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Korrespondenzadresse:
reinhard.sting@cvuas.bwl.de

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Chemisches und Veterinäruntersuchungsamt Stuttgart (CVUAS), Fellbach[1]
Konsiliarlabor für *Corynebacterium pseudotuberculosis* (DVG), Fellbach[2]
Wilhelma – Zoologisch-Botanischer Garten, Stuttgart[3]

Actinomycetes associated with abscess formation in a goat, a llama and two alpacas

Actinomyceten im Zusammenhang mit Abszessen bei einer Ziege, einem Lama und zwei Alpakas

Reinhard Sting[1,2], Anna Katharina Schwalm[1], Matthias Contzen[1], Marco Roller[3], Jörg Rau[1]

Abstract

The genera *Actinomyces* and *Schaalia*, both members of the bacterial order Actinomycetales, include relevant infectious agents that cause abscesses in small ruminants and New World camelids. Due to the high diversity of the Actinomycetales, detection of undescribed members of this order is to be expected. Novel actinomycetes species were cultivated from a goat, a llama and two alpacas suffering from abscesses with suspected caseous lymphadenitis (CLA). Analyses carried out on these isolates using MALDI-TOF MS and 16S rRNA gene sequencing revealed actinomycetes, presumably belonging to the bacterial genera *Actinomyces* and *Schaalia*. The data suggest that the caprine isolate is a undescribed *Actinomyces* species, while the isolates originating from a llama and two alpacas show a close relationship to each other within a unique *Schaalia* cluster, suggesting a host-adapted novel *Schaalia* species. Both methods proved equally suitable for reliable identification of known and of undescribed Actinomyces and *Schaalia* species. This study contributes to extending our knowledge about novel species belonging to the bacterial family of Actinomycetaceae (actinomycetes) associated with abscesses in goats and New World camelids. Precise identification of actinomycetes at species level is of high relevance in veterinary practice with regard to differentiation from caseous lymphadenitis and assessment of treatment success.

Keywords: small ruminant, New World camelids, pseudotuberculosis, *Actinomyces*, Actinomycetes, Actinomycetaceae, *Schaalia*

Zusammenfassung

Bakterien der Gattungen *Actinomyces* und *Schaalia* gehören zur Ordnung Actinomycetales und sind wichtige Erreger infektiöser Abszesse bei Wiederkäuern und Neuweltkameliden. Aufgrund des großen Artenreichtums der Actinomycetales sind neue und bisher nicht beschriebene Arten zu erwarten. So konnten wir neue Actinomyceten aus Abszessen einer Ziege, eines Lamas und zweier Alpakas mit Verdacht auf Pseudotuberkulose isolieren. Untersuchungen der Isolate mittels MALDI-TOF-Massenspektrometrie und Sequenzierungen des 16S rRNA-Gens ergaben Actinomyceten, die den Gattungen *Actinomyces* und *Schaalia* zugeordnet werden können. Die Ergebnisse weisen darauf hin, dass es sich bei dem Ziegen-Isolat um eine bisher noch nicht beschriebene *Actinomyces*-Spezies handelt. Die Isolate von dem Lama und den beiden Alpakas hingegen erwiesen sich als eng verwandt innerhalb eines gemeinsamen *Schaalia*-Clusters, was auf eine neue wirtsadaptierte *Schaalia*-Spezies hinweist. Beide Methoden erwiesen sich als geeignet, bekannte und bisher nicht beschriebene *Actinomyces*- und *Schaalia*-Spezies zuverlässig zu identifizieren.
Diese Studie trägt dazu bei, unsere Kenntnisse über neue Spezies der Familie der Actinomycetaceae (Actinomyceten) im Zusammenhang mit Abszessen bei Ziegen und Neuweltkameliden zu erweitern. Eine exakte Identifizierung von Actinomyceten ist unter dem Hintergrund der Differenzierung zur Pseudotuberkulose und der Einschätzung von Therapieerfolgen von großer Bedeutung.

Schlüsselwörter: Kleine Wiederkäuer, Neuweltkameliden, Pseudotuberkulose, *Actinomyces*, Actinomyzeten, Actinomycetaceae, *Schaalia*

Introduction

Abscess formation due to bacterial infections can lead to the development of severe debilitating or even life-threatening diseases in animals and humans. Among causative pathogens, bacteria of the order Actinomycetales are of special relevance for purulent lesions. Actinomycetales belong to the Actinobacteria, which represent a diverse phylum of gram-positive bacteria, comprising the major pathogen containing bacterial families *Actinomycetaceae*, *Corynebacteriaceae*, *Mycobacteriaceae* and *Nocardiaceae* (Nouioui et al. 2018). Certain members of the genus *Corynebacterium* are well known pathogens which spread within a herd and cause abscesses (Braga et al. 2006, Sprake and Gold 2012), whereas bacterial species of the family *Actinomycetaceae* (actinomycetes) with the genera *Actinomyces*, *Arcanobacterium*, *Trueperella*, and the recently described genus *Schaalia* (recently separated from the genus *Actinomyces*) usually affect individuals (Brown 2006, Fowler 1996, Nouioui et al. 2018). Therefore, detection of the causative agent and identification at species level is of particular importance if there is suspicion of caseous lymphadenitis (CLA) in goats, sheep, and camelids. The family *Actinomycetaceae* forms a large group of anaerobic or microaerophilic bacteria producing short, curved rods or branching filaments in varying degrees. Among these, the genera *Actinomyces* and *Schaalia* comprise many species which are widely distributed in the environment and have been isolated from natural habitats like soil, but also from humans and animals (Nouioui et al. 2018, Yassin 2014). Infections caused by actinomycetes have been recognised for some time (Smith 1918) and numerous species have since been described as causative agents of purulent and suppurative infections in various hosts (Nouioui et al. 2018, Yassin 2014). However, due to the phylogenetic diversity of the genus *Actinomyces* which subdivides into different clusters, lineages and groups, undescribed species are to be expected (Yassin 2014, Zhao et al. 2014). Accordingly, the taxonomy of the Actinobacteria has undergone revision, species have been transferred to other genera and novel species have been proposed and defined in the last decade (Nouioui et al. 2018, Yassin 2014, Zhao et al. 2014). In view of the large phylum of Actinobacteria encompassing numerous pathogenic and apathogenic bacterial species, precise identification at species level is crucial. This is of special relevance for the identification of bacterial pathogens causing abscesses that resemble CLA when considering the impact on control programs implemented for goats, sheep and camelids or assessment of successful treatment (Schumacher et al. 2009).

In this study, we describe the occurrence of abscesses suspicious for CLA in a goat, a llama and two alpacas caused by undescribed actinomycetes. These cases show that a broader spectrum of actinomycetes than previously known poses a determining cause of abscess formation and pyogenic lesions in small ruminants and New World camelids.

Material and methods

In November 2011, material from an abscess on the neck of a llama (*Lama glama*) was submitted for bacteriological examination.

In October 2018, an adult goat (*Caprae aegagrus hircus*) suffered from a neck abscess. Material for bacteriological examination was taken from the opened abscess.

In August 2019, an abscess was noticed on the shoulder and chest area of an alpaca (*Vicugna pacos*). After opening the abscess, material was taken for bacteriological examination.

In September 2019, another adult alpaca had three abscesses, two in the left cheek and one at the left jaw angle. The abscesses were opened by incision and a smooth, yellowish mass was recovered and sent to our laboratory for bacteriological examination.

The goat, the llama and the two alpacas lived all on separate farms without any direct contact.

All samples were submitted for bacteriological examination to clarify the suspicion of CLA. Bacteriological examination was carried out according to standard procedures. Abscess material was streaked on 5% sheep blood agar (Oxoid, Wesel, Germany) and MacConkey agar (BD BBL, Heidelberg, Germany) for aerobic incubation at 37°C for two days. In addition, Schaedler agar (BD) and Wilkins-Chalgren agar with amikacin and 7% sheep blood (BD) were inoculated and incubated anaerobically at 37°C for two days. The isolates were stored in our culture collection at -70°C using the Microbank™ system (Pro-Lab Diagnostics, Neston, Cheshire, U.K.).

For comparative studies, *Schaalia hyovaginalis* field isolates and the type strain DSM 10695 (DSMZ German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) were included in this study (Table 1).

All bacterial isolates were analysed by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) (Bruker Biotyper; Bruker Daltonik, Bremen, Germany), using the commercial database 8.468 augmented with additional reference entries created in this study. The Bruker Biotyper database 8.468 does not yet take into account the newer taxonomy of the revised genus *Actinomyces* (Nouioui et al. 2018).

The creation of new reference entries, so called main spectra projections (MSP), followed the instructions and standards of the manufacturer. These procedures and the software used have been described elsewhere in more detail (Pranada et al. 2016, Rau et al. 2016a). Further information on user-made additional MSP applied in this study (see Table 1) is shown in the MALDI-UP catalogue on https://maldi-up.ua-bw.de (Rau et al. 2016b).

For decoding of 16S rRNA gene sequences, PCR assays were carried out as described elsewhere (Contzen et al. 2011) using the primers 27f (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1522rN (5′-CAT GCG GCC GCA AGG AGG TGA TCC ARC CGC A-3′) according to Johnson (1994). The PCR products were sequenced on demand (Microsynth, Balgach, Switzerland) and the sequence data obtained was compared with sequence entries in GenBank (http://www.ncbi.nlm.nih.gov) using the Basic Local Alignment Search Tool for nucleotides (BLASTN) on the NCBI website (Pruitt et al. 2002).

MALDI-TOF MS and 16S rDNA dendrograms were created using *Actinomyces* and *Schaalia* reference isolates including the isolates which originated from the goat, llama and the two alpacas. In addition, a *Corynebacterium pseudotuberculosis* and a *Trueperalla pyogenes* strain were included (Table 1).

The MALDI-TOF MS dendrogram was created with the "BioTyper MSP Dendrogram Creation Standard

TABLE 1: *Data on the reference strains used in this study for cluster analysis of spectra obtained by MALDI-TOF MS and for construction of a phylogenetic tree based on 16S rRNA gene sequences*

Species	Isolate (culture collection no.)	Source	Acc. No. (culture collection no.)	Sequence (bp)	User-made MSP*
Actinomyces bovis	DSM 43014^T	cattle	NR_118899 (DSM 43014^T)	1379	yes
Actinomyces bowdenii	DSM 15435^T	dog	NR_041982 (CCUG 37421^T)	1513	
Actinomyces dentalis	DSM 19115^T	human	NR_025633.1 (DSM 19115^T)	1517	
Actinomyces israelii	DSM 43320^T	human	NR_114401 (JCM 12964^T)	1512	
Actinomyces naeslundii	DSM 43013^T	human	NR_113326 (JCM 8349^T)	1522	
Actinomyces oris	DSM 23056^T	human	NR_117358 (ATCC 27044^T)	1475	
Actinomyces ruminicola	DSM 27982^T	cattle	NR_043523 (B71^T)	1526	
Actinomyces urogenitalis	DSM 15434^T	human	NR_25364 (CCUG 38702^T)	1423	
Actinomyces viscosus	DSM 43327^T	hamster	NR_026228 (ATCC 15987^T)	1417	
Actinomyces weissii	DSM 24894^T	dog	NR_108476 (CCM 7951^T)	1379	yes
Corynebacterium pseudotuberculosis	DSM 20689^T	sheep	NR 119175.1 (NCTC 3450^T)	1446	
Schaalia canis	DSM 15536^T	dog	AJ243891 (CCUG 41706^T)	1428	yes
Schaalia cardiffensis	DSM 15803^T	human	NR_025521 (CCUG 44997^T)	1522	yes
Schaalia funkei	DSM 15537^T	human	NR_028960 (CCUG 42773^T)	1366	
Schaalia georgiae	DSM 6843^T	human	NR_026182 (DSM 6843^T)	1422	
Schaalia hyovaginalis	CVUAS 30200	sheep	MN864534	1494	yes
Schaalia hyovaginalis	CVUAS 4547.5	sheep	MN864532	1494	yes
Schaalia hyovaginalis	CVUAS 6234.5	pig	MN864533	1494	yes
Schaalia hyovaginalis	DSM 10695^T	pig	MN864535 (DSM 10695^T)	1497	yes
Schaalia meyeri	DSM 20733^T	human	X82451 (CIP 103148^T)	1441	
Schaalia odontolytica	DSM 19120^T	human	NR_041983 (CCUG 20536^T)	1412	
Schaalia radingae	DSM 9169^T	human	NR_026169 (ATCC 51856^T)	1429	
Schaalia suimastitidis	DSM 15538^T	pig	NR_025401 (CCUG 39276^T)	1416	
Schaalia turicensis	DSM 9168^T	human	NR_037020 (ATCC 51857^T)	1453	
Schaalia vaccimaxillae	DSM 15804^T	cattle	NR_025523 (DSM 15804^T)	1493	
Trueperella pyogenes	DSM 20630^T	pig	NR 117537.1 (ATCC 19411^T)	1293	yes

^{T} = type strain; * own reference spectra (MSP)
Further information is shown in the MALDI-UP catalogue on https://maldi-up.ua-bw.de.

Method", as provided by the manufacturer within the Biotyper software (vers. 3.1, Bruker), using the newly created MSP from this study and a collection of MSP from the commercial Bruker MBT Compass Reference Library that was released in April 2019 and contains 2,969 species and 8,468 MSP.

The phylogenetic tree was constructed using the neighbour-joining distance algorithm with standard settings in Geneious Prime 2020.1.1.

Results

Bacterial culture

The bacteria cultivated from the goat's abscess (isolate CVUAS 31303) grew in a whitish, chalk-like layer of discernible pinpoint-sized, non-haemolytic colonies under aerobic conditions. The bacteria appeared as catalase-positive, gram-positive, short, club-shaped rods. This primary culture was accompanied by an equivalent strong growth of the obligate anaerobic bacteria *Fusobacterium* (*F.*) *necrophorum* and *Prevotella* (*P.*) *heparinolytica* detected under anaerobic atmospheric conditions.

The bacteria originating from the abscess of the llama (isolate CVUAS 8688) and the alpacas (isolate CVUAS 31838 and CVUA 31845.2) showed profuse and strong growth of pinpoint-sized, non-haemolytic colonies on blood sheep agar after a two-days incubation period in an aerobic atmosphere. None of the three isolates showed catalase activity. Gram staining revealed gram-positive, short or slightly curved, club-shaped rods. Additionally, strong growth of *Trueperella* (*T.*) *pyogenes* and moderate growth of *Bibersteinia* (*B.*) *trehalosi* was observed with the llama's abscess. In contrast, the isolates originating from the alpacas grew in pure culture.

MALDI-TOF MS analyses

The isolate CVUAS 31303 (goat) could be distinguished from *Schaalia* isolates using MALDI-TOF MS and was located within the *Actinomyces* cluster (Fig. 1). The isolates originating from the llama (isolate CVUAS 8688) and the alpacas (isolates CVUAS 31838 and CVUAS

31845.2) exhibit a close relationship clustering on a branch belonging to a separate cluster close to several *Schaalia* (*S.*) species, and are therefore referred to as *Schaalia* species. In contrast, caprine, sheep and porcine *S. hyovaginalis* field isolates and the *S. hyovaginalis* type strain DSM 10695, which were identified and verified as *S. hyovaginalis* by MALDI-TOF MS, are located within a common cluster clearly separated from the isolates originating from the New World camelids (Fig. 1). The MALDI TOF mass-spectra of the isolates and several reference strains are available by exchange via the MALDI-TOF user platform (Rau et al. 2016b).

16S rRNA gene sequencing

In the 16S rDNA dendrogam, the goat isolate CVUAS 31303 showed a close relationship to *Actinomyces* species, including *Actinomyces bovis*. In contrast, the llama and alpaca isolates showed a close relationship in a common sub-cluster within a *Schaalia* cluster, but were clearly distinct from the cluster comprising sheep, caprine and porcine *S. hyovaginalis* field isolates including the type strain DSM 10695 (Fig. 2).

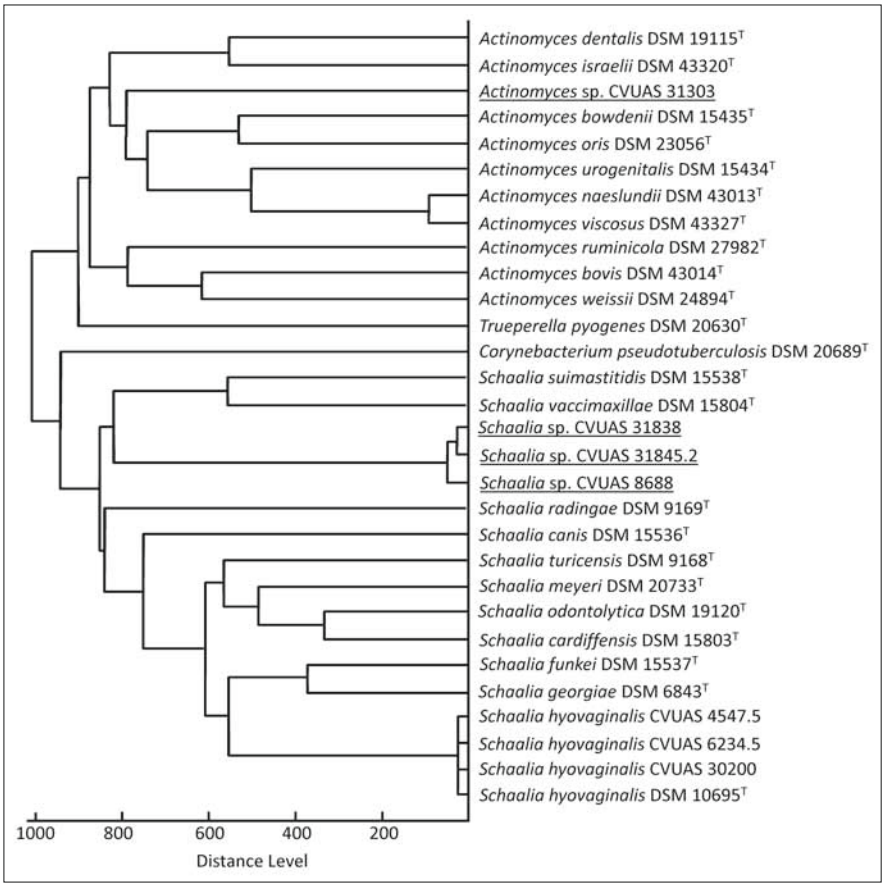

FIGURE 1: *MALDI-TOF MS dendrogram created by cluster analysis of spectra obtained by MALDI-TOF mass spectrometry including the isolates CVUAS 31303 (goat), CVUAS 8688 (llama), CVUAS 31845.2 (alpaca) and CVUAS 31838 (alpaca) in context of reference spectra. For details of the isolates and reference spectra used in this study see Table 1.*

For this dendrogram, the same isolates were used as for the MALDI-TOF mass spectrometry dendrogram (Fig. 1). For details of the isolates and sequences used in this study see Table 1 (Graphic: CVUA Stuttgart).

Discussion

A considerable proportion of abscess formation in small ruminants and camelids is caused by bacteria belonging to the phylum Actinobacteria. A prominent member of the order Actinomycetales is *Corynebacterium (C.) pseudotuberculosis*, the causative agent of CLA, which is highly relevant in small ruminants and camelids worldwide (Al-Harbi 2011, Al-Tuffyli and Shekhan 2012, de la Fuente et al. 2017, de Lima e Silva et al. 2016). Control programmes have been implemented in numerous countries worldwide to effectively combat CLA in small ruminants and camelids. Therefore, abscess formation resembling CLA has to be verified by identification of the pathogenic agent, which must be reliably distinguished from *C. pseudotuberculosis*. Numereous actinomycetes have been described as causative agents of suppurative lesions in humans and animals (Nouioui et al. 2018, Yassin 2014). Among the genera within the family Actinomycetaceae, the recently described novel genus *Schaalia* has been separated from the genus *Actinomyces* and reported in connection with abscess formation in humans and various animal species. Case reports have been published on abscess formation due to *Actinomyces* and *Schaalia* infections in different localisations such as the brain, subcutis or lymph nodes of goats (Alssahen et al. 2020, Hirai et al. 2007, Ndegwa et al. 2001, Oyekunle et al. 2010, Schumacher et al. 2009), New World camelids (Brown 2006, Fowler 1996), sheep (Alssahen et al. 2020, Collins et al. 2001, Foster et al. 2012), pigs (Hommez et al. 1991, Reichel and Wragg 2007), horses (Chung et al. 2018, Fielding et al. 2008), dogs (Hoyles et al. 2000, Song et al. 2015) and captive and free-ranging wildlife (Alssahen et al. 2020, Gamble and Clancy 2013, Wickhorst et al. 2017). The microorganisms reported in this study, which had been isolated from abscesses in a goat, a llama and two alpacas, attracted our attention because of their similarity to *Actinomyces* spp. and *Schaalia* spp., respectively. Further studies on these isolates using MALDI-TOF MS and 16S rDNA analysis initially yielded inconclusive results at species level, despite comprehensive databases that are available for evaluation procedures. However, the isolates could be assigned to bacteria of the genera *Actinomyces* (goat isolate) and *Schaalia* (llama and alpaca isolates). The isolate originating from the abscess in the goat is located within the MALDI-TOF MS and 16S rDNA *Actinomyces* cluster, but is separate to other concrete *Actinomyces* spp. In contrast, the isolates obtained from the llama and the alpacas show very close relationships to each other in a separate branch next to other *Schaalia* spp. in both the MALDI-TOF MS and 16S rDNA dendrogram. The close relationship of these actinomycetes in New World camelids, which were isolated at different times and places, suggest the presence of a novel host-adapted *Schaalia* species. Fowler (1996) also

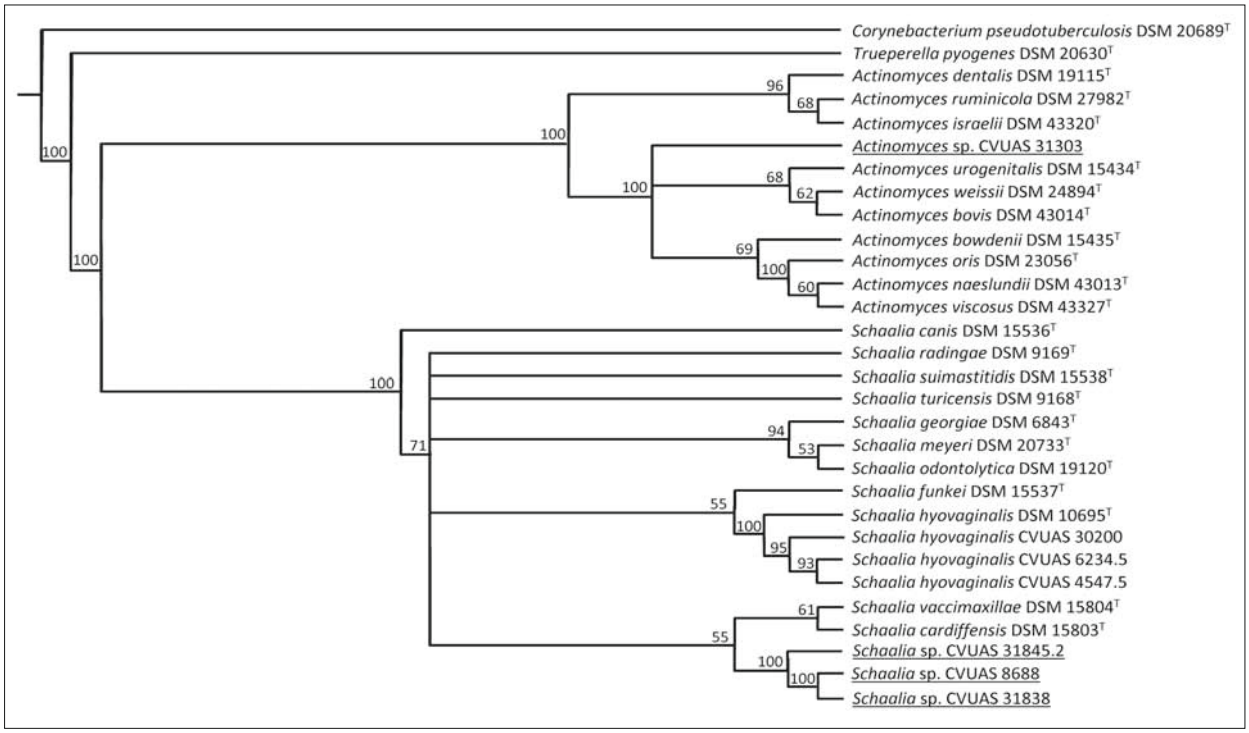

FIGURE 2: *Phylogenetic tree based on 16S rRNA gene sequences including the isolates CVUAS 31303 (goat, 1487 bp; GenBank Accession No. MN864531), CVUAS 8688 (llama, 1517 bp; MN864528), CVUAS 31845.2 (alpaca, 1517 bp; MN864529) and CVUAS 31838 (alpaca, 1517 bp; MN864530). Numbers indicate percent bootstrap support (100 replications).*
For this dendrogram, the same isolates were used as for the MALDI-TOF mass spectrometry dendrogram (Fig. 1). For details of the isolates and sequences used in this study see Table 1 (Graphic: CVUA Stuttgart).

reported on abscess formation in the throat region, lungs or liver and dental abscesses caused by *Actinomyces* sp. unique to New World camelids and provisionally called these isolates *Actinomyces* (lamae). The author pointed out that these bacterial isolates may be under-reported due to their morphological appearance as gram-positive, short rods which may be mistaken for cocci.

It is notable that the actinomycetes growth reported in this study was accompanied by *F. necrophorum* and *P. heparinolytica* (goat) or *T. pyogenes* and *B. trehalosi* (llama). However, no accompanying bacterial aerobic or anaerobic flora could be detected in the abscesses from the two alpacas. Other researchers also report growth of actinomycetes in pure cultures (Brown 2006, Chung et al. 2018, Foster et al. 2012, Oyekunle et al. 2010, Song et al. 2015) or growth in mixed cultures including *T. pyogenes*, *Fusobacterium* spp., *Bacteroides* spp. and *Prevotella* spp. or *Staphylococcus* spp. and *Streptococcus* spp. (Al-Harbi 2011, Collins et al. 2001, Foster et al. 2012, de la Fuente et al. 2017, Gamble and Clancy 2013, Roeder et al. 1989, Schumacher et al. 2009, Wickhorst et al. 2017).

The abscess wounds in the two alpacas were successfully treated by rinsing with iodine solution without additional antibiotic treatment. Unfortunately, no data is available on the treatment of the abscesses in the goat and the llama.

MALDI-TOF MS and 16S rRNA gene analyses suggest that the actinomycetes described in this study represent undesignated novel *Actinomyces* and *Schaalia* species. However, this assumption has to be proven by further comprehensive investigations to elucidate the taxonomic status of these interesting, obviously pathogenic actinomycetes.

Conclusion

Actinomycetes represent important bacteria associated with abscess formation. This report shows that so far undescribed actinomycetes are also responsible for abscess formation in addition to already described pyogenic bacteria. Precise and reliable identification at species level is therefore crucial with special reference to CLA (pseudotuberculosis), which is controlled in large-scale control programmess in many countries. Reliable identification of *Actinomyces* and *Schaalia* species and detection of undescribed species can be achieved by MALDI-TOF MS and 16S rDNA analyses.

The present study extends our current knowledge about novel actinomycetes associated with abscess formation in small ruminants and New World camelids.

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Conflict of interest statement

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of this paper.

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Address for correspondence
Dr. Reinhard Sting
Chemisches und Veterinäruntersuchungsamt Stuttgart
Schaflandstr. 3/3
70736 Fellbach
Germany
reinhard.sting@cvuas.bwl.de