolyticum* DSM 20595^T were determined. The targeted amplified DNA fragments were double sequenced on both strands and the sequences were deposited in the GenBank (National Center for Biotechnology Information) (Tab. 22). The phylogenetic

analysis was based on the utilization of the *fusA, tuf, metG* and *gyrA* partial gene sequences. The GenBank accession numbers of locus sequences obtained in this study are provided in Tab. 22.

Tab. 22: GenBank accession numbers of locus sequences of *T. pyogenes* isolates from three grey slender lorises and two *T. pyogenes* reference strains obtained in this study.

	T. pyogenes isolates and strains	*fusA*	*Tuf*	*metG*	*gyrA*
1	121018522	KJ605914	HG941714	HG941711	HG941706
2	121008157	KJ605913	HG941713	HG941710	HG530074
3	11-07-D-03394	KJ605912	HG941712	HG941709	HG941702
4	DSM 20630^T	KJ605911	HG941716	HG941708	HG941704
5	DSM 20594	KJ605910	HG941715	HG941707	HG941703

As described in 3.8.2.1 the oligonucleotide primers fusA-F and fusA-R were used for amplification of translation elongation factor G encoding gene *fusA* of *T. pyogenes*. An 828 bp amplicon was amplified from the three investigated *T. pyogenes* isolates obtained from the grey slender lorises and for the reference strains *T. pyogenes* DSM 20594, *T. pyogenes* DSM 20630^T and *A. haemolyticum* DSM 20595^T. The cluster analysis of the translation elongation factor G encoding gene *fusA* succeeded to subdivide the three investigated *T. pyogenes* strains and the reference strains (*T. pyogenes* DSM 20594, *T. pyogenes* DSM 20630^T and *A. haemolyticum* DSM 20595^T). The phylogenetic tree built with the *fusA* nucleotide sequences of a total of 746 nucleotide positions split the *T. pyogenes* strains in the present study into two groups. The first group (GI) comprised four strains and two subgroups. The first subgroup (GI.I) is clustering *T. pyogenes* 11-07-D-03394, *T. pyogenes* 121008157 and *T. pyogenes* 121018522 with 100% similarity and bootstrap supports of 97%. The second subgroup is clustering the reference strain *T. pyogenes* DSM 20630^T. The second group (GII) clustered the reference strain *T. pyogenes* DSM 20594 (Fig. 36A). The phylogenetic tree built with the FusA amino acid sequences of a total of 248 amino acid positions split the strains in the present study into in two groups. The first group (GI) comprised 3 strains and is clustering *T. pyogenes* 11-07-D-03394, *T. pyogenes* 121008157 and *T. pyogenes* 121018522 with 100% similarity. The second group (GII) is clustering the reference strains *T. pyogenes* DSM 20594 and *T. pyogenes* DSM 20630^T (Fig. 36B). Reference strain *A. haemolyticum* DSM 20595^T was assembled and clustered separately out of the group.

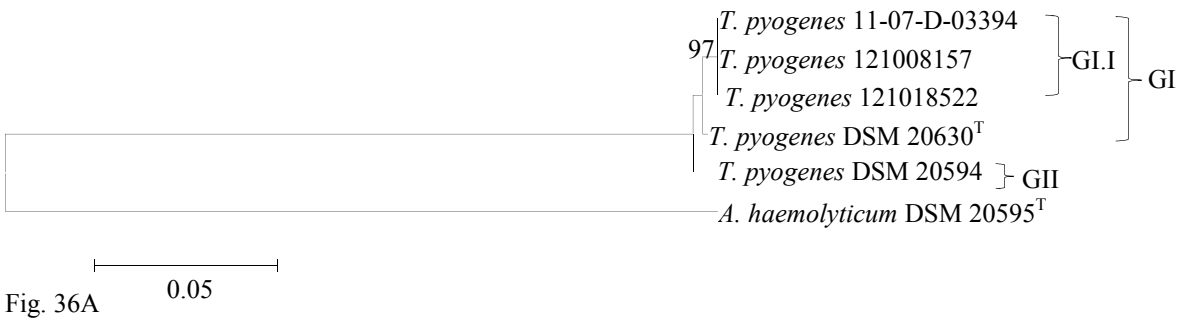


Fig. 36A

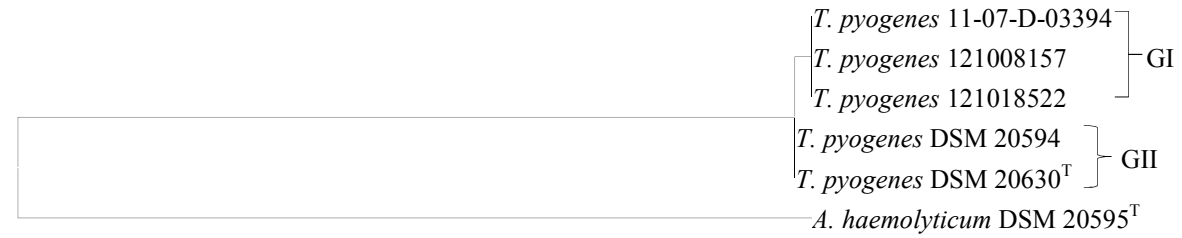


Fig. 36B

Fig. 36: Maximum-likelihood tree based on *fusA* nucleotide sequences of a total of 746 nucleotide positions (29A) and FusA amino acid sequences of a total of 248 amino acid positions (29B) of the investigated target gene and protein of the three *T. pyogenes* isolates of the three grey slender lorises origin, *T. pyogenes* DSM 20594, *T. pyogenes* DSM 20630^{T} and *A. haemolyticum* DSM 20595^{T}.

As described in 3.8.2.2 the oligonucleotide primers *tuf*-F and *tuf*-R were used for amplification of translation elongation factor Tu encoding gene *tuf* of *T. pyogenes*. An 796 bp amplicon was amplified from the investigated three *T. pyogenes* isolates of the grey slender loris origin and for the reference strains *T. pyogenes* DSM 20594, *T. pyogenes* DSM 20630^{T} and *A. haemolyticum* DSM 20595^{T}. The phylogenetic tree built with the *tuf* nucleotide sequences of a total of 795 nucleotide positions split the isolates of the present study into two groups. The first group (G-I) comprised four strains with final bootstrap support of 85% and is clustering two subgroups. The first subgroup (GI.I) is clustering *T. pyogenes* 11-07-D-03394, *T. pyogenes* 121008157 and *T. pyogenes* 121018522 with 100% similarity and bootstrap supports of 95%. The second subgroup is clustering reference strain *T. pyogenes* DSM 20630^{T}. The second group (G-II) comprised reference strain *T. pyogenes* DSM 20594 (Fig. 37A). The phylogenetic tree built with the Tuf amino acid sequences of a total of 265 amino acid positions with 100% similarity failed to split the strains of the present study into groups (Fig. 37B). The reference strain *A. haemolyticum* DSM 20595^{T} was assembled and clustered separately out of the group.

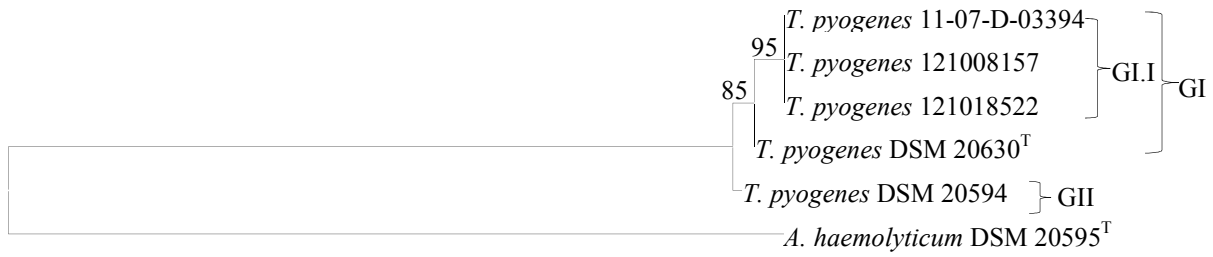


Fig. 37A

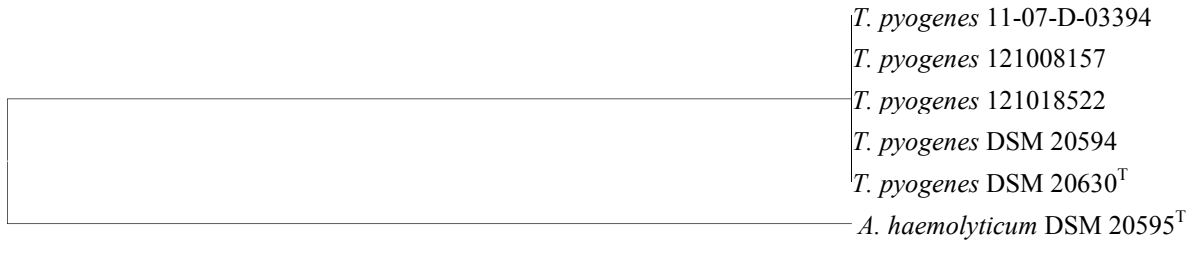
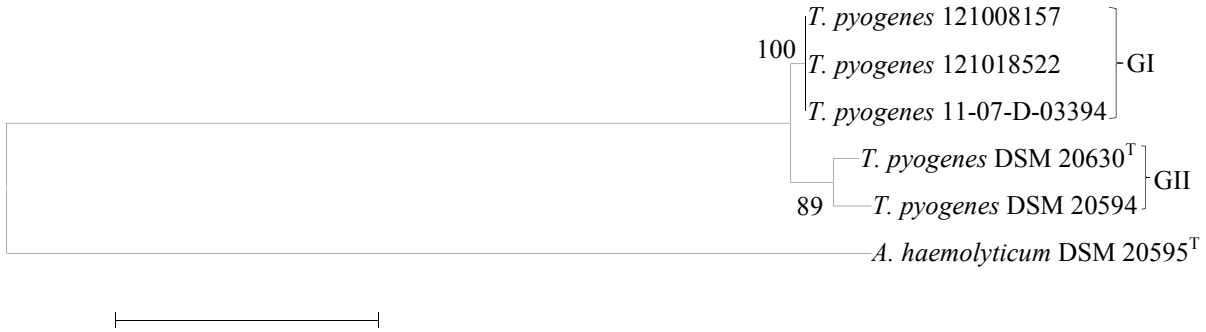


Fig. 37B

Fig. 37: Maximum-likelihood tree based on *tuf* nucleotide sequences of a total of 795 nucleotide positions (A) and Tuf amino acid sequences of a total of 265 amino acid positions (B) of the investigated target gene and protein of the three *T. pyogenes* isolates of the three grey slender lorises origin, *T. pyogenes* DSM 20594, *T. pyogenes* DSM 20630^T and *A. haemolyticum* DSM 20595^T.

As described in 3.8.2.3 the oligonucleotide primers *metG*-F and *metG*-R were used for amplification of methionyl-tRNA synthetase encoding gene *metG* of *T. pyogenes*. An 837 bp amplicon was amplified from the investigated three *T. pyogenes* isolates of the grey slender loris origin and for the reference strains *T. pyogenes* DSM 20594, *T. pyogenes* DSM 20630^T and *A. haemolyticum* DSM 20595^T. The phylogenetic tree built with *metG* nucleotide sequences of a total of 836 nucleotide positions split the strains of the present study into two groups, with final bootstrap supports for each group of 100% and 89%, respectively. The first group (G-I) is clustering *T. pyogenes* 11-07-D-03394, *T. pyogenes* 121008157 and *T. pyogenes* 121018522 with 100% similarity and bootstrap supports of 100%. The second group (G-II) is clustering the reference strains *T. pyogenes* DSM 20630^T and *T. pyogenes* DSM 20594 with final bootstrap support of 89% (Fig. 38A). The phylogenetic tree built with the Tuf amino acid sequences of a total of 278 amino acid positions split the investigated strains in the present study into two groups. The first group (G-I) is clustering *T. pyogenes* 11-07-D-03394, *T. pyogenes* 121008157 and *T. pyogenes* 121018522 with 100% similarity. The second group (G-II) is clustering the reference strains *T. pyogenes* DSM 20630^T and *T.*

pyogenes DSM 20594 (Fig. 38B). The reference strain *A. haemolyticum* DSM 20595^T was assembled and clustered separately out of the group.



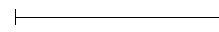
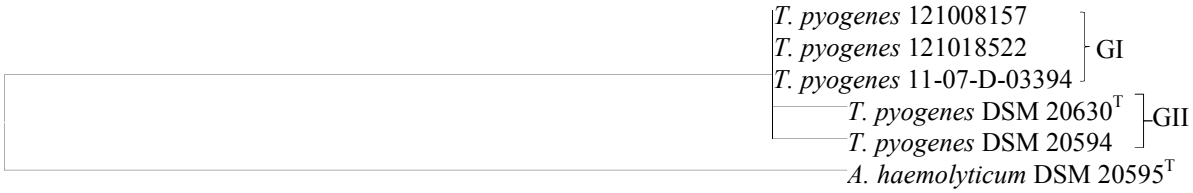


Fig. 38A



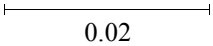


Fig. 38B

Fig. 38: Maximum-likelihood tree based on *metG* nucleotide sequences of a total of 836 nucleotide positions (A) and MetG amino acid sequences of a total of 278 amino acid positions (B) of the investigated target gene and protein of the three *T. pyogenes* isolates of the three grey slender lorises origin, *T. pyogenes* DSM 20594, *T. pyogenes* DSM 20630^T and *A. haemolyticum* DSM 20595^T.

As described in 3.8.2.4 the oligonucleotide primers GyrA-F and GyrA-R were used for amplification of DNA gyrase (subunit A) encoding gene *gyrA* of *T. pyogenes*. An 937 bp amplicon was amplified from the investigated three *T. pyogenes* isolates of the three grey slender lorises origin and for the reference strains *T. pyogenes* DSM 20594, *T. pyogenes* DSM 20630^T and *A. haemolyticum* DSM 20595^T. The phylogenetic tree built with *gyrA* nucleotide sequence of a total of 937 nucleotide positions split the strains in the present study into two groups, with final bootstrap supports of 60%. The first group (GI) comprised four isolates and is clustering *T. pyogenes* 11-07-D-03394, *T. pyogenes* 121008157 and *T. pyogenes* 121018522 in a separate subgroup with 100% similarity and bootstrap supports of 100%. This group assembled the reference strain *T. pyogenes* DSM 20630^T. The second group (GII) included the reference strain *T. pyogenes* DSM 20594 (Fig. 39A). The phylogenetic tree built with the GyrA amino acid sequences of a total of 312 amino acid positions with 100% similarity failed to split the strains of the present study into groups (Fig. 39B). Reference strain *A. haemolyticum* DSM 20595^T was assembled and clustered separately out of the group.

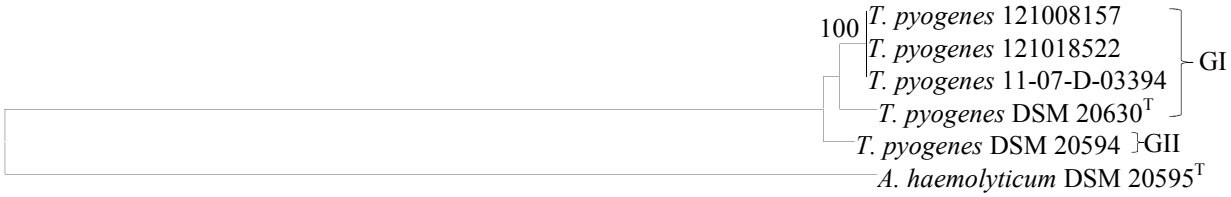


Fig: 39 A

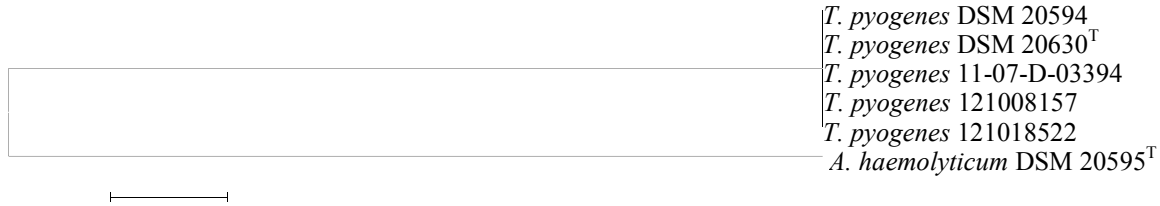


Fig: 39 B

Fig. 39: Maximum-likelihood tree based on *gyrA* nucleotide sequences of a total of 937 nucleotide positions (A) and GyrA amino acid sequences of a total of 312 amino acid positions (B) of the investigated target gene and protein of the three *T. pyogenes* isolates of the three grey slender lorises origin, *T. pyogenes* DSM 20594, *T. pyogenes* DSM 20630^{T} and *A. haemolyticum* DSM 20595^{T}.

All four partially sequenced nucleotide sequences and amino acid sequences of the housekeeping genes were concatenated in the following order: *fusA-tuf-metG-gyrA* and FusA-Tuf-MetG-GyrA with a nucleotide sequence of 3,314 bp and an amino acid sequence of 1,103 sites. The cluster analyses of the phylogenetic trees of concatenated sequences succeeded to subdivide the three investigated *T. pyogenes* isolates of the grey slender loris origin and the reference strains (*T. pyogenes* DSM 20594, *T. pyogenes* DSM 20630^{T} and *A. haemolyticum* DSM 20595^{T}). The concatenated tree built with the nucleotide sequence with 3,314 bp clustered the investigated strains and isolates in two groups. The first group (GI) comprised the three isolates of the three grey slender loris origin with 100% similarity and bootstrap supports of 100%. The second group (G-II) comprised the reference strains *T. pyogenes* DSM 20594 and *T. pyogenes* DSM 20630^{T} (Fig. 40A). The average GC content of the concatenated sequences of the four housekeeping genes ranged from 61.2% to 61.4% within the *T. pyogenes* species and 54.7% for the reference strain *A. haemolyticum* DSM 20595^{T} (Tab. 23). The isolates presented a related interspecies genome size and GC content, indicating a homogeneity within the *T. pyogenes* species. A pairwise comparison of MLSA among the *T. pyogenes* species revealed sequence distance between 0% and 0.25% (Tab. 24). The concatenated tree built with the amino acid sequence with 1,103 sites succeeded to cluster the three investigated *T. pyogenes* isolates of the grey slender lorises origin and the

reference strains (*T. pyogenes* DSM 20594, *T. pyogenes* DSM 20630^T and *A. haemolyticum* DSM 20595^T) in two groups. The first group (GI) comprised the three isolates of the three grey slender loris origin with 100% similarity and bootstrap supports of 87%. The second group (G-II) comprised the reference strains *T. pyogenes* DSM 20594 and *T. pyogenes* DSM 20630^T (Fig. 40B).

Fig. 40 A

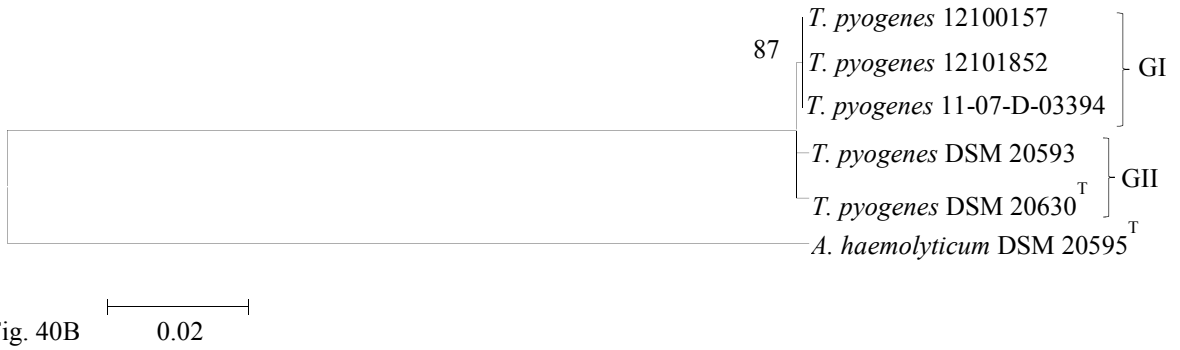


Fig. 40B

Fig. 40: Phylogenetic analysis based on concatenated partial *fusA-tuf-metG-gyrA* nucleotide sequences of a total of 3,314 nucleotide positions (A) and FusA-Tuf-MetG-GyrA amino acid sequences of a total of 1,103 amino acid positions (B) of the three investigated target genes of the three *T. pyogenes* of isolates of the grey slender lorises origin, *T. pyogenes* DSM 20594, *T. pyogenes* DSM 20630^T and *A. haemolyticum* DSM 20595^T.

The percentage of amino acids sequence similarity among *T. pyogenes* species and *A. haemolyticum* DSM 20595^T is ranging from 80.3% to 80.4%, while within the *T. pyogenes* species it ranges from 99.6% to 100% (Tab. 25). Translation of the concatenated nucleotide sequences in to protein sequences of the selected *T. pyogenes* isolates showed identical content of 14 amino acids and a variation in the content of six amino acids (Tab. 26). The analysis of the amino acid compositions of the concatenated FusA-Tuf-MetG-GyrA of the three *T. pyogenes* isolates of the grey slender loris origin, *T. pyogenes* DSM 20594 and *T. pyogenes* DSM 20630^T showed 20 different amino acids. They are identical in the

compositions percentage of 14 different amino acids. These similarities were reported in alanine (Ala) with 8.26%, cysteine (Cys) with 0.82%, phenylalanine (Phe) with 2.45%, glycine (Gly) with 7.53%, histidine (His) with 2%, lysine (Lys) with 5%, leucine (Leu) with 9.26%, proline (Pro) with 4.54%, glutamine (Gln) with 3.36%, arginine (Arg) with 6.54%, threonine (Thr) with 5.17%, valine (Val) with 8.08%, tryptophan (Trp) with 1% and tyrosine (Tyr) with 2.9%. The comparison of the amino acid compositions revealed variations in the content of 6 amino acids; aspartic acid (Asp) between 8.26% and 8.44%, glutamic acid (Glu) between 8.26% and 8.44%, isoleucine (Ile) between 6.35% and 6.44%, methionine (Met) between 2.27% and 2.36%, asparagine (Asn) between 3.27% and 3.44% and serine (Ser) between 4.26% and 4.45%. The reference strain *A. haemolyticum* DSM 20595^{T} showed the same 20 amino acids as species *T. pyogenes,* in which three amino acids were identical in composition with the species *T. pyogenes* (Ala, Glu, Thr and Trp) and had 17 different amino acid profiles in composition with the species *T. pyogenes* (Cys, Asp, Phe, Gly, His, Ile, Lys, Leu, Met, Asn, Pro, Gln, Arg, Ser, Val and Tyr).

Tab. 23: The average percent of molar GC (guanine-cytosine) content of the concatenated sequences for the four locus sequences obtained in this study of the three *T. pyogenes* of isolates of the grey slender loris origin, *T. pyogenes* DSM 20594, *T. pyogenes* DSM 20630^{T} and *A. haemolyticum* DSM 20595^{T}.

	Isolates or strains	G mol%	C mol%	G+C mol%
1	*T. pyogenes* 121018522	29.7	31.6	61.3
2	*T. pyogenes* 121008157	29.7	31.6	61.3
3	*T. pyogenes* 11-7-D-03394	29.7	31.6	61.3
4	*T. pyogenes* DSM 20630^{T}	29.8	31.5	61.3
5	*T. pyogenes* DSM 20594	29.8	31.5	61.3
6	*A. haemolyticum* DSM 20595^{T}	27.8	26.9	54.7

Tab. 24: Average pairwise distances calculated using Kimura two-parameter model of the concatenated sequences for the four locus sequences of the three *T. pyogenes* of isolates of the grey slender loris origin, *T. pyogenes* DSM 20594, *T. pyogenes* DSM 20630^T and *A. haemolyticum* DSM 20595^T obtained in this study.

	Isolates or strains	1	2	3	4	5	6
1	*T. pyogenes* 121018522						
2	*T. pyogenes* 121008157	0					
3	*T. pyogenes* 11-7-D-03394	0	0				
4	*T. pyogenes* DSM 20630^T	0.010	0.010	0.010			
5	*T. pyogenes* DSM 20594	0.012	0.012	0.012	0.010		
6	*A. haemolyticum* DSM 20595^T	0.249	0.249	0.249	0.250	0.249	

Tab. 25: Percentage of amino acids composition similarity between the protein sequences for the investigated target gene of the three *T. pyogenes* of isolates of the grey slender loris origin, *T. pyogenes* DSM 20594, *T. pyogenes* DSM 20630^T and *A. haemolyticum* DSM 20595^T.

	Isolates or strains	1	2	3	4	5	6
1	*T. pyogenes* 121018522						
2	*T. pyogenes* 121008157	100					
3	*T. pyogenes* 11-7-D-03394	100	100				
4	*T. pyogenes* DSM 20630^T	99.7	99.7	99.7			
5	*T. pyogenes* DSM 20594	99.7	99.7	99.7	99.6		
6	*A. haemolyticum* DSM 20595^T	80.3	80.3	80.3	80.4	80.3	

Tab. 26: Content (mol%) of the amino acids composition in the concatenated protein sequences derived from partially sequenced *FusA*, *Tuf*, *MetG* and *GyrA* genes of the three *T. pyogenes* of isolates of the grey slender loris origin, *T. pyogenes* DSM 20594, *T. pyogenes* DSM 20630^T and *A. haemolyticum* DSM 20595^T.

	Isolates or strains	Ala	Cys	Asp	Glu	Phe	Gly	His	Ile	Lys	Leu	Met	Asn	Pro	Gln	Arg	Ser	Thr	Val	Trp	Tyr
1	*T. pyogenes* 121018522	8.26	0.82	8.44	8.26	2.45	7.53	2.00	6.35	4.99	9.26	2.36	3.36	4.54	3.36	6.53	4.36	5.17	8.08	1.00	2.90
2	*T. pyogenes* 121008157	8.26	0.82	8.44	8.26	2.45	7.53	2.00	6.35	4.99	9.26	2.36	3.36	4.54	3.36	6.53	4.36	5.17	8.08	1.00	2.90
3	*T. pyogenes* 11-7-D-03394	8.26	0.82	8.44	8.26	2.45	7.53	2.00	6.35	4.99	9.26	2.36	3.36	4.54	3.36	6.53	4.36	5.17	8.08	1.00	2.90
4	*T. pyogenes* DSM 20630^T	8.26	0.82	8.26	8.44	2.45	7.53	2.00	6.35	4.99	9.26	2.36	3.27	4.54	3.36	6.53	4.45	5.17	8.08	1.00	2.90
5	*T. pyogenes* DSM 20594	8.26	0.82	8.26	8.35	2.45	7.53	2.00	6.44	4.99	9.26	2.27	3.45	4.54	3.36	6.53	4.36	5.17	8.08	1.00	2.90
6	*A.haemolyticum* DSM 20595^T	8.26	0.64	9.17	8.35	2.72	7.62	2.27	5.72	5.08	9.53	2.54	2.90	4.36	3.09	6.81	3.90	5.17	8.35	1.00	2.54

alanine (Ala), cysteine (Cys), aspartic Acid (Asp), glutamic Acid (Glu), phenylalanine (Phe), glycine (Gly), histidine (His), isoleucine (Ile), lysine (Lys), leucine (Leu), methionine (Met), asparagine (Asn), proline (Pro), glutamine (Gln), arginine (Arg), serine (Ser), threonine (Thr), valine (Val), tryptophan (Trp), tyrosine (Tyr).

Tab. 27: A summarized table comparing the PCR mediated amplification of the various putative virulence factor encoding genes of the investigated *T. pyogenes* isolates and the *T. pyogenes* reference strains of the present study.

Origin of the *T. pyogenes* isolates or reference strains, respectively	Number of PCR- positive isolates or reference strains, respectively								
	n	*plo* (704 bp)*	*cbpA* (327 bp)	*nanH* (781 bp)	*nanP* (150 bp)	*fimA* (605 bp)	*fimC* (843 bp)	*fimE* (775 bp)	*tet(W)* (1,843 bp)
Bovine mastitis	57	57**	1	39	48	57	53	52	36
Bovine mastitis (Gelatinase negative)	1	1	0	0	0	1	1	1	1
Bovine cervical swabs	14	14	1	14	14	14	13	14	9
Grey Slender Lorises	3	3	0	3	0	3	3	3	3
∑	75	75 (100%)	2 (2.6%)	56 (74.6%)	62 (82.6%)	75 (100%)	70 (92%)	70 (92%)	49 (65%)
T. pyogenes DSM 20594	1	1	0	1	1	1	1	1	0
T. pyogenes DSM 20630^T	1	1	1	1	1	0	1	1	1

n = Number of isolates: * = expected amplicon size; ** = number of isolates with the respective property.

5 Discussion

T. pyogenes is known as a commensal and an opportunistic pathogen for various host species, causing different diseases as mastitis, metritis, liver abscessation and pneumonia. *T. pyogenes* associated with bovine mastitis causes serious economic problems in commercial dairy milk production. The collection of the *T. pyogenes* isolates of the present study gave the opportunity to investigate the phenotypic and genetic diversity of this species and to determine epidemiological relationships of infections with this bacterial pathogen.

5.1 Phenotypic and genotypic properties of *T. pyogenes* isolated from bovine mastitis

All 57 *T. pyogenes* isolates investigated in the present study were identified and classified to genus *Trueperella* as *T. pyogenes*. All 57 isolates exhibited a complete zone of hemolysis after cultivation on sheep blood agar under microaerobic conditions in a candle jar. However, the investigated *T. pyogenes* isolates did not show an enhanced hemolysis after cultivation on rabbit blood. Differences in the size of the hemolytic zone after cultivation on sheep and rabbit blood agar could be observed for *A. haemolyticum* and to some degree for *A. pluranimalium* (Ülbegi-Mohyla et al., 2009; Ülbegi-Mohyla, 2010; Hijazin, 2012a). The hemolysis of *T. pyogenes* is well known to be caused by the pore forming toxin pyolysin. Pyolysin (PLO) of *T. pyogenes* is a member of the cholesterol-dependent cytolysin toxin (CDC) family (Billington et al., 1997). PLO binding to the cholesterol containing membranes induces membrane alteration and defacement (Billington et al., 2000). This effect could be seen as circular-like and arc-like structured pores on the red blood cells (RBCs) causing lyses of RBCs of humans and of a variety of animal species (Smyth and Duncan, 1978; Lämmler and Blobel, 1988; Ding and Lämmler, 1996; Billington et al, 1997). PLO is a primary virulence factor of *T. pyogenes* and its cytotoxic effect plays an important role against 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 for laboratory animals.

The 57 *T. pyogenes* isolates derived from bovine milk samples of the present study showed a synergistic CAMP-like reaction with *S. aureus* ß-hemolysin and *R. equi* and *A. haemolyticum* as

indicator strains respectively, and no reverse CAMP reaction in the zone of staphylococcal ß-hemolysin. However, no synergistic or antagonistic CAMP-like reaction could be observed with *S. agalactiae* as indicator strain. The present results agreed with previous reports (Fraser, 1964; Lämmler and Blobel, 1988; Ülbegi-Mohyla et al., 2009). The CAMP reaction was first described by Christie, Atkins and Munch-Petersen (1944) as a synergistic hemolytic reaction of CAMP-factor of *S. agalactiae* belonging to serological group B cultivated in the zone of incomplete staphylococcal β-hemolysin. Later on, the CAMP-reaction was widely used in identification of different types of bacteria (Fraser, 1964; Skalka et al., 1979; Linder, 1984; Hebert and Hancock, 1985; Lämmler and Blobel, 1987, 1988). In 2009, Ülbegi-Mohyla inquired CAMP-like activities of bacteria of genus *Arcanobacterium* and *Trueperella* and showed that the various synergistic hemolytic reactions could be used as additional criteria for identification of bacteria of genus *Arcanobacterium* and *Trueperella*.

All 57 *T. pyogenes* isolates of the present study were positive for the enzymes β-D-glucuronidase, α-D-glucosidase, N-acetyl-β-D-glucosaminidase and 56 isolates positive for β-D-galactosidase activity. However, one (1.8%) isolate was only weakly positive in this assay. Detection of this enzyme activity could be accomplished with 4-methylumbelliferyl-conjugated substrates previously used for biochemical characterization of *T. pyogenes* (Ülbegi, 2010; Hijazin, 2012a). Among the 57 *T. pyogenes* isolates three (5.7%) isolates were weakly positive for the enzyme α-mannosidase. However, Carlson and Kontiainen (1994) reported that all *T. pyogenes* (n=30) were α-mannosidase negative. Hijazin (2012a) found 19.6% of 51 *T. pyogenes* α-mannosidase positive. All 57 *T. pyogenes* isolates were catalase negative. This is matching with other studies (Collins et al., 1982b, Ramos et al., 1997, Lawson et al., 2001, Hoyles et al., 2002, Johnson et al., 2003, Lehnen et al., 2006, Azuma et al., 2009; Ülbegi, 2010).

All *T. pyogenes* isolates of the present study were positive for the enzyme DNase using DNase test agar. These results corresponded to previous results (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; Ülbegi, 2010; Hijazin, 2012a). All 57 *T. pyogenes* isolates had the liquefaction capability of Loeffler medium and hydrolysed casein. These results corresponded with other studies (Narayanan 1998; Ülbegi, 2010; Hijazin, 2012a). The amylase enzyme activity was detected only in four (7%) *T. pyogenes* isolates of the present study and the reference strain *T. pyogenes* DSM 20630^{T} of porcine origin. However, Hijazin (2012) reported

that all *T. pyogenes* isolates from bovine origin were amylase negative. In addition, all 57 *T. pyogenes* isolates showed a cross-reaction with streptococcal serogroup G-specific antiserum. This also corresponded to previous results (Lämmler and Blobel, 1988; Lämmler and Hartwigk, 1995).

Prior to the MALDI-TOF MS analysis of the present study comparable cultivation conditions were applied for all investigated bacteria. The MALDI-TOF MS analysis was performed with all 57 *T. pyogenes* isolates and 12 reference strains representing ten species of genera *Arcanobacterium* and *Trueperella*. The isolates and the reference strains were cultivated on sheep blood agar plates for 48 h at 37 °C under microaerobic conditions in a candle jar. Sample processing for the isolates and the reference strains included the application of a protein extraction step prior to analysis using Bruker ethanol formic acid extraction procedure. The protein extraction prior to MALDI-TOF MS analysis led to a significant improvement in the number of isolates which could be identified to both genus and species level (Alatoom et al., 2011). Moreover, the protein extraction improve the generation of specific spectral fingerprints which led to an accurate microbial identification and characterization of the investigated strains (Bizzini et al., 2011). In the present study, the MALDI-TOF MS fingerprint analysis identified all 57 *T. pyogenes* isolates to the species level. The 57 *T. pyogenes* isolates and the 12 reference strains representing ten species of genera *Trueperella* and *Arcanobacterium* were identified to the species level through comparing the total main spectrum profile (MSP) for each strain against the new acquired MSPs from the 12 reference strains of genera *Trueperella* and *Arcanobacterium* and against the reference library entries using the MALDI Biotyper version 3.1 software package. The interpretation of the MALDI-TOF MS fingerprint analysis was based on several recently published studies, where authors rated log (score) values ≥ 2.0 as probable identification at genus and species level. Otherwise, log (score) values ≥ 1.7 and < 2.0 were rated as identification at genus level and the 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).

In this study, the 57 isolates and the 2 reference strains were identified as *T. pyogenes* and were separated from other reference strains of genera *Trueperella* and *Arcanobacterium* in different clusters by using of MALDI-TOF MS. As reported in several studies, differences between protein spectra determined by MALDI-TOF MS can be used for identification and individual strain

typing of different bacterial species (Besséde et at., 2011; Mellmann et al., 2008; Murray, 2010; Nagy et al., 2009; Seibold et al., 2010; Seng et al., 2009). As shown in the present study, MALDI-TOF MS proved to be a rapid and cost saving technique for identifying and typing of *T. pyogenes* isolates from bovine orgin. However, as shown above for the two isolates *T. pyogenes* 1584/22 and *T. pyogenes* 1183/105, the discriminatory power of MALDI-TOF MS was found to be insufficient for subtyping of *T. pyogenes* isolates to the level of distinct clones. Limitations and drawback of MALDI-TOF MS subtyping were also reported for other pathogens such as *S. aureus* or *E. coli* (Wolters et al., 2011; Lartigue, 2013; Sandrin et al., 2013; Lasch et al., 2014;).

It was reported by Curk et al. (1994), Beattie et al. (1998) and Grunert et al. (2013) that FT-IR spectra of bacterial isolates belonging to the same genus have a similar feature. This allows to differentiate them from spectra of bacteria representing other genera. FT-IR spectroscopy had already been used as a tool for classification of *Listeria* and *Yersinia* species (Janbu et al., 2008; Kuhm et al., 2009; Wortberg et al., 2012), Coryneform bacteria (Oberreuter et al., 2002) and for a large number of other clinically relevant pathogens (Samuels et al., 2009; Contzen et al., 2011; Grunert et al., 2013). Most of the studies used an internal validation model. However, the present study represents both an internal validation model and an external validation model. The external validation of the *T. pyogenes* typing by FT-IR spectroscopy was performed with an independent sample set consisting of 57 *T. pyogenes* isolates that were not included in the calibration procedure. The internal validation was performed with a small heterogeneous database of reference strains from three genera (*Actinomyces, Trueperella* and *Arcanobacterium*) which will reinforce the correct identification with higher values rather than in an external validation model. Three isolates were required to create the *T. pyogenes*-module. This mirrors the limited intra-species variation of infrared spectra of this species, which can also be shown in the cluster analysis of the *Trueperella* isolates used in this study. In the dendrogram, it is shown that the distance of the infrared-spectra of the *T. pyogenes* isolate variations are far away from all the other known *Trueperella* species, to separate the species *T. pyogenes* unequivocally in this environment. Noticeable in this context is the clear division of the genus in two branches. One branch comprises the type strains of the four species *T. abortisuis*, *T. bernardiae*, *T. bialowiezensis* and *T. bonasi*. The second, very close branch was formed by all *T. pyogenes* reference strains and the 57 isolates from the mastitis cases, independent from the observed variations in phenotype and genotype. This shows the slight resolution for single isolates in IR

analysis in this isolate set. Up to an exception, the *T. pyogenes* isolates of the present study were obtained from different animals in a wide diversity of farms and locations. Merely three isolates were recovered from one cow. Conspicuously, these three isolates clump together in a special sub branch. The cluster analysis turned out to be a useful tool for differentiating the bacterial isolates in the present study, as it revealed relationships among their FT-IR spectra. Because of the small difference in heterogeneity to the next neighbour isolates, the suitability for a meaningful isolate differentiation cannot be estimated from this limited data-set. In another context and for other Gram-positive bacteria, like *Bacillus cereus* or *Staphylococcus aureus*, contamination route analysis succeeded by use of FT-IR (Rau et al., 2009; Johler et al., 2013). Based on the database containing well defined isolates of the various species of genus *Trueperella*, *Arcanobacterium* and *Actinomyces,* the FT-IR spectroscopy appeared to be comparable to the previously described MALDI-TOF MS (Hijazin et al., 2012b) and appears to be a promising tool for rapid and reliable identification of *T. pyogenes* in routine diagnosis. In several laboratories FT-IR spectroscopy has become the first choice method for differentiation of various bacterial species. However, FT-IR spectroscopy will not replace but complement for the classical phenotypical and genotypical diagnostic systems useful for characterization of *T. pyogenes*.

The genotypic identification of the *T. pyogenes* of the present study was performed by amplification of species-specific regions of the 16S-23S rDNA intergenic spacer region (ISR) and superoxide dismutase A encoding gene *sodA* of *T. pyogenes.* The ISR has been examined in a wide range of eubacteria and can be used to generate specific PCR primers related to the microorganism of interest (Barry et al., 1991; Tilsala-Timisjärvi and Alatossava, 1997). In 1995, Kostman designed a pair of primer to amplify the ISR of several bacterial pathogens including *Staphylococcus aureus, Enterococcus faecium, E. coli* and *Enterobacter species* (Kostman et al., 1995). That was followed by another research by Chanter et al. (1997) on Lancefield group C streptococci by designing a pair primer for identification and sub-specific typing of *Streptococcus equi, S. zooepidemicus, S. equisimilis and S. dysgalactiae*. Other researchers have developed species specific oligonucleotide primers which were used for identification of *T. pyogenes, T. bernardiae, T. bialowiezensis, T. bonasi, A. haemolyticum* and *A. hippocoleae* (Hassan et al., 2008; Ülbegi, 2010). In the present study, the 57 *T. pyogenes* isolates and both *T. pyogenes* reference strains proved PCR-postive using the ISR species specific oligonucleotide primers described by Ülbegi (2010) and Ülbegi et al. (2010).

Many research addressed the superoxide dismutase A encoding gene *sodA* as a good discriminatory tool for different mycobacterial species (Zolg and Philippi-Schulz, 1994), various streptococcal type strains (Poyart et al., 1998), enterococcal type strains (Poyart et al., 2000) and coagulase-negative staphylococcal (CNS) type strains (Poyart et al., 2001). In 2010, Ülbegi developed a new oligonucleotide primer pair which successfully helped in amplification, sequencing and differentiation of gene *sodA* of species of genera *Trueperella* and *Arcanobacterium* (not *T. bialowiezensis*). Furthermore, in 2011 the *sodA* species specific oligonucleotide primers were successfully used in genotypic identification of 61 *T. pyogenes* isolated from bovine mastitis and from various other origins (Hijazin et al., 2011). In the present study, the 57 *T. pyogenes* isolates from bovine mastitis and the reference strains *T. pyogenes* DSM 20594 and *T. pyogenes* DSM 20630^{T} were successfully identified genotypically by amplification of a species specific region of the *sodA* gene of *T. pyogenes*.

Both ISR and *sodA* species specific oligonucleotide primer showed a high specificity for *T. pyogenes* with no recorded cross reaction with other control strains (Ülbegi, 2010; Hijazin, 2012). This confirms that both the designed ISR and *sodA* species specific oligonucleotide primers can be used for reliable genotypic identification of *T. pyogenes.*

T. pyogenes is additionally characterized by producing a variety of known and putative virulence factors that include pyolysin (PLO) and other factors promoting adhesion to host cells (CbpA, NanH, NanP, FimA, FimC and FimE) (Zastempowska and Lassa, 2012). The haemolytic exotoxin pyolysin is the primary virulence factor of *T. pyogenes* (Billington et al., 1997; Ding and Lämmler 1996; Jost et al., 1999). In 1997, Billington et al. had succeeded in sequencing the pyolysin encoding gene (*plo*) with a nucleotide sequence of 1,605 bp (Billington et al., 1997). Later on, another study showed that a *T. pyogenes* mutant deficient in the production of PLO resulted in the reduction of the virulence effect in contrast to the wild type *T. pyogenes.* However, this effect was reversed by transferring the *plo* gene in *trans* to the PLO mutant deficient isolate, suggesting that PLO is necessary for the *T. pyogenes* survival and pathogenesis (Jost et al., 1999). Subsequent studies have shown that all *T. pyogenes* isolates expressed the pyolysin encoding gene *plo* (Ertaş et al., 2005; Jost and Billington, 2005; Silva et al., 2008; Hijazin et al., 2011; Zastempowska and Lassa, 2012; Belser et al., 2015). In the present study all 57 *T. pyogenes* harbored gene *plo* using the oligonucleotide primer developed by Ülbegi et al. (2010). These

results showed that the pyolysin encoding gene *plo* is a useful molecular marker for genotypic identification of *T. pyogenes.*

T. pyogenes collagen-binding protein CbpA encoded by gene *cbpA* was firstly described by Esmay et al. (2003). Besides the neuraminidase, CbpA is the second *T. pyogenes* protein involved in adhesion to collagen rich tissues. Esmay et al. (2003) reported that a *cbpA* negative mutant shows a retarded adhesion property to both HeLa and 3T6 cells. However, this mutant property can be reversed by introducing a replicating plasmid containing the *cbpA* gene (Esmay et al., 2003). The presence of gene *cbpA* in *T. pyogenes* from bovine origin is variable, Esmay et al. (2003) reported that the gene *cbpA* is present in 49%, Silva et al. (2003), in 100% and Hijazin et al. (2011) and in 7% of the investigated strains, respectively. Moreover, Hijazin *T. pyogenes* isolated from small ruminants and wild ruminants, horse, rabbit and rat did not carry gene *cbpA*. (Hijazin, 2012a). In the present study gene, *cbpA* could be found in one (1.8%) of the 57 *T. pyogenes* isolates of bovine origin. Reference strain *T. pyogenes* DSM 20594 of bovine origin was negative for gene *cbpA,* while *T. pyogenes* DSM 20630^{T} was positive (Ülbegi et al., 2010; Hijazin et al., 2011).

The *T. pyogenes* neuraminidase encoding genes *nanH* and *nanP* were originally cloned and sequenced by Jost et al. (2001, 2002b). The neuraminidase plays a role in adhesion of the pathogen to mucosal surfaces of the mediating host cell (Jost et al., 2001). However, *T. pyogenes* mutants in one or both neuraminidases did not affect the adherence properties to epithelial cells but reduced there adherence properties to HeLa cells (Jost et al., 2002b). Jost and Billington (2005) reported that all *T. pyogenes* isolates carried *nanH* and 64.2% *nanP*, Silva et al. (2008) reported that all *T. pyogenes* isolates carried *nanH* and *nanP* and Hijazin et al. (2011) reported that 29% of the *T. pyogenes* isolates were positive for *nanH* and 65.5% for *nanP.* In the present study 39 (68%) of the *T. pyogenes* isolates were positive for *nanH,* 48 (84%) isolates positive for *nanP* and 35 (61.4%) isolates positive for both genes. Both reference strains *T. pyogenes* DSM 20594 and *T. pyogenes* DSM 20630^{T} were positive for *nanH* and *nanP* (Silva et al., 2008; Hijazin et al., 2011).

Fimbriae are appendages that play a role in the adherence of several *Corynebacterium* spp. and *T. pyogenes* (Yanagawa and Honda 1976; Ton-That et al., 2004). In this study, the *T. pyogenes* fimbriae encoding gene *fim*A was amplified using oligonucleotide primers developed by Silva et al. (2008) and was positive for all 57 (100%) *T. pyogenes* isolates from bovine mastitis.

According to Santos et al., (2010) the presence of *fim*A appeared to be variable in *T. pyogenes* isolates from non metritic cows (64.1%) and from metritic cows (90.9%). *T. pyogenes fim*A was reported for 94% of the *T. pyogenes* isolates described by Jost and Billington (2005) and for 100% of the *T. pyogenes* described by Silva et al. (2008), Hijazin et al. (2011) and Zastempowska and Lassa (2012).

T. pyogenes fimbriae encoding gene *fimC,* also amplified using the oligonucleotide primers developed by Silva et al. (2008), was positive for 53 (93%) of the *T. pyogenes* isolates from cases of bovine mastitis. Presence of *fimC* in isolates of other studies varied between 87.6% (Zastempowska and Lassa, 2012), 94% (Hijazin, 2012a) and 100% (Silva et al., 2008). *T. pyogenes* fimbriae encoding gene *fimE,* also amplified using the oligonucleotide primer developed by Silva et al. (2008), could be observed for 52 (91.2%) of the *T. pyogenes* isolates of the present study. The frequency of *fimE* in strains of other studies varied between 91% (Zastempowska and Lassa, 2012) and 100% (Silva et al., 2008; Hijazin, 2012a).

The tetracycline resistance gene *tet*(*W*) of the *T. pyogenes* of the present study was amplified using the oligonucleotide primer sequence described by Billington and Jost (2006) yielding a positive rection in 35 (61.4%) of the *T. pyogenes* isolates. Reference strain *T. pyogenes* DSM 20630^{T} was *tet*(*W*) positive, *T. pyogenes* DSM 20594 *tet*(*W*) negative. In previous studies the tetracycline resistance gene *tet*(*W*) could be observed in 42% (Billington and Jost, 2006) or 85.5% (Zastempowska and Lassa, 2012) of the *T. pyogenes* isolates.

According to the present studies there seemed to be no predominant genotype of *T. pyogenes* associated with bovine mastitis. Comparably, Silva et al. (2008) concluded that all *T. pyogenes* may have an equal potential to induce bovine clinical metritis.

The used PCR-mediated identification of the *T. pyogenes* revealed no cross reactivity with the control strains representing other species of genera *Arcanobacterium* and *Trueperella*, indicating that the used targets could be used for molecular identification of this species.

MLSA is known as a powerful tool for discrimination, classification and phylogenetic analysis of several bacterial isolates. In addtion, MLSA is highlighted as a diversity method in comparison to other conventional phenotypic and genotypic methods (Maiden et al., 1998: Wertz et al., 2003; Zeigler, 2003). The present study displays the first MLSA scheme focusing on genera *Trueperella* and *Arcanobacterium.* The four housekeeping genes (*fusA-tuf-metG-gyrA)* used in this study were previously used for various other bacterial spieces (Henssge et al., 2009; Joseph

and Forsythe, 2012; Mun et al., 2013; Sulyok et al., 2014). The MLSA study of the present investigation was performed with 14 selected *T. pyogenes* isolates. The 14 *T. pyogenes* represented isolates obtained from different locations, the same location, from different animals and from the same animal. Using MLSA the 14 *T. pyogenes* isolates could be clearly differentiated into five groups.

On the basis of partial gene *fusA* sequences all *T. pyogenes* isolates and the reference strains *T. pyogenes* DSM 20594 and *T. pyogenes* DSM 20630^T were clearly differentiated, forming distinct branches. At the intergenus level the gene *fusA* sequence similarity was 70.8% for *A. haemolyticum* DSM 20595^T. At the intraspecies level the isolates of the same species had at least 98.5% gene *fusA* sequence similarity. The nucleotide sequences of gene *fusA* of the 14 selected *T. pyogenes* isolates showed variations in GC contents (60.1 mol% to 60.7 mol%) compared to 52.2 mol% for the reference strain *A. haemolyticum* DSM 20595^T. Translation of the DNA sequence of the gene *fusA* of the 14 selected *T. pyogenes* isolates to protein sequences showed some variations in the amino acid composition. The amino acid composition of the FusA proteins from the 14 selected *T. pyogenes* isolates varied in four different amino acids (aspartic acid with 7.41% to 7.82%, glutamic acid with 8.64% to 9.05%, asparagine with 2.88% to 3.29% and threonine with 3.70% to 4.12%). FusA protein and the gene *fusA* of the *T. pyogenes* isolates from the same animal (*T. pyogenes* 336/1, *T. pyogenes* 336/2 and *T. pyogenes* 336/4) had 100% nucleotide and amino acid sequence similarity, a similarity value which was not found with other isolates. The results of the *fusA* analysis supported the result and the conclusion that the three *T. pyogenes* strains isolated from one animal represent a single clone. Thus, the gene *fusA* can be used as parameter for discrimination of single strains of the species *T. pyogenes*. Gene *fusA* already showed the molecular evolution of *Cronobacter* spp. (Joseph and Forsythe, 2012) and succeeded in characterization and discrimination of *Citrobacter pasteurii* as a novel species of the genus (Clermont et al., 2015).

On the basis of partial sequences of gene *tuf*, the 14 selected *T. pyogenes* isolates and the reference strains *T. pyogenes* DSM 20594 and *T. pyogenes* DSM 20630^T of the present study could be differentiated from reference strain *A. haemolyticum* DSM 20595^T. At the intergenus level the gene *tuf* sequence similarity with the selected *T. pyogenes* isolates was at maximum 83.1% for *A. haemolyticum* DSM 20595^T. At the intraspecies level the isolates of the same species had at least 99.2% gene *tuf* sequence similarity. The DNA nucleotide sequences of gene

tuf of the 14 selected *T. pyogenes* isolates showed variations in the GC contents (60.7 mol% to 60.9 mol%) and a GC contents of 54.6 mol% for the reference strain *A. haemolyticum* DSM 20595^T. Translation of the DNA sequences of gene *tuf* to the protein sequences of the 14 selected *T. pyogenes* isolates showed variations in the amino acid composition. The amino acid composition of the Tuf proteins from the selected *T. pyogenes* isolates varied in four separate amino acids (Phenylalanine with 1.98 to 2.34, Glutamic acid with 8.98% to 8.59%, Asparagine with 5.47% to 6.25% and Threonine with 6.64% to 5.86%). However, again *T. pyogenes* 336/1, *T. pyogenes* 336/2 and *T. pyogenes* 336/4 shared the same nucleotide sequences, GC profile and amino acid composition. Gene *tuf* has already been used in differentiating five *Flavobacterium* species isolated from freshwater sources and clearly classified them from other 37 strains of the same species (Mun et al., 2013).

The partial gene *metG* sequences of all *T. pyogenes* isolates and the reference strains *T. pyogenes* DSM 20594 and *T. pyogenes* DSM 20630^T were clearly different and enabled forming of distinct branches. At the intergenus level, the gene *metG* sequence similarity with the selected *T. pyogenes* species and *A. haemolyticum* DSM 20595^T was at minimum 74.05% and at maximum with 75%. At the intraspecies level, the isolates of the same species had at least 98.02% gene *metG* sequence similarity. The nucleotide sequences of gene *metG* of the selected *T. pyogenes* isolates showed variations in the GC content (60.8 mol% to 61.4 mol%) compared to 53.7 mol% for the reference strain *A. haemolyticum* DSM 20595^T. Translation of the DNA sequences of gene *metG* of the selected *T. pyogenes* isolates to protein sequences showed variations in the amino acid composition. The amino acid composition of the MetG proteins from the selected *T. pyogenes* isolates varied in eight separate amino acids (aspartic acid with 8.92% to 9.29%, glutamic acid with 5.95% to 6.32%, isoleucine with 4.46% to 4.83%, methionine with 2.23% to 2.6%, asparagine with 4.46% to 4.83%, serine with 4.83% to 5.2%, threonine with 5.2% to 5.57 and tyrosine with 2.97% to 3.45%). According to partial gene *metG* and MetG protein sequences the three isolates *T. pyogenes* 336/1, *T. pyogenes* 336/2 and *T. pyogenes* 336/4 again had 100% nucleotide and amino acid sequence similarity among each other and 100% amino acid sequence similarity to *T. pyogenes* 843/2 and the reference strain *T. pyogenes* DSM 20594. However, gene *metG* showed a high discriminatory capability and in studying the molecular evolution of *Actinomyces oris* and *Actinomyces johnsonii* by differentiating them from *Actinomyces naeslundii* (Henssge et al., 2009).

Using partial sequences of the DNA gyrase (subunit A) encoding gene *gyrA* the selected 13 *T. pyogenes* isolates and the reference strains *T. pyogenes* DSM 20594 and *T. pyogenes* DSM 20630^T could be clearly differentiated into distinct branches. At the intergenus level the gene *gyrA* sequence similarity with the selected *T. pyogenes* isolates and *A. haemolyticum* DSM 20595^T was at maximum 72.4%. At the intraspecies level, the isolates of the same species had at least 97.9% gene *gyrA* sequence similarity. The DNA nucleotide sequences of gene *gyrA* of the selected *T. pyogenes* isolates showed variations in the GC contents (62.5 mol% to 63 mol%) comparable to 57.8 mol% of the reference strain *A. haemolyticum* DSM 20595^T. Translation of the DNA sequences of gene *gyrA* of the selected *T. pyogenes* isolates to protein sequences showed variations in the amino acid composition. The amino acid composition of the GyrA proteins from the selected *T. pyogenes* isolates varied in six separate amino acids (alanine with 7.93 mol% to 7.59 mol%, aspartic acid with 8.62 mol% to 8.97 mol%, glutamic acid with 9.31 mol% to 8.97 mol%, histidine with 2.41 mol% to 2.07 mol%, lysine with 4.48 mol% to 4.82 mol% and valine with 7.59 mol% to 7.93 mol%). Depending on gene *gyrA* and GyrA protein sequence the three isolates *T. pyogenes* 336/1, *T. pyogenes* 336/2 and *T. pyogenes* 336/4 had 100% nucleotide and amino acid sequence similarity. However, these isolates shared the same amino acid similarity profile with another six *T. pyogenes* isolates showing that gene *gyrA,* at least on the nucleotide sequence level, is a good discrimination tool for *T. pyogenes*. The DNA gyrase (subunit A) encoding gene *gyrA* already showed a high level of genetic diversity and discriminatory capability for *Klebsiella pneumoniae* (Brisse and Verhoef, 2001), *Campylobacter jejuni* and *Campylobacter coli* (Ragimbeau et al., 2014) and *Actinomyces naeslundii* and *Actinomyces oris* (Henssge et al., 2009).

The phylogenetic relationships of the concatenated nucleotide sequences of the four housekeeping genes indicated that the selected *T. pyogenes* investigated in the present study are highly diverse. However, *T. pyogenes* 1065/41 from location 35 was excluded from this analysis because the DNA gyrase (subunit A) encoding gene *gyrA* of this isolate could not be amplified. The concatenated tree built with partial nucleotide sequences of the four genes of all 13 *T. pyogenes* isolates and the reference strains *T. pyogenes* DSM 20594 and *T. pyogenes* DSM 20630^T are clustered in five groups. The first group (GI) integrated the three *T. pyogenes* isolates (*T. pyogenes* 336/1, *T. pyogenes* 336/2 and *T. pyogenes* 336/4) which were isolated from location 48 and the same cow (5134) and separate them from the other *T. pyogenes* (GI.I) indicating a

cross-infection of these isolates between the udder quarters. Also, the first group (GI) clustered *T. pyogenes* 843/2 from location16 and the reference strain *T. pyogenes* DSM 20594 which are from different geographic locations. The second group (GII) integrated the *T. pyogenes* 506/74 from location 31 and reference strain *T. pyogenes* DSM 20630^T which were also obtained from different locations. The third group (GIII) clustered five isolates: *T. pyogenes* 1065/47 from location 35, *T. pyogenes* 1800/9 from location 16, *T. pyogenes* 220/1 from location 21 and *T. pyogenes* 1512/21 and *T. pyogenes* 59/11 from location 15. The isolates of the third group (GIII) were clearly separated from each other although *T. pyogenes* 1512/21 and *T. pyogenes* 59/11 were obtained from the same farm (W) but from different cows (21 and 52). The fourth group (GIV) clustered *T. pyogenes* 58/4 from location 21 with *T. pyogenes* 1220/112 from location 1. The nucleotide sequence of isolate *T. pyogenes* 887/77 from location 31 was unique and assembled alone in group GV. The GC content of the concatenated nucleotide sequences pattern of *T. pyogenes* isolates showed that *T. pyogenes* 336/1, *T. pyogenes* 336/2 and *T. pyogenes* 336/4 have the same GC content separate to the other investigated *T. pyogenes* isolates in the present study.

The analyses of the concatenated amino acid sequences were conducted using the four genes of all 13 *T. pyogenes* isolates and the reference strains *T. pyogenes* DSM 20594 and *T. pyogenes* DSM 20630^T. The concatenated tree built with partial amino acid sequences of the four genes of all *T. pyogenes* isolates and the reference strains *T. pyogenes* DSM 20594 and *T. pyogenes* DSM 20630^T clustered the isolates in four groups. The first group (GI) clustered *T. pyogenes* 336/1, *T. pyogenes* 336/2, *T. pyogenes* 336/4 from location 48 and the reference strain *T. pyogenes* DSM 20594 with 100% sequence similarity and *T. pyogenes* 887/77 from location 16. The second group (GII) integrated *T. pyogenes* 1220/112 from location 1 and *T. pyogenes* 887/77 from location 16. The third group (GIII) clustered five isolates: *T. pyogenes* 59/11 from location 15, *T. pyogenes* 220/1 from location 21, *T. pyogenes* 506-74 from location 31, 1065/47 from location 35 and the reference strain *T. pyogenes* DSM 20630^T which could be clearly differentiated from others. *T. pyogenes* 1800/9 from location 16 clustered separately in the fourth group (GIV).

Depending on the various genetic markers the MLSA results of the present study showed that the selected *T. pyogenes* and the two *T. pyogenes* reference strains belong to different clonal complexes with distinct high interspecies resolution. However, there were isolates from the same

location but from different animals which were clearly separated by MLSA indicating that they belong to a different clonal complex.

MLSA had been described by several authors as a valuable tool for monitoring and characterizing various bacterial species (Bolt et al., 2010; Chen et al., 2010; Eisenberg et al., 2015; Henssge et al., 2009; Octavia et al., 2013; Zhang et al., 2013). However, the present study is considered as the first MLSA analysis for genus *Trueperella* and *Arcanobacterium.* The objective of this study was to develop a discriminative tool for studing the molecular epidemiology of *T. pyogenes* isolated from bovine mastits and to identify isolate relationships by exploring the diversity and geographic distribution of the *T. pyogenes* originated from single and different locations. This technique appears to be a valuable molecular tool for outbreak investigations. However, the different housekeeping genes used in the present study were not equally in segregation and separation of the investigated *T. pyogenes* isolates into separate genospecies.

The *T. pyogenes* isolates from mastitis cases had several putative virulence factor encoding genes in different combinations. These results, along with the DNA fingerprinting in a newly established MLSA, showed that the investigated *T. pyogenes* mastitis cases of the present study are mainly caused by different bacterial clones without any relationship among each other.

5.2 Phenotypic and genotypic properties of the gelatinase negative *T. pyogenes* 754B isolated from bovine mastitis

An unusual *T. pyogenes* isolate (*T. pyogenes* 754B) from bovine mastitis which was obviously negative in various proteolytic reactions was further characterized phenotypically and genotypically. *T. pyogenes* 754B was identified phenotypically by determination of hemolysis and CAMP-like hemolytic reactions, by using the API Coryne test system and various other phenotypical tests. *T. pyogenes* 754B showed the typical properties of *T. pyogenes*. *T. pyogenes* 754B was positive for the enzymes activity of pyrrolidonyl arylamidase, alkaline phosphatase, β-D-glucuronidase, β-galactosidase, α-D-glucosidase, N-acetyl-β-D-glucosaminidase and amylase, showed degradation of D-glucose, D-ribose, D-maltose, D-lactose, D-saccharose and glycogen, and a cross reaction with streptococcal serogroup G specific antiserum. *T. pyogenes* 754B was negative for the enzymes activity of α-galactosidase, β-glucosidase, pyrazinamidase, urease, catalase, α-mannosidase, for nitrate reduction, esculin hydrolysis, and for degradation of D-mannitol. However, this isolate showed an unusual phenotypic property namely the absence of gelatinase activity. The extracellular proteolytic activity is generally typical for *T. pyogenes* and is widely used for phenotypic identification of this species (Bisping and Amtsberg 1988; Lämmler 1990; Lämmler and Hartwigk 1995; Hijazin et al., 2011).

Determination of gelatinase enzyme activity of *T. pyogenes* 754B of the present study was determined with the API Coryne test system and additionally using a gelatin agar plate method described by Smith and Goodner (1958) and by a gelatin stab method described by Zimbro et al., 2009. *T. pyogenes* 754B was gelatinase negative in all these tests. In addition this isolate was negative for caseinase enzyme activity and showed no groove formation in Loeffler medium, indicating a lack of proteolytic activity.

The first isolation and characterization of an extracellular protease from *T. pyogenes* was performed by Schaufuss et al. (1989b). Later on, Takeuchi et al. (1995) investigated extracellular gelatinase and caseinase activity of *T. pyogenes*. In 2005, Jost and Billington mentioned that *T. pyogenes* proteases hydrolyse the peptide bonds of host proteins and recycle the amino acids as nutrients.

The unusual *T. pyogenes* isolate 754B of the present study could also be identified to the species level by MALDI-TOF MS. This isolate matched with a log (score) value of 2.36 with reference

strain *T. pyogenes* DSM 20594 and with a log (score) value of 2.07 with *T. pyogenes* DSM 20630^T.

A genotypic identification of *T. pyogenes* 754B was performed by sequencing the 16S rRNA gene and, as described previously by Sammra et al. (2014a) as novel molecular target, by sequencing glyceraldehyde-3-phosphate dehydrogenase encoding gene *gap* and, as described by Hijazin et al. (2011), by amplification of *T. pyogenes* specific parts of 16S-23S rDNA intergenic spacer region (ISR) and *T. pyogenes* specific parts of superoxide dismutase A encoding gene *sodA*. Amplification of the known and putative virulence factor encoding genes revealed that *T. pyogenes* 754B carried gene *plo*, the genes *fimA*, *fimC* and *fimE* and tetracycline resistance encoding gene *tet*(*W*) but not gene *cbpA* and both neuraminidases encoding genes *nanH* and *nanP*. The presence and absence of known and putative virulence factor encoding genes of *T. pyogenes* 754B of the present study generally corresponded to previously investigated *T. pyogenes* isolates of various origins (Jost and Billington, 2005; Silva et al., 2008; Santos et al., 2010; Hijazin et al., 2011). However, in contrast to the present study, none of the *T. pyogenes* investigated by Silva et al. (2008) were negative for both neuraminidases encoding genes *nanH* and *nanP*. However, *T. pyogenes* negative for both *nanH* and *nanP* were also observed by Hijazin et al. (2011).

Despite of the negative proteolytic reactions of the *T. pyogenes* 754B investigated in the present study, it could reliably be classified phenotypically and genotypically as *T. pyogenes*. However, whether the lack of proteolytic activity of this strain is caused by a mutation of the respective gene or by a reduced expression of the proteolytic enzyme is not known at present. The importance of bacterial proteases as virulence factors has been pointed out by Travis et al. (1995). However, comparable to the *T. pyogenes* mutant deficient in production of the pore forming cytolysin pyolysin which displayed reduced virulence (Jost et al., 1999) or the reduced adherence properties of a *T. pyogenes nanH* and *nanP* double mutant (Jost et al., 2002b), the *T. pyogenes* 754B described in the present study is considered as the first *T. pyogenes* isolate with no detectable proteolytic activity. These results might serve in future challenge tests investigating the role of proteases of this species.

5.3 Phenotypic and genotypic properties of *T. pyogenes* isolated from bovine genital tract

All 14 *T. pyogenes* isolated from bovine genital tract displayed a complete zone of hemolysis after cultivation on sheep blood agar and no enhancement of hemolysis after cultivation on rabbit blood agar. The 14 *T. pyogenes* showed synergistic CAMP-like reactions with *S. aureus* ß hemolysin, *R. equi* and *A. haemolyticum* but not with *S. agalactiae* as indicator strain and no reverse CAMP reaction in the zone of staphylococcal ß-hemolysin. In addition, all 14 *T. pyogenes* isolates were positive for the enzymes β-D-glucuronidase, β-galactosidase, α-D-glucosidase, N-acetyl-β-D-glucosaminidase, DNase and amylase, for liquefaction of Loeffler agar and the enzyme caseinase and showed a cross-reaction with streptococcal serogroup G-specific antiserum. The 14 *T. pyogenes* isolates were negative for the enzymes β-glucosidase, α-mannosidase and catalase. This, as mentioned before, generally corresponded to other studies investigating *T. pyogenes* (Bisping and Amtsberg, 1988; Carlson and Kontiainen, 1994; Lämmler and Hartwigk, 1995; Hijazin et al., 2011; Hijazin, 2012). Detection of the enzyme amylase of the 14 *T. pyogenes* isolated from cervical swabs of bovines revealed that two *T. pyogenes* from endometritis and two *T. pyogenes* from apparently healthy cows were positive for this enzyme. It was of interest that the production of amylase seemed to be enhanced among *T. pyogenes* isolated from cervical swabs of bovines (26.6%) compared to the *T. pyogenes* isolated from milk (6.9%). The difference in the production of amylase between the two groups appeared to be significant ($P = 0.039$). Corresponding to Hijazin (2012a), the reference strain *T. pyogenes* DSM 20594 of bovine origin was amylase negative and the reference strain *T. pyogenes* DSM 20630^T of porcine origin was amylase positive. It was of interest that among the 14 *T. pyogenes* 9 isolates (64.3%) had identical phenotypical properties as bovine reference strain *T. pyogenes* DSM 20594 and 4 isolates (28.7%) had identical phenotypical properties as porcine reference strain *T. pyogenes* DSM 20630^T.

In addition, all 14 *T. pyogenes* could be identified to the speices level by MALDI-TOF MS fingerprint. The comparison of the log (score) values from the MALDI-TOF MS fingerprint analysis of the 14 *T. pyogenes* strains among each other showed the lowest log (score) value of 2.5 to a maximal log (score) value of 2.9. This very high log score values indicated very similar mass spectra. However, MALDI-TOF MS did not allow a reliable differentiation between the

two groups of isolates. The MSPs of all 14 *T. pyogenes* were matched to the MSPs of the reference strains *T. pyogenes* DSM 20630^T and *T. pyogenes* DSM 20594. The comparison of the log (score) values of the 14 *T. pyogenes* strains with reference strain *T. pyogenes* DSM 20594 showed the lowest log (score) value of 2.1 to a maximal log (score) value of 2.4. However, with *T. pyogenes* DSM 20630^T the strains displayed the lowest log (score) value of 1.9 to a maximal log (score) values of 2.2. These results showed that the 14 *T. pyogenes* isolated from bovine cervical swabs seemed to be slightly more related with the bovine reference strain *T. pyogenes* DSM 20594. According to the present results, MALDI-TOF MS seemed to be a powerful and accurate tool to quickly identify *T. pyogenes*. However, based on the present study design MALDI-TOF MS cannot be recommend for typing of *T. pyogenes.* This technique wasn't able to distinguish significant differences between the two groups of the *T. pyogenes* isolated from bovine genital tract. However, MALDI-TOF MS succeeded in differentiation between pathogenic and non-pathogenic *Leptospira* spp. (Xiao et al., 2015).

Using the primer pair described by Ülbegi (2010) and Ülbegi et al. (2010) for amplification of ISR all 14 *T. pyogenes* isolated from bovine genital tract investigated in the present study could be identified genotypically. The *sodA* species specific oligonucleotide primer developed by Hijazin (2012a) were also used in the current study and could successfully identify all 14 investigated *T. pyogenes* isolates and the two *T. pyogenes* reference strains. According to Hijazin (2012a) there was no recorded cross reactivity using the ISR or *sodA* species specific oligonucleotide primers with the control strains from other species of genera *Arcanobacterium* and *Trueperella*. These results showed that both genes could be used for molecular identification of *T. pyogenes*. Amplification of putative virulence genes reveled that all 14 *T. pyogenes* were positive for the genes *plo, nanH, nanP, fimA, fimC* and *fimE*. Gene *cbpA* could be observed in one isolate (7%) from metritic cows. However, Santos et al. (2010) reported about the presence of *cbpA* in 3% of the *T. pyogenes* isolated from metritic cows but not in non metritic cows. Esmay et al. (2003) reported about the presence of gene *cbpA* in 49%, Silva et al. (2008) in 100% and Hijazin et al. (2011) in 7% of the *T. pyogenes* strains. Santos et al. (2010) described that gene *fimA* could be found in 90.9% of the *T. pyogenes* isolated from metritic cows and in 64.1% of the *T. pyogenes* isolated from non metritic cows.

The tetracycline resistance gene *tet*(*W*) in *T. pyogenes* was amplified using the oligonucleotide primer described by Billington and Jost (2006). In the current study the tetracycline resistance

gene *tet*(*W*) could be amplified in 9 (64.3%) of the *T. pyogenes* isolated from bovine genital tract. The tetracycline resistance gene *tet*(*W*) could be amplified in 3 (21.4%) of the *T. pyogenes* isolated from metritic cows. However, gene *tet*(*W*) could be amplified in all six *T. pyogenes* isolated from non metritic cows. Tetracycline resistance gene *tet*(*W*) was reported in previous studies for 42% of the investigated *T. pyogenes* isolates (Billington and Jost, 2006), for 18.2% of *T. pyogenes* isolated from metritis cows and for 51.3% of the investigated *T. pyogenes* isolated from non metritic cows (Zastempowska and Lassa, 2012). Other authors reported that the majority of isolates of bovine origin have the tetracycline resistance gene *tet*(*W*) but none of the isolates of porcine origin (Billington et al., 2002). There are three functional genetic elements which carry *tet(W)* in *T. pyogenes* isolates. These elements are transferable to other isolates but differ from those elements that carry *tet(W)* in other species (Billington and Jost, 2006).

The presented phenotypical and genotypical analysis did not detect a clonal homogencity of the two groups of *T. pyogenes* isolated from bovine genital tract, respectively. In addition, no characteristic property could be determined which seems to be responsible for the development of the disease, indicating that additional criteria might be responsible for onset and etiopathology of bovine metritis.

5.4 Phenotypic and genotypic properties of *T. pyogenes* isolated from three grey slender lorises

Grey slender lorises are a primate species from the Lorisidea family whose taxonomy is currently under revision. There habitat is eastern and southern India as well as Sri Lanka (Perera, 2008). Grey slender lorises are primarily insectivorous nocturnal animals with loose social interactions. They forage on trees in dry zone forests where they also sleep in aggregations of several animals. At Frankfurt Zoo they are kept in pairs in the nocturnal animal house.

The first six year old grey slender loris was found dead in the terrarium 2011. Later on, two other grey slender lorises kept in the same terrarium died in 2012 and *T. pyogenes* could be found as one of the major pathogens. In the present study the *T. pyogenes* isolated from these three grey slender lorises were identified phenotypically and genotypically and investigated for epidemiological relationships by various genotypical tests. The three *T. pyogenes* isolated from grey slender lorises exhibited a complete zone of hemolysis after cultivation on sheep blood agar under microaerobic conditions in a candle jar. No enhancement of hemolysis could be observed after cultivation of the bacteria on rabbit blood agar. The three *T. pyogenes* isolated from grey slender lorises showed a synergistic CAMP-like reaction with *S. aureus* ß-hemolysin and *R. equi* and *A. haemolyticum* as indicator strains, respectively and no reverse CAMP reaction in the zone of staphylococcal ß-hemolysin. However, no synergistic or antagonistic CAMP-like reaction could be observed with *S. agalactiae* as indicator strain. These results, as mentioned before (5.1, 5.2, and 5.3), come in line with several previous studies (Lämmler and Blobel, 1988; Ülbegi-Mohyla et al., 2009).

The phenotypic identification and further characterization of the *T. pyogenes* isolates from three grey slender lorises origin showed a positive reaction for the enzymes pyrrolidonyl arylamidase, alkaline phosphatase, β-D-glucuronidase, β-D-galactosidase, α-D-glucosidase, N-acetyl-β-D-glucosaminidase, DNase and amylase, for degradation of D-glucose, D-ribose, D-maltose, D-lactose, D-saccharose and glycogen, for liquefaction of Loeffler agar and for the enzyme caseinase which is also corresponding with previous studies (see 5.1; 5.2; 5.3). In addtion, the *T. pyogenes* isolates from three grey slender lorises origin showed a negative

reaction for the enzymes pyrazinamidase, β-glucosidase, urease, catalase α-galactosidase, α-mannosidase and for esculin hydrolysis.

The three *T. pyogenes* isolates also displayed a cross-reaction with group G-specific antiserum, which is considered as typical characteristic of *T. pyogenes* (see 5.1, 5.2, 5.3). This initial biochemical screening and analysis of the three *T. pyogenes* isolated from the grey slender lorises origin exhibited the same phenotypic profile with 100% similarity among each other and to *T. pyogenes* DSM 20630^T of porcine orgin.

MALDI-TOF MS has been widely used in routine laboratory practice for microbial identification because of its economical and diagnostic benefits (Decristophoris et al., 2011; Gaia et al., 2011; Hijazin et al., 2012b). In the present study, MALDI-TOF MS has been successfully used to identify the *T. pyogenes* isolates from three grey slender lorises origin and twelve reference strains of genera *Trueperella* and *Arcanobacterium*, also including the two *T. pyogenes* reference strains. The three *T. pyogenes* isolates could successfully be identified to the species level by matching there MSPs against the spectral database library using the MALDI Biotyper 3.1 software package. The three *T. pyogenes* isolates could be identified to species level with a log (score) value of 2.59 and 2.53 among each other and with log (score) values between 2.2 and 2.54 with the two reference strains *T. pyogenes* DSM 20630^T and *T. pyogenes* DSM 20594. As mentioned before log (score) values ≥ 2.0 are rated as identification of bacteria to species level (Mellmann et al., 2008; Barbuddhe et al., 2008; Ilina et al., 2009; Lartigue et al., 2009; Marklein et al., 2009; Nagy et al., 2009). MALDI-TOF MS is already accepted as identification tool to genus and species level (Lynn et al., 1999; Bernardo et al., 2002; Ruelle et al., 2004; Rupf et al., 2005; Murray, 2010; Bizzini et al., 2011; Clark et al., 2013; Hrabák et al., 2013). However, MALDI-TOF MS as discriminatory tool below the species level are still under altercation and did not reach, as also shown for the three grey slender lorises isolates of the present study, the desired level as subtyping method for different species (Wolters et al., 2011; Lartigue, 2013; Sandrin et al., 2013; Lasch et al., 2014).

The genotypic identification of the three *T. pyogenes* isolated from three grey slender lorises was performed by analysis of the 16S rRNA gene and gene *gap*. It is well known that the 16S rRNA gene sequence is a good indicator of phylogenetic relationships among bacteria at the intra- and interspecies level. In this study, the 16S rRNA gene sequence analysis and the sequence of gene *gap* showed a very good identification of the three *T. pyogenes* isolated from three grey slender

lorises as *T. pyogenes*. Also the dendrogram analysis of the 16S rRNA gene sequences and gene *gap* sequences of these isolates together with the 16S rRNA and gene *gap* sequences of the reference strains of genera *Trueperella* and *Arcanobacterium* obtained from NCBI GenBank allowed a clear identification as *T. pyogenes*. But as expected, the analysis of 16S rRNA gene sequence alone was not sufficient to discriminate the three *T. pyogenes* isolates from grey slender lorises and the reference strains *T. pyogenes* DSM 20594 and *T. pyogenes* DSM 20630^T. In addition, sequencing of gene *gap* was not sufficient to discriminate the three *T. pyogenes* isolated from the grey slender lorises and the reference strain *T. pyogenes* DSM 20594. However, these findings showed that gene *gap* seems to be not an appropriate genetic marker to subtype the isolates of the present study.

As additional genetic marker the three *T. pyogenes* isolated from the grey slender lorises could successfully be identified by genotypic amplification of ISR and gene *sodA* using the primer pairs described by Ülbegi (2010) and Hijazin (2012a), respectively.

The three *T. pyogenes* were additionally postive for the putative virulence genes *plo, nanH, fimA, fimC* and *fimE*. However, the three *T. pyogenes* were negative for the genes *nanP* and *cbpA*. Moreover, the tetracycline resistance gene *tet*(*W*) could be amplified for all three *T. pyogenes* isolates. The presence or absence of the eight genes encoding putative virulence factors (*plo*, *cbpA, nanH, nanP, fimA, fimC, fimE*) and *tet*(*W)* of the three *T. pyogenes* isolated from the grey slender lorises and the two reference strains *T. pyogenes* DSM 20594 and *T. pyogenes* DSM 20630^T displayed three phylogroups. Group A assembled the three *T. pyogenes* isolated from the grey slender lorises, group B the reference strain *T. pyogenes* DSM 20594 and group C the reference strain *T. pyogenes* DSM 20630^T. The phenotypic and genotypic characterization described in the present study revealed that the three *T. pyogenes* isolated from the grey slender lorises acquired the same phenotypic and genotypic profile.

There are dispersed repetitive sequences separating longer single-copy DNA sequences in both prokaryotic and eukaryotic genomes. Genomic fingerprinting by polymerase chain reaction techniques use oligonucleotide primers complementary to repetitive sequences (rep-PCRs) distributed randomly throughout the genome, which amplifies numerous regions of targeted DNA surrounded by the repetitive sequences. These results in amplification patterns specific for each individual strain finally form a genomic fingerprinting of the microbe (Versalovic et al., 1991; Rademaker and de Bruijn, 1997; Mohapatra et al., 2007). The rep-PCRs fingerprinting is

depending on conserved repetitive sequences which are divided into four types: the repetitive extragenic palindromic (REP) sequences, the enterobacterial repetitive intergenic consensus (ERIC) sequences, the BOX sequences and the polytrinucleotide $(GTG)_5$ sequences (Versalovic et al., 1991; Versalovic et al., 1994).

The Random Amplified Polymorphic DNA (RAPD) is a PCR based technique used for identifying genetic variations by using of a single arbitrary primer in a PCR reaction. The amplification of genomic DNA with this arbitrary primer leads to distinct DNA products which can be used as genetic markers (Williams et. al., 1990; Welsh and McClelland, 1990). The RAPD-PCR has already been used for epidemiological subtyping of different bacteria (Stephan et al., 1994;, Eisen et al., 1995: Hijazin et al., 2013; Kämpfer et al., 2013).

The genotypic differentiation of the three *T. pyogenes* isolates from the grey slender lorises was performed with these different genomic fingerprinting methods. The PCR fingerprinting by using rep-PCRs and by RAPD-PCR discriminated the three *T. pyogenes* isolates from the grey slender lorises and the reference strains *T. pyogenes* DSM 20594 and *T. pyogenes* DSM 20630^T into three genotype profiles. Based on the results of cluster analysis and amplification pattern, $(GTG)_5$–PCR was found to be the most suitable method for molecular typing of the three *T. pyogenes*, followed by ERIC-PCR and BOX-PCR. The $(GTG)_5$–PCR appeared to be better than other genomic fingerprinting tools for the analysis of the *T. pyogenes* samples because it was less complex and had more discriminative properties. The REP-PCR was excluded because the bands were in low number and showed little discrimination power (data not shown).

The present investigation represents the first comparative study to evaluate the efficacy of three rep-PCR methods in molecular discrimination of *T. pyogenes* isolates. Additionally, this is also the first study to assess the potential of RAPD-PCR in tracking the genomic fingerprinting of *T. pyogenes* isolates obtained from an exotic origin.

The REP-PCR and ERIC-PCR were already used for subtyping *E. coli* isolated from cows with clinical mastitis while ERIC-PCR showed a better discriminatory power than REP-PCR (Lipman et al., 1995). REP-PCR and BOX-PCR were also used to discriminate *E. coli* isolates of various origins (human, duck, geese, chicken, pig, sheep and cow) while BOX-PCR showed a superior discriminatory efficacy than REP-PCR (Dombek et al., 2000). In another study REP-PCR and ERIC-PCR were used for subtyping *E. coli* isolates of different origins (McLellan et al., 2003). According to Leung et al. (2004) ERIC-PCR proved to be not successful for subtyping *E. coli*.

Mohapatra et al. (2007) used REP-PCR, ERIC-PCR, ERIC2-PCR, BOX-PCR and $(GTG)_5$-PCR in differentiation of *E. coli* isolates while $(GTG)_5$-PCR showed a respected discriminant function. $(GTG)_5$-PCR also showed a superior discriminatory power in comparison to REP-PCR, ERIC-PCR and BOX-PCR in subtyping different microbes like *Lactobacillus* (Gevers et al., 2001) *Enterococcus* (Švec et al., 2005) and *Salmonella* (Rasschaert et al., 2005). ERIC-PCR, BOX-PCR and REP-PCR were also evaluated in differentiating the genetic relatedness of clinical *Stenotrophomonas maltophilia* isolates of human origin while ERIC-PCR depicted less discriminatory power than BOX-PCR and REP-PCR (Lin et al., 2008). The RAPD-PCR technique was already used for differentiation and for analysis of the genetic structure of closely related bacteria of *S. aureus* (Chiang et al., 2014), *S. epidermidis* (Wieser and Busse 2000) and *E. coli* (Kar et al., 2015). The wide use of the RAPD-PCR is due to the fact that this method is more sensitive in detecting genetic diversity than 16s rRNA sequencing because it depends on the whole genome (van Rossum et al., 1995). According to Thong et al. (2002) PCR fingerprinting subtyping methods are widely used for DNA typing and the differentiation between them is depending on several evaluation criteria like reproducibility, discriminatory power and cost and ease of interpretation. Till now Pulsed-field gel electrophoresis (PFGE) is the "gold standard" molecular typing method and well known for its reproducibility. However, Pulsed-field gel electrophoresis is an expensive, more complex and time consuming method. The PCR fingerprinting subtyping methods described before offer alternative techniques that can be applied on a small or a large number of strains which can be easily interpreted (Olive and Bean, 1999).

The novel MLSA mentioned before (5.1) could also be used for epidemiological typing of the *T. pyogenes* isolated from the three grey slender lorises. Again the fragments *fusA* (746 nt), *tuf* (795 nt), *metG* (836 nt) and *gyrA* (937 nt) of the *T. pyogenes* were sequenced.

On the basis of gene *fusA* sequences, the three *T. pyogenes* isolates of grey slender lorises origin and the reference strains *T. pyogenes* DSM 20594 and *T. pyogenes* DSM 20630^T of the present study were genetically segregated. At the intergenus level the gene *fusA* sequence similarity was at maximum 70.5% for *A. haemolyticum* DSM 20595^T. At the intraspecies level, the isolates of the same species had at least 99.3% gene *fusA* sequence similarity. The three *T. pyogenes* isolates of grey slender lorises origin shared 100% gene *fusA* sequence similarity among each other and a similarity of 99.5% and 99.3%, respectively to the reference strains *T. pyogenes* DSM 20594 and

T. pyogenes DSM 20630^T. The analysis of the intragenomic GC content of the investigated *T. pyogenes* isolates showed that the three investigated *T. pyogenes* isolates and the reference strain *T. pyogenes* DSM 20630^T have the same percentage with 60.1% and the reference strain *T. pyogenes* DSM 20594 a GC content of 60.5 mol%. However, the pairwise comparison of the gene *fusA* MLSA among the *T. pyogenes* strains showed that the three investigated *T. pyogenes* isolates from three grey slender lorises have 100% similarity among each other and a sequence similarity of 99.5% and 99.3% to the reference strains *T. pyogenes* DSM 20594 and *T. pyogenes* DSM 20630^T, respectively. Translation of the partial DNA sequence of gene *fusA* to the protein sequence revealed 18 amino acids. The three investigated *T. pyogenes* isolates and the reference strains *T. pyogenes* DSM 20594 and *T. pyogenes* DSM 20630^T showed the same amino acid composition in 16 positions. However, the amino acid compositions of the FusA proteins of the *T. pyogenes* isolates varied in two amino acids (glutamic acid with 8.9% or 9.3% and asparagine with 8.1% or 7.7%, respectively). The three *T. pyogenes* isolates of grey slender lorises origin have the same composition percentage of glutamic acid (8.9%) and asparagine (8.1%) which is different to the reference strains (9.3% and 7.7%, respectively). The percentage of amino acids composition similarity among the three investigated *T. pyogenes* isolates from three grey slender was 100% among each other and 99.6% to the reference strains *T. pyogenes* DSM 20594 and *T. pyogenes* DSM 20630^T.

On the basis of gene *tuf* sequence the three *T. pyogenes* isolates of grey slender lorises origin differed from the reference strains *T. pyogenes* DSM 20594 and *T. pyogenes* DSM 20630^T. At the intergenus level, the gene *tuf* sequence similarity was at maximum 82.3% for *A. haemolyticum* DSM 20595^T. At the *T. pyogenes* intraspecies level, the isolates of the same species had at least 99.3% gene *tuf* sequence similarity. The three *T. pyogenes* isolates of grey slender lorises origin shared 100% gene *tuf* sequence similarity among each other and to the reference strains *T. pyogenes* DSM 20594 and *T. pyogenes* DSM 20630^T a sequence similarity of 99.3% and 99.6%, respectively. The nucleotide sequence of gene *tuf* of the selected five *T. pyogenes* isolates showed variations in GC content (60.5 mol% to 60.8 mol%) and 54.6 mol% for the reference strain *A. haemolyticum* DSM 20595^T. The three *T. pyogenes* isolates of grey slender lorises origin shared a GC content of 60.5 mol% , the reference strains *T. pyogenes* DSM 20594 and *T. pyogenes* DSM 20630^T of 60.6% and 60.8%, respectively. Translation of the DNA sequences of gene *tuf* to

protein sequence of the selected five *T. pyogenes* isolates showed no variations in the amino acid composition and did not show any discriminatory power.

On the basis of gene *metG* sequence the three *T. pyogenes* isolates of grey slender lorises origin and the reference strains *T. pyogenes* DSM 20594 and *T. pyogenes* DSM 20630^T of the present study could be differentiated. At the intergenus level, the gene *metG* nucleotide sequence similarity was at maximum 74.76% for *A. haemolyticum* DSM 20595^T. At the intraspecies level the three *T. pyogenes* isolates of grey slender lorises origin had 100% gene *metG* sequence similarity and a sequence similarity of 98.2% and 98.4% to the reference strains *T. pyogenes* DSM 20594 and *T. pyogenes* DSM 20630^T, respectively. The nucleotide sequences of gene *metG* of the selected *T. pyogenes* isolates showed variations in GC contents (60.5 mol% to 61 mol%) and 53 mol% for reference strain *A. haemolyticum* DSM 20595^T. The three *T. pyogenes* isolates of grey slender lorises origin shared a GC content of 60.8 mol%. The reference strains *T. pyogenes* DSM 20594 and *T. pyogenes* DSM 20630^T had GC contents of 60.5 mol% and 61 mol%, respectively. Translation of the DNA sequences of gene *metG* to the correspondent protein sequence of the five selected *T. pyogenes* isolates showed variations in the amino acid composition. According to the partial MetG protein sequence the three *T. pyogenes* isolates had 100% amino acid sequence similarity among each other and 99.28% amino acid sequence similarity to the reference strains *T. pyogenes* DSM 20594 and *T. pyogenes* DSM 20630^T. Translation of the partial DNA sequences of gene *metG* to MetG protein sequences of the five selected *T. pyogenes* isolates revealed 20 amino acid positions. The investigated three *T. pyogenes* isolates and the reference strains *T. pyogenes* DSM 20594 and *T. pyogenes* DSM 20630^T showed an identical amino acid composition in 15 positions. However, the amino acid composition of the MetG proteins from the three *T. pyogenes* isolates and the *T. pyogenes* reference strains varied in five amino acids (glycine with 5.8% to 6.12%, aspartic acid with 9.7% to 9.6%, isoleucine 4.7% to 5%, methionine 2.2% to 2.5% and asparagine 4.3% to 5%). The three *T. pyogenes* isolates of grey slender lorises origin had the same composition percentage of aspartic acid (9.7%) and asparagine (4.7%) among each other, which was different to the reference strains. The three *T. pyogenes* isolates of grey slender lorises origin and reference strain *T. pyogenes* DSM 20594 had the same composition percentage of glycine with 5.8%, the reference strain *T. pyogenes* DSM 20630^T with 6.12%. The three *T. pyogenes* isolates of grey slender lorises origin and the reference strain *T. pyogenes* DSM 20630^T had the same composition

percentage of isoleucine with 4.7% and methionine with 2.5%, the reference strain *T. pyogenes* DSM 20594 with 5.4% and 2.16%, respectively.

Using the DNA gyrase (subunit A) encoding gene *gyrA* sequences the three *T. pyogenes* isolates of grey slender lorises origin and the reference strains *T. pyogenes* DSM 20594 and *T. pyogenes* DSM 20630^T formed distinct branches and could be clearly differentiated. At the intergenus level the gene *gyrA* sequence similarity between the three *T. pyogenes* and *A. haemolyticum* DSM 20595^T was at maximum 72.7%. At the *T. pyogenes* intraspecies level the three *T. pyogenes* isolates of grey slender lorises origin had 100% gene *metG* sequence similarity among each other and a sequence similarity of 98.5% and 98.7% to the reference strains *T. pyogenes* DSM 20594 and *T. pyogenes* DSM 20630^T, respectively. The three *T. pyogenes* isolates of grey slender lorises origin shared the same GC content with 63.3 mol% while the reference strains *T. pyogenes* DSM 20594 and *T. pyogenes* DSM 20630^T had a GC content of 63 mol%. Translation of the partial DNA sequences of gene *gyrA* to GyrA protein sequence of the five selected *T. pyogenes* isolates revealed 20 amino acids. The investigated three *T. pyogenes* isolates and the reference strains *T. pyogenes* DSM 20594 and *T. pyogenes* DSM 20630^T showed an identical amino acid composition in all 20 positions. The amino acid composition of the GyrA protein did not allow a subtyping of the investigated *T. pyogenes* isolates.

The concatenated tree built with partial nucleotide sequences of the four genes of the *T. pyogenes* isolates and the reference strains *T. pyogenes* DSM 20594 and *T. pyogenes* DSM 20630^T clustered in two groups. The first clade (GI) included the three *T. pyogenes* isolates of grey slender lorises origin with 100% pairwise nucleotide sequence similarity among each other. The second clade (GII) included the two reference strains *T. pyogenes* DSM 20594 and *T. pyogenes* DSM 20630^T with 99% pairwise nucleotide sequence similarity to the first clade (GI). The nucleotide composition (GC content) of the four investigated target genes (*fusA-tuf-metG-gyrA*) of the three *T. pyogenes* of the grey slender lorises origin, *T. pyogenes* DSM 20594 and *T. pyogenes* DSM 20630^T exhibited an identical GC content of 61.3 mol%. However, percentage in the content of Guanine and Cytosine were identical for the three *T. pyogenes* isolates of grey slender lorises orgin. (29.7 mol%, 31.6 mol% respectively) and for the two reference strains *T. pyogenes* DSM 20594 and *T. pyogenes* DSM 20630^T (29.8 mol% and 31.5 mol% respectively).

The analyses of the concatenated amino acid sequences were conducted using the four genes of the three *T. pyogenes* isolates and the reference strains *T. pyogenes* DSM 20594 and *T. pyogenes*

DSM 20630^T. The phylogenetic analysis based on concatenated partial FusA, Tuf, MetG and GyrA amino acid sequences clades the three *T. pyogenes* of grey slender lorises origin and the two reference strains *T. pyogenes* DSM 20594 and *T. pyogenes* DSM 20630^T of the present study into two clades. The first clade (GI) included the three *T. pyogenes* isolates of grey slender lorises origin with 100% amino acid identity among each other. The second clade (GII) included the two reference strains *T. pyogenes* DSM 20594 and *T. pyogenes* DSM 20630^T with 99.7% amino acid identity compared to the first clade (GI). Translation of the DNA sequences of the concatenated nucleotide sequences of the three *T. pyogenes* isolates of grey slender lorises origin and the two reference strains *T. pyogenes* DSM 20594 and *T. pyogenes* DSM 20630^T to partial protein sequences revealed 20 different amino acids.

The amino acid composition of the three *T. pyogenes* isolates and the two reference strains *T. pyogenes* DSM 20594, *T. pyogenes* DSM 20630^T were identical in 14 amino acids and showed variations in six separate amino acids (aspartic acid, glutamic acid, asparagine, isoleucine, methionine and serine). The three *T. pyogenes* isolated from grey slender lorises showed identical amino acid compositions for aspartic acid, glutamic acid, asparagine (8.44%, 8.26% and 3.36% respectively), the two reference strains *T. pyogenes* DSM 20594 and *T. pyogenes* DSM 20630^T showed identical amino acid composition for aspartic acid (8.26%) but a variation in the amino acid composition for glutamic acid (8.35% and 8.44%) and asparagine (3.45% and 3.27%). The three *T. pyogenes* isolated from grey slender lorises and the reference strain *T. pyogenes* DSM 20594 showed identical amino acid composition for serine with 4.4% but not with the reference strain *T. pyogenes* 20630^T (4.5%). The three *T. pyogenes* isolated from grey slender lorises and the reference strain *T. pyogenes* DSM 20630^T showed identical amino acid composition for isoleucine with 6.35% and methionine 2.4% but not with the reference strain *T. pyogenes* 20594 (6.4% and 2.3%, respectively).

The several genetic marker of the presented MLSA scheme showed clearly that the three *T. pyogenes* isolates of grey slender lorises origin and the two *T. pyogenes* reference strains belong to three different clonal complexes, respectively. The results of the present investigation which represents the first detailed epidemiological study of *T. pyogenes* of this origin clearly indicated that all three *T. pyogenes* isolates which contributed with other potentially pathogenic bacteria to the septicemia of the three grey slender lorises, respectively had a common clonal origin. However, whether the cross infection between the animals with is *T. pyogenes* strain, which

seems to be constantly present in Frankfurt Zoo over a certain period of time, occurred because of direct contact of the animals or a lack of disinfection of the animal facility after the detection of the first or the second case remains unclear.

6 Summary

In the present study *T. pyogenes* isolated from milk of mastitic dairy cattle (n=57, n=1), from genital tract of metritic and apparently healthy dairy cattle (n=14) and from fatal infections of three grey slender lorises (n=3) together with 12 reference strains representing ten species of genera *Trueperella* and *Arcanobacterium* could be identified and characterized individually by using phenotypical methods, by MALDI-TOF MS fingerprinting, by FT-IR spectroscopy and by various genotypic techniques investigating the molecular targets 16S rRNA, ISR, *sodA* and *gap*. The genotypic techniques also included the amplification of the putative virulence factor encoding genes *plo*, *cbpA*, *nanH*, *nanP*, *fimA*, *fimC*, *fimE* and *tet(W)*. A collection of the investigated *T. pyogenes* from milk of mastitic dairy cattle were subjected to epidemiological studies using MLSA and the *T. pyogenes* from fatal infections of three grey slender lorises using rep-PCRs , RAPD-PCR and MLSA.

The *T. pyogenes* of mastitic origin were isolated in a period of 3 years and were mainly isolated together with various other bacteria from milk of mastitic dairy cattle with varying clinical symptoms. However, *T. pyogenes* seemed to be the major causative agent. The phenotypic properties, also including MALDI-TOF MS analysis and the newly described FT-IR spectroscopy and the genotypic methods, allowed a reliable identification and further characterization of the bacteria of this origin. The *T. pyogenes* of mastitis origin possessed several putative virulence factor encoding genes in varying combinations. These results together with the genomic fingerprinting with a newly established MLSA revealed that bovine mastitis in farms caused by *T. pyogenes* is mainly caused by individual bacterial clones without relation to each other.

The 14 *T. pyogenes* isolated from genital tract of metritic and apparently healthy dairy cattle and from the three grey slender lorises could also be identified phenotypically and genotypically. However, the distribution of virulence factor encoding genes of the *T. pyogenes* isolated from bovine genital tract revealed no significant differences between diseased and apparently healthy animals indicating that additional criteria might be responsible for onset and etiopathology of bovine metritis.

In contrast to the *T. pyogenes* from mastitis origin and from bovine genital tract the three *T. pyogenes* isolated from grey slender lorises displayed identical phenotypical and genotypical

properties. The latter could also be demonstrated by genomic fingerprinting using three different rep-PCRs, by RAPD-PCR and by MLSA. These results showed that the fatal infection of the three grey slender lorises were caused by a cross infection of a single *T. pyogenes* clone. However, the route of infection of the three grey slender lorises at Frankfurt Zoo remains unclear. In the present study *T. pyogenes* of bovine and grey slender lorises origin could reliably be identified by several phenotypic and genotypic methods and further characterized by determination of putative virulence factor encoding genes and by novel DNA fingerprinting procedures. All these techniques might help to improve a future identification and characterization of *T. pyogenes* and might help to determine epidemiological relationships of these bacteria in animal or human infections.

7 Zusammenfassung

In der vorliegenden Studie wurden 57 *Trueperella (T.) pyogenes*-Stämme, isoliert aus Milch von an Mastitis erkrankten Milchkühen (n=57, n=1), *T. pyogenes*-Stämme, isoliert aus dem Genitaltrakt von an Metritis erkrankten und offensichtlich gesunden Milchkühen (n=14) und drei *T. pyogenes*- Stämme, isoliert von tödlichen Infektionskrankheiten von drei Grauen Schlankloris, zusammen mit 12 Referenzstämme von zehn Arten der Gattungen *Trueperella* und *Arcanobacterium* mithilfe phänotypischer Methoden, durch MALDI-TOF MS und FT-IR-Spektroskopie und durch verschiedene genotypische Techniken durch Nachweis der molekularen Zielstrukturen 16S rRNA, ISR, *sodA* und *gap* untersucht. Die genotypischen Methoden enthielten desweiteren die Amplifizierung der mutmaßlichen Virulenzfaktor-kodierenden Gene *plo*, *cbp*A, *nan*H, *nan*P, *fim*A, *fim*C, *fim*E und *tet*(*W*). Eine Auswahl der untersuchten *T. pyogenes*-Stämme, isoliert von Milch von Kühen mit Mastitis, wurde in epidemiologischen Untersuchungen mittels MLSA und die *T. pyogenes,* isoliert von tödlichen Infektionen der drei Grauen Schlankloris, mittels rep-PCR, RAPD-PCR und MLSA weitergehend analysiert.

Die aus Mastitis isolierten *T. pyogenes*-Stämme wurden überwiegend zusammen mit verschiedenen anderen Bakterienarten aus der Milch von Kühen mit Mastitis mit unterschiedlicher klinischen Symptomatik in einem Zeitraum von 3 Jahren isoliert. *T. pyogenes* schien bei den Mastitisfällen allerdings der Hauptinfektionserreger zu sein. Die phänotypischen Untersuchungen, darunter auch die MALDI-TOF MS-Analysen und die neu beschriebene FT-IR-Spektroskopie und die genotypischen Verfahren, erlaubten eine zuverlässige Identifizierung und weitergehende Charakterisierung der Bakterien diesen Ursprungs. Die *T. pyogenes*-Isolate mit Herkunft Mastitis besaßen mehrere mutmaßlich Virulenzfaktor-kodierende-Gene in unterschiedlichen Kombinationen. Diese Ergebnisse, zusammen mit dem DNA-Fingerprinting in einer neu etablierten MLSA, ergaben, dass *T. pyogenes*-Mastitiden in Betrieben hauptsächlich von unterschiedlichen Erregerklonen ohne Beziehung zueinander verursacht werden.

Die 14 *T. pyogenes*-Stämme die aus dem Genitaltrakt von an Metritis-erkrankten und offensichtlich gesunden Milchkühen sowie von den drei Grauen Schlankloris isoliert wurden, konnten ebenso phänotypisch und genotypisch identifiziert werden. Das Vorkommen der Virulenzfaktor-kodierenden Gene bei *T. pyogenes,* isoliert aus dem Genitaltrakt von Rindern, ergab jedoch keine signifikanten Unterschiede zwischen kranken und offensichtlich gesunden

Tieren, sodass zusätzliche Kriterien für die Entstehung und den Krankheitsverlauf von Rindermetritiden verantwortlich zu sein scheinen.

Im Gegensatz zu den *T. pyogenes*-Stämmen, isoliert von Kühen mit Mastitis und aus dem Genitaltrakt von Rindern, zeigten die drei *T. pyogenes*-Isolate, isoliert von den Grauen Schlankloris identische phänotypische und genotypische Eigenschaften. Letzteres konnte auch durch DNA-Fingerprinting unter Verwendung von drei verschiedenen rep-PCRs, durch RAPD-PCR und durch MLSA nachgewiesen werden. Diese Ergebnisse zeigten, dass die tödlichen Infektionen der drei Grauen Schlankloris durch eine Kreuzinfektion mit einem einzelnen *T. pyogenes*-Klons verursacht wurden. Der Weg der Infektion der drei Grauen Schlankloris im Frankfurter Zoo bleibt allerdings unklar.

In der vorliegenden Studie wurden *T. pyogenes*-Isolate, isoliert von Rindern und von Grauen Schlankloris, durch unterschiedliche phänotypische und genotypische Verfahren identifiziert und durch Bestimmung mutmaßlicher Virulenzfaktor-kodierender Gene und durch neue DNA-Fingerprinting-Verfahren weitergehend charakterisiert. Diese Techniken könnten helfen in Zukunft die Identifizierung und Charakterisierung von *T. pyogenes* zu verbessern und epidemiologische Zusammenhänge bei tierischen oder menschlichen Infektionen mit diesen Bakterien eingehender zu analysieren.

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