Phenotypic and Genotypic Analysis of an *Arcanobacterium pluranimalium* Isolated from a Muskox (*Ovibos moschatus*)

Siti Gusti Ningrum[1*], Wyanda Arnafia[1], Osama Sammra[2], Mazen Alssahen[2], Jörn-Peter Wickhorst[2], Abdulwahed Ahmed Hassan[3], Christoph Lämmler[2], Ellen Prenger-Berninghoff[4], Markus Timke[5], Amir Abdulmawjood[6]

Abstract
The present study was designed to characterize an *Arcanobacterium pluranimalium* strain isolated from a muskox (*Ovibos moschatus)* phenotypically, by MALDI-TOF MS analysis and genotypically using various molecular targets. The phenotypic properties, the MALDI-TOF MS analysis and sequencing the 16S rRNA gene, the β subunit of bacterial RNA polymerase encoding gene *rpoB*, the glyceraldehyde 3-phosphate dehydrogenase encoding gene *gap,* the elongation factor tu encoding gene *tuf* and the pluranimaliumlysin encoding gene *pla* allowed a successful identification of the isolated strain as *A. pluranimalium*. Gene *pla* could also be detected by a previously described loop-mediated isothermal amplification (LAMP) assay. This is first report on the isolation and characterization of *A. pluranimalium* originated from a Muskox.

Keywords
Arcanobacterium pluranimalium — muskox — MALDI-TOF MS — 16S rDNA — *rpoB*

[1] *Department of Animal Disease and Veterinary Public Health, Bogor Agricultural University, Bogor, 16680, Indonesia*
[2] *Institut für Pharmakologie und Toxikologie, Justus-Liebig-Universität Giessen, Schubertstr. 81, 35392 Giessen, Germany*
[3] *Department of Veterinary Public Health, College of Veterinary Medicine, University of Mosul, Mosul, Iraq*
[4] *Institut für Hygiene und Infektionskrankheiten der Tiere, Justus-Liebig-Universität Giessen, 35392 Giessen, Germany*
[5] *Entwicklung Bioanalyse, Bruker Daltonik GmbH, Fahrenheitstraße 4, 28359 Bremen, Germany*
[6] *Institut für Lebensmittelqualität und-sicherheit, Stiftung Tierärztliche Hochschule Hannover, 30173 Hannover, Germany*
***Corresponding author**: sitiningrum10@gmail.com

Introduction

Arcanobacterium pluranimalium, belonging to family *Actinomycetaceae*, was initially characterized with two strains isolated from a dead harbor porpoise and a dead fallow dear (14). Further studies described the presence of this bacterial species from a dog with pyoderma (23), from a juvenile giraffe following necropsy (19), from ovine specimen on 33 occasions and from a milk sample of a cow with mastitis (7). Cases of bovine mastitis associated with *A. pluranimalium* were also described by Moser et al. (17), Balbutskaya et al. (3) and Wickhorst et al. (25). The present study gives a first report on the isolation and detailed characterization of an *A. pluranimalium* isolated from a muskox.

Material and Methods

The isolate investigated in the present study was obtained from a 19-year-old female muskox from Zoo of Cologne, Germany, which, after suffer from severe weakness, was euthanized in January 2015. Postmortem analysis revealed a diffuse catarrhal gastroenteritis, a *stomatitis profunda*, a diffuse fatty liver, and arthrosis of tarsal and carpal joints. Bacterial investigations yielded the isolation of *A. pluranimalium* and several other bacteria from the liver in low number and the isolation of aerobic bacilli, α-hemolytic streptococci and *Micrococcus* sp. from the intestine, also in low numbers.

The *A. pluranimalium* isolate was characterized by hemolysis on sheep blood agar, CAMP-like reaction, Reverse CAMP reaction, Api-Coryne test system (Biomerieux, Nürtingen, Germany), tablet containing substrates (Rosco Diagnostica A/S, Taastrup, Denmark), 4-methylumbelliferyl conjugated substrates (Sigma, Steinheim, Germany), catalase, Serolysis on Loeffler agar, caseinase and amylase determination according to previous studies (19, 23, 25). The reference strain used as positive control was obtained from Justus Liebig University bacterial collection.

MALDI-TOF MS analyses were performed according to the extraction protocol of previous study (3). A few colonies of freshly cultured bacteria were suspended into 75% ethanol. After centrifugation, the pellet was resuspended in 30 μl 70 % formic acid and with the same volume of pure acetonitrile. The suspension was centrifuged and 1 ml of the supernatant was transferred to a polished steel MALDI target plate (Bruker Daltonik) and allowed to dry at room temperature. The sample was overlaid with 1 ml matrix (10 mg α-cyano-4-hydroxy-cinnamic acid ml^{-1} in 50 % acetonitrile/2.5 % trifluoroacetic acid). Mass spectra were acquired using a microflex mass spectrometer (Bruker Daltonik) in the linear mode and a mass range of 2–20 kDa using the automated functionality of flexControl 3.0 software (Bruker Daltonik). At least 20 raw spectra were used

Table 1. Oligonucleotide primer sequences and PCR conditions

Oligonucleotide primers	Sequences	Program*	Size of PCR product (bp)	References
16S rDNA UNI-L 16S rDNA UNI-R	5'-AGAGTTTGATCATGGCTCAG-3' 5'-GTGTGACGGGCGGTGTGTAC-3	1	1352	9
*rpo*B-F *rpo*B-R	5'-CGWATGAACATYGGBCAGGT-3' 5'-TCCATYTCRCCRAARCGCTG-3'	2	406	24
gap-F *gap*-R	5'-TCGAAGTTGTTGCAGTTAACGA-3' 5'-CCATTCGTTGTCGTACCAAG-3'	3	784	20
tuf-F *tuf*-R	5'-GGACGGTAGTTGGAGAAGAATGG-3' 5'-CCAGGTTGATAACGCTCCAGAAGA-3'	4	796	25
pla-F *pla*-R	5'-GTTGATCTACCAGGATTGACGC-3' 5'-TTGTCGGGGTGTCCATTGCC-3'	5	283	3

*PCR program:

1: x1 (10 min at 95°C), x30 (30 s at 95°C, 60 s at 58°C, 60 s at 72°C), x1 (7 min at 72°C),

2: x1 (10 min at 95°C), x35 (30 s at 94°C, 30 s at 37°C, 120 s at 72°C), x1 (10 min at 72°C),

3: x1 (3 min at 94°C), x30 (30 s at 94°C, 40 s at 50°C, 60 s at 72°C), x1 (5 min at 72°C),

4: x1 (3 min at 94°C), x30 (45 s at 94°C, 40 s at 57°C, 60 s at 72°C), x1 (7 min at 72°C),

5: x1 (3 min at 94°C), x30 (45 s at 94°C, 30 s at 57°C, 60 s at 72°C), x1 (7 min at 72°C).

to generate a main spectrum. The main spectrum of the *A. pluranimalium* analysed in the present study was matched to the database included in the MALDI Biotyper 2.0 software package and to the newly acquired main spectra of reference strains representing six species of the genera *Arcanobacterium*. The software calculates a similarity score [log (score)] by calculation of a value considering the proportion of matching peaks between the unknown spectrum and the main spectrum of the database, the frequency of peaks in multiple measurements as well as the consistency of the peak intensities between these spectra. The logarithmized score values range from 0 (no similarity) to 3 (absolute identity). Log (score) values $\geq$2.3 are rated as highly probable species identification. Log (score) values 1.8-2.299 are considered as identification of microorganisms on the genus level and probable species level. Log (score) values 1.6-1.799 are considered as identification of microorganisms at least on the genus level. Log (score) values $<$1.6 indicate that a spectrum is not suitable for identification by the MALDI Biotyper.

16s rDNA, the β subunit of bacterial RNA polymerase encoding gene *rpoB*, the glyceraldehyde 3-phosphate dehydrogenase encoding gene *gap*, the elongation factor tu encoding gene *tuf* and the pluranimaliumlysin encoding gene *pla* were sequenced as described by Hassan et al. (9), Ülbegi-Mohyla et al. (24), Sammra et al. (20), Wickhorst et al. (25) and Balbutskaya et al. (3). A detailed description of the primer sequences and the temperature programs is listed in Table 1.

The presence of gene *pla* was determined with a previously described loop-mediated isothermal amplification (LAMP) assay (2). This was performed using a heat block and subsequent detection of the LAMP product in an agarose gel and by using a real-time fluorometer (Genie II®, Optigene, UK).

Results

The bacterial strain investigated in the present study could be identified phenotypically and genotypically as *A. pluranimalium*. The phenotypical test revealed the typical biochemical properties of this species (Table 2). According to MALDI-TOF MS analysis the investigated strain could be identified to the species level matching to type strain *A. pluranimalium* DSM 13483 with a log (score) value of 2.689 (Figure 1).

Sequencing 16S rDNA, the β subunit of bacterial RNA polymerase encoding gene *rpoB,* the glyceraldehyde 3-phosphate dehydrogenase encoding gene *gap*, the elongation factor to encoding gene *tuf* and pluranimaliumlysin encoding gene *pla* revealed a sequence identity of 99.9%, 99.8%, 99.9% 99.6%, and 99.6% to the respective sequences of type strain *A. pluranimalium* DSM 13483.

Typical dendrograms of the sequencing results of 16S rDNA and the genes *rpoB*, *gap*, *tuf* and *pla* are shown in Figure 2, 3 and 4. The species-specific gene *pla* of *A. pluranimalium* 230/15 of the present study could also successfully be detected by using a LAMP assay. The *pla* LAMP products could be detected as amplification signal using a real-time fluorometer (Figure 5).

Discussion and conclusion

A. pluranimalium strain 230/15 investigated in the present study was identified by determination of hemolysis, CAMP-like hemolytic reactions and by biochemical properties. The biochemical properties of the investigated strain showed the typical characteristics of this species.

Comparable to previously conducted MALDI-TOF MS analysis investigating bacteria of genus *Arcanobacterium, Trueperella* and *A. pluranimalium* of various origins (3, 10, 19, 25), the investigated strain of the present study could be clearly identified to the species level. MALDI-TOF MS has already been shown by numerous authors to constitute as rapid and reliable method for identification of various microorganisms (4, 18, 22).

In addition, several molecular targets have been exploited for molecular identification of this strain. The determination of sequences of various molecular targets is an important tool for identification of bacteria and for phylo-

Table 2. Biochemical properties of *A. pluranimalium* 230/15 investigated in the present study and type strain *A. pluranimalium* DSM 13483.

Biochemical properties	*A. pluranimalium* 230/15	*A. pluranimalium* DSM 13483**
Hemolysis on sheep blood agar	+	+
CAMP−like reaction with:*		
Staphylococcus aureus β−hemolysin	+	+
Streptococcus agalactiae	−	−
Rhodococcus equi	+	+
Reverse CAMP reaction	−	−
Nitrate reduction	−[1]	−[1]
Pyrazinamidase	+[1]	+[1]
Pyrrolidonyl arylamidase	(+)[1]	+[1]
Alkaline phosphatase	−[1]	−[1]
β−Glucuronidase	+[1,2,3]	+[1,2,3]
β−Galactosidase	−[1,2,3]	−[1,2,] (+)[3]
α−Glucosidase	−[1,2,3]	−[1,2,3]
β−Glucosidase	+[2]	+[2]
N−Acetyl−β−Glucosaminidase	−[1,3]	−[1,3]
Esculin	+[1]	+[1]
Urease	−[1]	−[1]
Gelatine	+[1]	+[1]
Fermentation of:		
D−Glucose	+[1]	+[1]
D−Ribose	+[1]	+[1]
D−Xylose	−[1]	−[1]
D−Mannitol	−[1]	−[1]
D−Maltose	(+)[1]	(+)[1]
D−Lactose	−[1]	−[1]
D−Saccharose	−[1]	−[1]
Glycogen	−[1]	−[1]
Catalase	+	+
Serolysis on Loeffler agar	−	−
Caseinase	+	+
Amylase	+	+

The reactions are shown as follows:

*synergistic CAMP−like reaction with indicator strains;

**results mostly obtained from Ülbegi−Mohyla et al. (23);

+: positive reaction; (+): weak positive reaction; −: negative reaction.

[1]Api−Coryne test system (Biomerieux, Nürtingen, Germany);

[2]tablet containing substrates (Rosco Diagnostica A/S, Taastrup, Denmark);

[3]4−methylumbelliferyl conjugated substrates (Sigma, Steinheim, Germany).

genetic studies (8). Sequencing the 16S rDNA is a rDNA-based universal target among bacteria and large enough for bioinformatic purposes. Sequencing 16S rDNA of *A. pluranimalium* 230/15 of the present study showed a sequence similarity of 99.9% to 16S rDNA of type strain *A. pluranimalium* DSM 13483. The 16S rDNA contains various specific regions (5) and values of $\geq$ 99% similarity of 16S rDNA sequence were assigned as suitable cut-off for bacterial species identification and $\geq$ 97% for bacterial identification at genus level (6).

A molecular identification to the species level could also be conducted by amplification and sequencing the housekeeping genes *rpoB, gap* and *tuf* encoding the β subunit of bacterial RNA polymerase, the glyceraldehyde 3-phosphate dehydrogenase and the elongation factor tu. All three genes revealed a sequence identity of more than 99% indicating their usefulness as molecular targets. Khamis et al. (11) showed that gene *rpoB* could be used for phylogenetic analysis of species of genus *Corynebacterium*. In addition, gene *gap* has been widely used as target gene for molecular identification of various species of genus *Arcanobacterium* (20, 21) and genus *Staphylococcus* (8). The application of *tuf* gene analysis for molecular identification has already been evaluated for various bacterial species of genus *Enterococcus, Streptococcus,* coagulase-negative *Staphylococcus* and *Lactococcus* (15).

The investigated *A. pluranimalium* strain obtained from a muskox is also carrying the potential virulence factor pluranimaliumlysin encoding gene *pla*. Sequencing gene *pla* of *A. pluranimalium* 230/15 showed a high sequence similarity (99.6%) to *pla* gene of type strain *A. pluranimalium* DSM 13483. Gene *pla* has already been described as novel target for molecular identification of *A. pluranimalium* (17). Balbutskaya et al. (3), Risse et al. (19) and Wickhorst et

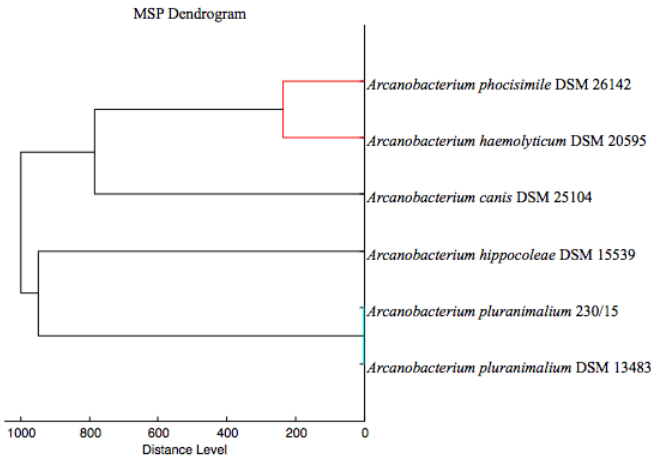

Figure 1. A score-oriented dendrogram of MALDI-TOF MS spectra profiles of *A. pluranimalium* 230/15, type strain *A. pluranimalium* DSM 13483 and some reference strains of genus *Arcanobacterium*

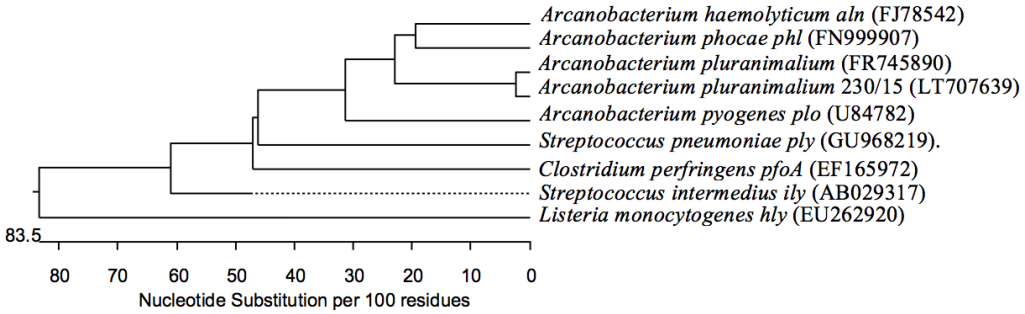

Figure 2. Dendrogram analysis of 16S rDNA of *A. pluranimalium* 230/15, type strain *A. pluranimalium* DSM 13483 and other species of genus *Arcanobacterium* obtained from NCBI GenBank. *Accession number

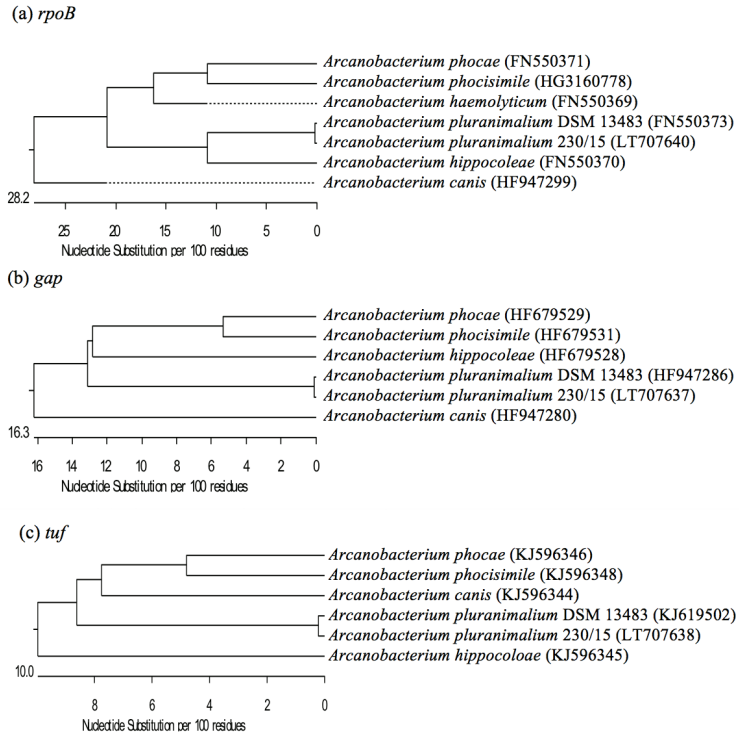

Figure 3. Dendrogram analysis of the genes *gap* (a) *rpoB* (b) and *tuf* (c) of *A. pluranimalium* 230/15, type strain *A. pluranimalium* DSM 13483 and other species of genus *Arcanobacterium* obtained from NCBI GenBank

al. (25) also showed that gene *pla* of *A. pluranimalium* seems to be constantly present in all strains of this species

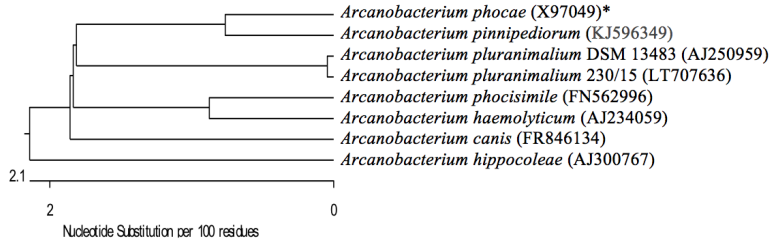

Figure 4. Dendrogram analysis of pluranimaliumlysin encoding gene *pla* of *A. pluranimalium* 230/15, *pla* of *A. pluranimalium* DSM 13483 and the genes *aln* of *A. haemolyticum, phl* of *A. phocae*, *plo* of *T. pyogenes*, *ily* of *Streptococcus intermedius*, *ply* of *Streptococcus pneumoniae*, *pfoA* of *Clostridium perfringens*, *hly* of *Listeria monocytogenes* obtained from NCBI GenBank

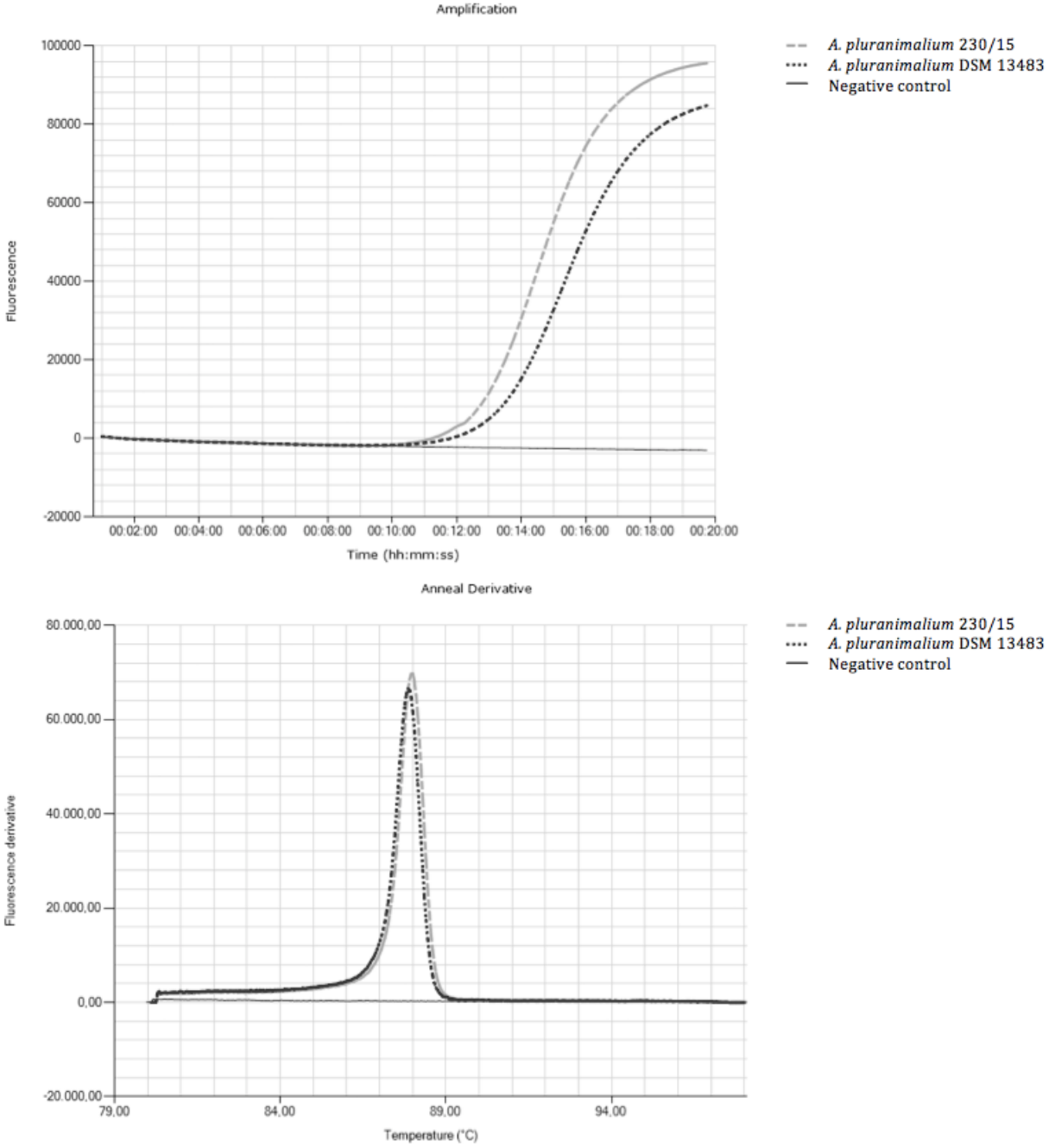

Figure 5. LAMP amplification signal of *A. pluranimalium* 230/15, the type strain *A. pluranimalium* DSM 13483 as positive control as well as a negative control (a). The melting curve (anneal derivation) of the same amplicons (b)

and could be used for molecular identification. Comparable to previous studies (2, 25) this species-specific gene *pla* of *A. pluranimalium* could also be detected successfully by using a *pla* LAMP assay. Comparable LAMP assays have already been used to identify *Leptospira* species (13, 16), *Ersypelothrix rhusiopathiae* (26), *Streptococcus equi* subsp. *zooepidemicus* (12) and ostrich meat (1). These authors described LAMP as a powerfool tool that can be used as an alternative to PCR-based methods since it has a high sensitivity and specificity, a shorter reaction time and a comparably low susceptibility for inhibitors.

The present study gives a first report on the isolation and characterization of *A. pluranimalium* from this origin. However, little is known about the pathogenic importance of the strain.

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Fenotipska i genotipska analiza *Arcanobacterium pluranimalium* izoliranog od mošusnog goveda (*Ovibos moschatus*)

Sažetak

Uvod i ciljevi

Arcanobacterium pluranimalium je prvi put izoliran 2001. godine iz uginule obalne pliskavice i uginulog jelena lopatara. Kasnije je izoliran iz psa s piodermom, juvenilne žirafe nakon obdukcije, iz 33 ovčija uzorka i uzorka mlijeka krave s mastitisom. Još uvijek nije jasno koje životinje su prijemćive ili koji su sistemi podložniji infekciji prouzrokovanoj *A. pluranimalium*. Cilj ovog istraživanja je karakterizacija *A. pluranimalium* izoliranog iz mošusnog goveda (*Ovibos moschatus*).

Materijal i metode

U ovom istraživanju je kao uzorak korištena jetra eutanazirane ženke mošusnog goveda, stare 19 godina. Obdukcijom su ustanovljeni: difuzni kataralni gastroenteritis, stomatitis profunda, masna jetra te artroza tarzalnih i karpalnih zglobova. *A. pluranimalium* je izoliran u malom broju, zajedno s nekoliko drugih bakterija. Za fenotipsku karakterizaciju izolata korišteni su krvni agar s ovčijom krvi za dokazivanje hemolize, CAMP reakcija, reverzna CAMP reakcija, API-Coryne, tablete sa supstratima, 4-methylumbelliferyl konjugirani suspstrati, katalaza, seroliza na Loeffler agaru, determinacija kazeinaze i amilaze, te pomoću MALDI-TOF MS analize i sekvencioniranjem 16S rRNA gena, β subjedinica bakterijske RNA polimeraze kodirane genom *rpoB,* gliceraldehid 3-fosfat dehidrogenaza kodirana genom *gap,* faktor elongacije tu kodiran genom *tuf* i pluranimaliumlizin kodiran genom *pla.* Nadalje, gen *pla* je istražen metodom petlje posredovanog izotermskog umnažanja (*eng*. loop-mediated isothermal amplification -LAMP assay), za koju su korišteni termoblok, detekcija LAMP produkta u agaroza gelu i *real-time* fluorometrom.

Rezultati i interpretacija

Fenotipskom analizom su ustanovljene tipične karakteristike ove vrste. *A. pluranimalium* izolat je bio katalaza, kazeinaza i amilaza pozitivan, te negativan na Loeffler agaru. Rezultati biohemijskog testiranja su također bili pozitivni za pirazinamidazu, pirolidonil arilamidazu, β-glukuronidazu, β-glukozidazu, eskulin, želatin, te ferementaciju glukoze, riboze i maltoze. CAMP test je rezultirao pojačanom hemolizom u reakciji sa *S. aureus* i *R. equi,* odnosno bez hemolize sa *S. agalactiae.* MALDI-TOF MS analiza je rezultirala identifikacijom vrste i podudaranjem s *A. pluranimalium* DSM 13483 sojem sa log vrijednosti 2.689. Nadalje, sekvencioniranje 16S rDNA, β subjedinica bakterijske RNA polimeraze kodirane genom *rpoB,* gliceraldehid 3-fosfat dehidrogenaza kodirana genom *gap,* faktor elongacije tu kodiran genom *tuf* i pluranimaliumlizin kodiran genom *pla* su rezultirali identitetom sekvenci od 99.9%, 99.8%, 99.9% 99.6% i 99.6% s odgovarajućim sekvencama *A. pluranimalium* DSM 13483 soja.

Glavni zaključak

Svi geni su imali identitet sekevenci viši od 99%, indicirajući njihovu iskoristivost kao ciljanih pri molekularnoj karakterizaciji. *A. pluranimalium* soj izoliran iz mošusnog goveda također ima potencijalni faktor virulencije, pluranimaliumlizin kodiran genom *pla.* Gen *pla* specifičan za vrstu *A. pluranimalium* 230/15 se također mogao uspješno dokazati pomoću LAMP metode. Ovo istraživanje predstavlja prvu izolaciju *A. pluranimalium* iz mošusnog goveda i njegovu karakterizaciju.