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Short Communication

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Phenotypic and genotypic approach to characterize a *Trueperella pyogenes* strain isolated from an Eurasian lynx (*Lynx lynx*)

Phänotypische und genotypische Charakterisierung eines Trueperella pyogenes-Stamms, isoliert von einem Eurasischen Luchs (Lynx lynx)

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Summary

In the present study, a single *Trueperella (T.) pyogenes* strain isolated from a pneumonic lung of an Eurasian lynx (*Lynx lynx*) was identified and further characterized by phenotypical investigations, by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), by Fourier Transform Infrared Spectroscopy (FT-IR) and genotypically by detection of *T. pyogenes* chaperonin-encoding gene *cpn60* with a previously developed loop-mediated isothermal amplification (LAMP) assay and by sequencing the 16S ribosomal RNA (rRNA) gene, the 16S-23S rDNA intergenic spacer region (ISR), the elongation factor tu encoding gene *tuf*, the β-subunit of bacterial RNA polymerase encoding gene *rpoB* and by sequencing the virulence factor pyolysin encoding gene *plo*.
The present study gives a detailed characterization of a single strain of the species *T. pyogenes* from an Eurasian lynx. However, the route of infection of the Eurasian lynx with the bacterial pathogen remains unclear.

Keywords: 16S rRNA gene, 16S-23S rDNA intergenic spacer region (ISR), *tuf, rpoB*, pyolysin

Zusammenfassung

In den vorliegenden Untersuchungen konnte ein einzelner *Trueperella (T.) pyogenes*-Stamm, isoliert von einer veränderten Lunge eines Eurasischen Luchses (*Lynx lynx*), identifiziert und weitergehend charakterisiert werden. Und zwar durch phänotypische Untersuchungen, durch Matrix-assistierte Laser-Desorption-Ionisierung mit Flugzeitanalyse (MALDI-TOF MS) und durch Fourier-Transform-Infrarotspektroskopie (FT-IR), ferner genotypisch durch Nachweis des Chaperonin-kodierenden Gens *cpn60* mit einem kürzlich entwickelten Loop-vermittelten isothermalen Amplifikations(LAMP)-Nachweisverfahren, im Weiteren durch Sequenzierung des 16S ribosomalen RNA(rRNA)-Gens, der 16S-23S rDNA Intergenic Spacer Region, des Elongationsfaktor Tu-kodierenden Gens *tuf*, des die β-Untereinheit der bakteriellen Polymerase-kodierenden Gens *rpoB* und durch Sequenzierung des den Virulenzfaktor Pyolysin-kodierenden Gens *plo*.
Die vorliegende Studie zeigt eine detaillierte Charakterisierung eines *T. pyogenes*-Stamms, isoliert von einem Eurasischen Luchs. Der Infektionsweg des Eurasischen Luchses mit dem bakteriellen Krankheitserreger bleibt allerdings unklar.

Schlüsselwörter: 16S rRNA-Gen, 16S-23S rDNA Intergenic Spacer Region (ISR), *tuf, rpoB,* Pyolysin

Introduction

Within genus *Trueperella* (*T.*) five species were distinguished currently: *T. pyogenes*, *T. abortisuis*, *T. bernardiae*, *T. bonasi* and *T. bialowiezensis* (Yassin et al. 2011). *T. pyogenes* is a worldwiide known pathogen of domestic ruminants and pigs, causing mastitis, abortion and a variety of pyogenic infections (Lämmler and Hartwigk 1995). As summarized by Jost and Billington (2005), this bacterial pathogen is also able to cause diseases in a large number of various animal species including antelopes, wildebeest, gazelles, deer, reindeer, bison, camels, elephants, horses, macaws, chicken and turkeys and also in companion animals such as dogs and cats (Billington et al. 2002). *T. pyogenes* infections in humans are rare (Gahrn-Hansen and Frederiksen 1992) and these infections are often a result of occupational exposure.

In 2010, Ülbegi-Mohyla et al. characterized phenotypically and genotypically two *T. pyogenes* strains isolated from septicaemia of a gecko and a bearded dragon, Eisenberg et al. (2012) a *T. pyogenes* strain isolated in pure culture from a facial abscess of a grey slender loris of Frankfurt Zoo (Frankfurt am Main, Germany), Al-Tarazi et al. (2012) *T. pyogenes* recovered from lung abscesses of one-humped camels and Wickhorst et al. (2017a) a *T. pyogenes* strain isolated from a brain abscess of an adult roebuck.

T. pyogenes possesses a number of known and putative virulence factors that may contribute to its pathogenic potential. A well characterized virulence factor is pyolysin, a haemolysin which is also cytolytic for immune cells (Jost and Billington 2005). The present study was designed to identify and further characterize a *T. pyogenes* strain isolated from a Eurasian lynx (*Lynx lynx*).

Material and Methods

A male 11-year-old Eurasian lynx (*Lynx lynx*) kept in a game park did not appear at the feeding place for three days and was subsequently found dead in its enclosure. The necropsy was performed at the Chemisches und Veterinäruntersuchungsamt Westfalen, Bochum (CVUA-Westfalen). The animal was in moderate body condition weighing 20.2 kg. Gross examination revealed an exudative pyogranulomatous pleuropneumonia with approximately two litres of reddish cloudy pleural exudation in both pleural cavities containing sulfur granules (Fig. 1A, B). Both lung lobes showed severe compression atelectasis. A traumatic lesion could neither detected in the thoracic wall nor in the oesophagus. *T. pyogenes* was isolated from lung in mixed culture with *Pasteurella multocida* and *Escherichia coli*. The *T. pyogenes* lung isolate was further characterized in detail.

Phenotypic analyses were performed by conventional biochemical assays and the API-Coryne System (BioMérieux, Nürtingen, Germany) as described (Al-Tarazi et al. 2012, Hijazin et al. 2011, Nagib et al. 2014, Ülbegi-Mohyla et al. 2010). In addition, the bacterial strain was identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS, Bruker Biotyper) using the commercial database version MBT 7,854 (Wickhorst et al. 2019) and by Fourier Transform Infrared Spectroscopy (FT-IR) (Nagib et al. 2014).

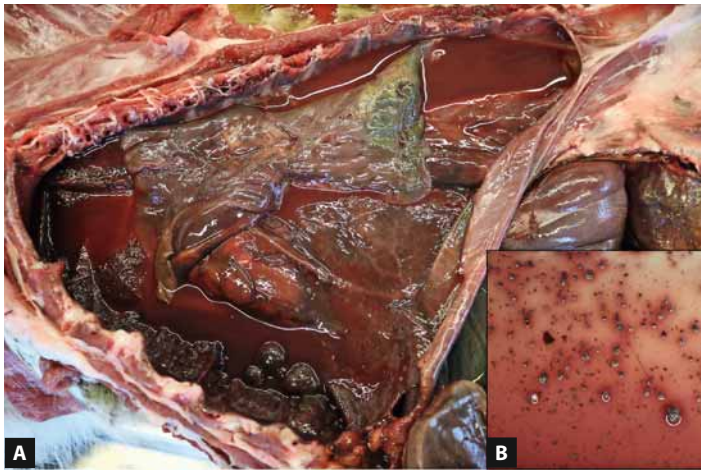

FIGURE 1: *European lynx with severe exudative pleuropneumonia (A). Sulfur granules in thoracic fluid (B). (Photos: Christoph Lämmler, JLU Gießen)*

The presence of *T. pyogenes* chaperonin-encoding gene *cpn60* was determined with a previously described loop-mediated isothermal amplification (LAMP) assay. This was performed using a real-time fluorometer (Genie II®, OptiGene, Horsham, UK) (Abdulmawjood et al. 2016, Wickhorst et al. 2017a).

A further genotypic analysis was conducted by sequencing the 16S ribosomal RNA (rRNA) gene (Hassan et al. 2009), the 16S-23S rDNA intergenic spacer region (ISR) (Hassan et al. 2009, Wickhorst et al. 2017a), by sequencing the elongation factor tu-encoding gene *tuf* (Wickhorst et al. 2017a, b) and by sequencing the β subunit of bacterial RNA polymerase encoding gene *rpoB* (Ülbegi-Mohyla et al. 2010, Wickhorst et al. 2017a). The bacterial strain was also characterized by amplification and sequencing of pyolysin-encoding gene *plo* (Eisenberg et al. 2012, Hijazin et al. 2011, Ülbegi-Mohyla et al. 2010).

Results and Discussion

T. pyogenes S 1276/1/18 investigated in the present study showed a narrow zone of haemolysis on 5% sheep blood agar and CAMP-like reactions in the zone of staphylococcal β-haemolysin and with *Rhodococcus hoagii* as indicator strain. In addition, the isolate could be identified phenotypically with a commercial identification system. The biochemical properties were almost identical to *T. pyogenes* DSM 20630^T and *T. pyogenes* DSM 20594 described previously (Eisenberg et al. 2012, Hijazin et al. 2011, Ülbegi-Mohyla et al. 2010). The isolate gave positive reactions for pyrrolidonyl arylamidase, alkaline phosphatase, β-glucuronidase, β-galactosidase, α-glucosidase, N-acetyl-β-glucosaminidase and negative reactions for nitrate reduction and pyrazinamidase. *T. pyogenes* S 1276/1/18 hydrolysed gelatine and esculin, but not urea, fermented D-glucose, D-ribose, D-xylose, D-maltose, D-lactose, D-saccharose, but not D-mannitol and glycogen. In addition, the isolate showed a negative catalase reaction and a positive reaction on Löffler agar. A positive reaction on Löffler agar is typical for *T. pyogenes* and widely used for phenotypic identification of this species (Bisping and Amtsberg 1988, Eisenberg et

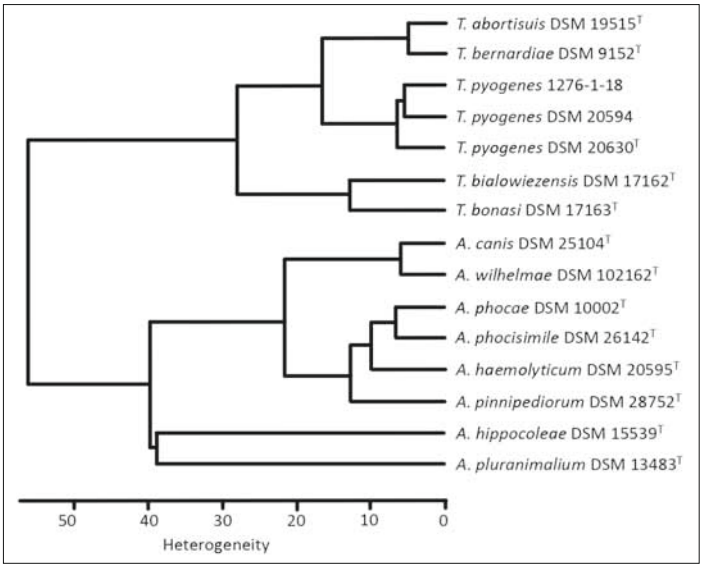

FIGURE 2: *Dendrogram of infrared spectra of T. pyogenes S1276/1/18 investigated in the present study in comparison with T. pyogenes DSM 20630T and T. pyogenes DSM 20594 and with other species of genera Trueperella and Arcanobacterium. Cluster analysis of FT-IR was performed by using the second derivatives of the spectra in the spectral ranges of 500 to 1800 cm^{-1} and 2800 to 3000 cm^{-1}. Ward's algorithm was applied. (Graphic: Christoph Lämmler, JLU Gießen)*

al. 2012, Hijazin et al. 2011, Lämmler 1990, Lämmler and Hartwigk 1995, Wickhorst et al. 2017a). The species identification of *T. pyogenes* S 1276/1/18 could be confirmed by MALDI-TOF MS analysis with a log (score) value of 2,14 for the first, and 2,108 for the second hit, according to the current decision rules of the manufacturer. Similar to the present results MALDI-TOF MS had already been shown to be a rapid and reliable technique for identification of bacteria of genera *Arcanobacterium* and *Trueperella*, also including *T. pyogenes* (Hijazin et al. 2012, Wickhorst et al. 2017a).

FT-IR spectroscopy, a promising technique for rapid and reliable identification of bacterial microorganisms, had already been used as tool for classification of *Listeria* (Janbu et al. 2008) and *Yersinia* species (Kuhm et al. 2009) and for a large number of other clinically relevant pathogens (Contzen et al. 2011, Grunert et al. 2013, Samuels et al. 2009), also including *T. pyogenes* isolated from bovine mastitis (Nagib et al. 2014). The infrared spectra of *T. pyogenes* S 1276/1/18 of the present study were analysed by the method described before (Nagib et al. 2014). Cluster analyses revealed spectra which were closely related to the type strain *T. pyogenes* DSM 20630T and to *T. pyogenes* DSM 20594 and clearly separated from available spectra of other species of the genus *Trueperella* and the genus *Arcanobacterium* (Fig. 2).

Comparable to the previously described LAMP assay for detection of gene *cpn60* of *T. pyogenes* of various origins (Abdulmawjood et al. 2016, Wickhorst et al. 2017a), the species-specific gene *cpn60* of the *T. pyogenes* strain of the present investigation could be successfully identified by using this *cpn60* specific LAMP assay, indicating that this method allows a low-cost and reliable identification of *T. pyogenes*.

The *cpn60* LAMP product could be amplified using a real-time fluorometer (Fig. 3).

T. pyogenes S 1276/1/18 was also identified by sequencing the 16S rRNA gene, ISR, the genes *tuf* and *rpoB* and the putative virulence factor pyolysin encoding gene *plo*. *T. pyogenes* S 1276/1/18 and the type strain *T. pyogenes* DSM 20630T showed sequence similarities of the 16S rRNA gene (acc. no.: MN135984, X79225), ISR (MN164031, EU194563) and for the genes *tuf* (MN163266, HG941716), *rpoB* (MN163265, FN550375) and *plo* (MN163264, U84782) of 99.6%, 100%, 100%, 98.5%, and 99.8%, respectively. A typical dendrogram analyses of ISR and for gene *rpoB* is presented in Figure 4.

Gene *plo* expresses the cholesterol-dependent pyolysin, which is well known as a major virulence factor of this species (Billington et al. 1997, Ding and Lämmler 1996, Jost and Billington 2005) and could be used for molecular identification of this species, because it is generally present in all *T. pyogenes* isolates (Billington et al. 2002, Ertaş et al. 2005, Ülbegi-Mohyla et al. 2010). A phylogenetic analysis of the amino acid sequences of pyolysin (PLO) of *T. pyogenes* S1276/1/18 of the present study, PLO of the reference strain *T. pyogenes* DSM 20630T (AAC45754), phocaelysin (PHL) of *A. phocae* (SMR98720), arcanolysin (ALN) of *A. haemolyticum* (ACV96715) and of other pore forming toxins including streptolysin O (SLO) of *Streptococcus pyogenes* (BAB41212), intermedilysin (ILY) of *Streptococcus intermedius* (BAA89790), pneumolysin (PLY) of *Streptococcus pneumoniae* (ADF28298) and listeriolysin O (HLY) of *Listeria monocytogenes* (NP_463733) obtained from NCBI GenBank revealed a close relationship of PLO of *T.*

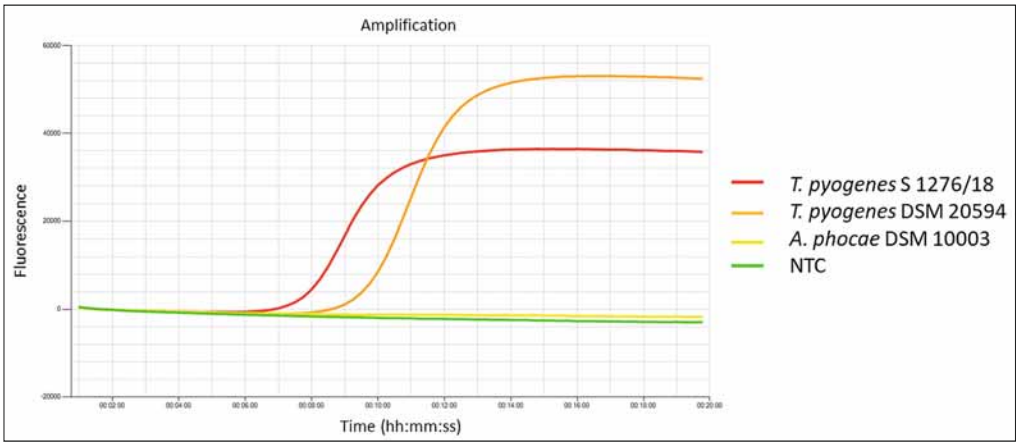

FIGURE 3: *Typical real-time amplification signal of T. pyogenes cpn60 LAMP products of T. pyogenes S 1276/1/18 and T. pyogenes DSM 20549. Negative reaction of A. phocae DSM 10003 and a negative control. (Graphic: Christoph Lämmler, JLU Gießen)*

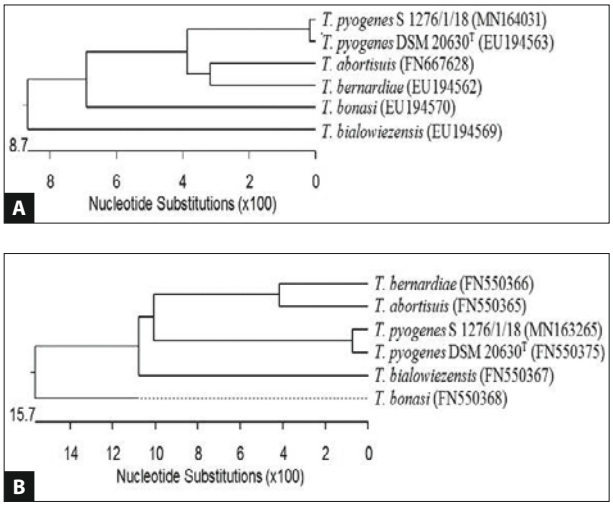

FIGURE 4: *Dendrogram analysis of ISR (A) and rpoB (B) of T. pyogenes S 1276/1/18 and T. pyogenes DSM 20630T and from other species of genus Trueperella obtained from NCBI Genbank using the Clustal W method of DNASTAR/Lasergene MegAlign program (version 8.0.2). (Graphic: Christoph Lämmler, JLU Gießen)*

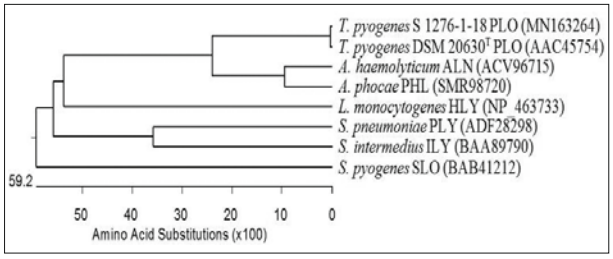

FIGURE 5: *Phylogenetic relationships among amino acid sequences of PLO of T. pyogenes S1276/1/18 of the present study, PLO of reference strain T. pyogenes 20630T, ALN of Arcanobacterium haemolyticum, PHL of Arcanobacterium phocae, HLY of Listeria monocytogenes, PLY of Streptococcus pneumoniae, ILY of Streptococcus intermedius and SLO of Streptococcus pyogenes obtained from NCBI GenBank. (Graphic: Christoph Lämmler, JLU Gießen)*

pyogenes S 1276/1/18 to PLO of *T. pyogenes* DSM 20630T (99.5% amino acid similarity) and a less pronounced relation to the other pore forming toxins (Fig. 5).

T. pyogenes S 1276/1/18 was isolated from a lung of an adult lynx, which suffered from an exudative pleuropneumonia. The strain was identified phenotypically, by MALDI-TOF MS and FT-IR analyses, and genotypically by detection of various species-specific targets. However, the importance of the *T. pyogenes* strain, which was isolated in mixed culture, for the clinical picture of the adult lynx and the route of infection of this bacterial pathogen remains unclear. The MALDI TOF mass-spectrum of *T. pyogenes* S 1276/1/18 and further information for the isolates used for comparison are available via the MALDI-TOF user platform (http://www.maldi-up.ua-bw.de; Rau et al. 2016).

Conflict of Interest

The authors declare no conflict of interest.

Ethical approval

Authors assure to have met common international ethical guidelines during the genesis of the above work, the underlying research and its publication.

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Authors' contribution

Konzeption oder Design der Arbeit: MA, CL.
Datenerhebung: MA, MP, JR, AH, MPlö, AA.
Datenanalyse und Interpretation: MA, MP, JR, AH, OS, EP-B.
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