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Identification and Characterization of *Arcanobacterium canis* from Companion Animals in Germany and The United Kingdom

Siti Gusti Ningrum[1], Antonia Kreitlow[2], Christoph Lämmler[3], Ellen Prenger-Berninghoff[3], Christa Ewers[3], Geoffrey Foster[4], Madeleine Plötz[2], Amir Abdulmawjood[2*]

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[1]Faculty of Veterinary Medicine, Universitas Wijaya Kusuma Surabaya, Surabaya, Jl. Dukuh Kupang XXV No.54, Dukuh Kupang, Surabaya, 60225; Indonesia; sitiningrum@uwks.ac.id (SGN)

[2]Institute of Food Quality and Food Safety, University of Veterinary Medicine Hannover, Bischofsholer Damm 15, 30173 Hannover, Germany; antonia.kreitlow@tiho-hannover.de (AK), Madeleine.Ploetz@tiho-hannover.de (MP)

[3]Institute of Hygiene and Infectious Diseases of Animals, Justus-Liebig-Universität Giessen, Frankfurter Straße 85-89, 35392 Gießen, Germany; Christoph.Laemmler@vetmed.uni-giessen.de (CL), Ellen.Prenger-Berninghoff@vetmed.uni-giessen.de (EP-B), Christa.Ewers@vetmed.uni-giessen.de (CE)

[4]SRUC Veterinary Services, An Lochran, 10 Inverness Campus, Inverness, IV2 5NA United Kingdom; Geoffrey.Foster@sruc.ac.uk (GF)

*Corresponding author: Amir Abdulmawjood; Institute of Food Quality and Food Safety, University of Veterinary Medicine Hannover, Bischofsholer Damm 15, 30173 Hannover, Germany; Amir.Abdulmawjood@tiho-hannover.de (AA)

Abstract: *Arcanobacterium canis* is a novel species of the *Arcanobacterium* most closely related to *A. haemolyticum*. This study aims to characterize two A. *canis* isolates recovered from companion animals, specifically the claw of a cat and a vaginal swab from a dog. This study used real-time PCR to characterize *A. canis* isolated from companion animals. Two isolates of *A. canis* were recovered from purulent material from the claw of an 11-year-old cat in Germany and a vaginal swab of a dog in the United Kingdom. The samples were characterized phenotypically and genotypically. Both isolates were analyzed using culture methods, biochemical analysis, MALDI-TOF MS, real-time PCR amplification and sequencing of the 16S rRNA gene, and *rpoB, gap*, and *tuf* genes. The findings showed that the isolates P5197-15 and M214-96-1 obtained from companion animals were successfully characterized and confirmed to species level by real-time PCR amplification and sequencing of the 16S rRNA gene, as well as the genes of *rpoB*, *gap*, and *tuf*. This study seeks to comprehensively understand the characteristics of *A. canis* isolates obtained from companion animals. Such knowledge is essential for accurate diagnosis, treatment, and control of infections caused by this pathogen in veterinary medicine. Additionally, it contributes to the

broader understanding of the genetic diversity and characteristics of *A. canis*, which can have implications for public health and animal well-being.

Keywords: *Arcanobacterium canis;* one health; 16S rRNA gene; *rpoB*; *gap*; *tuf*

1. Introduction

Arcanobacterium canis, is a bacterium that is becoming increasingly important among pets in Germany and the United Kingdom. The investigation of *A. canis* in animal companions, including felines and canines, carries substantial significance [1] within the field of this pathogen research. It is crucial to thoroughly understand the microbial environment that characterizes the connection between human and animal well-being. Gaining knowledge about pathogen investigation can provide significant perspectives on the mechanisms of disease dissemination, evolutionary processes, and the potential for zoonotic transmission [2]. Identifying and characterizing this pathogen from companion animals can provide valuable insights into their potential implications for human health.

Arcanobacterium comprises a group of asporogenous facultatively anaerobic Gram-positive rods [3]. These bacteria are commonly found in the microbiota of various animals, including humans, and are generally harmless. However, some species, such as *Arcanobacterium haemolyticum*, can lead to diseases, i.e., pneumonia, acute pharyngitis, and scarlet fever-like rash in humans [4] and other animals. Like several species in diverse environments, like those belonging to the *Corynebacterium* and *Trueperell*a genera, *Arcanobacterium* species are typically non-pathogenic [5–7]. Still, they can occasionally take advantage of unusual access to tissues, such as through wounds [5,8] or when the host's defenses are weakened [9,10].

Taxonomic revision of this genus was conducted in 2011 [11]. In 2012, a novel species of the genus *Arcanobacterium,* most genetically similar to *A. haemolyticum, was* described as *A. canis.* This species could be isolated from mixed infections with several other bacteria from companion animals [12]. Further characteristics of *A. canis* have been studied by previous studies using amplification and sequencing of the 16S rRNA gene, as well as the *rpoB* gene encoding the RNA polymerase beta subunit and the *gap* gene encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [13]. The isolates investigated were recovered from the external otitis of a seven-year-old female English bulldog (*A. canis* DSM 25104^{T}) [12]. Additionally, the investigation conducted by Osama et al. [13] reported the isolation and characterization of *A. canis* from a nine-year-old Doberman's sperm (*A. canis* 2308), the paw of an eight-year-old female English bulldog suffering from otitis (*A. canis* P3542), the peritoneal effusion of a dog (*A. canis* ZT11003002), and necrotizing fasciitis in a cat (*A. canis* ZT12020010).

Furthermore, the evolving roles of *Arcanobacterium* as an emerging human pathogen have been a subject of discussion [14]. Continuing the search for novel sources of

Arcanobacteria pathogens is of paramount importance. *A. canis*, a significant *Arcanobacteria* found in companion animals, remains relatively understudied. Hence, it is worthwhile to research *A. canis*, given their potential risk of infecting animals and humans.

This study aims to identify and characterize *Arcanobacterium* isolates originating from the vaginal specimens of dogs in Germany and the claw samples of cats in the UK. The isolates were subjected to culture techniques, biochemical analysis, and MALDI-TOF MS to establish their phenotypic characteristics. Additionally, an optimized real-time PCR method focusing on the 16S rRNA gene and the molecular markers rpoB, gap, and tuf was utilized to determine partial genome sequences from the isolates, facilitating subsequent bioinformatic analyses.

2. Materials and Methods

2.1. Bacterial isolates

The isolate P5197-15 was collected from purulent material of the claw of an 11-year-old cat during routine microbiological diagnostic tests at the Institute of Hygiene and Infectious Diseases of Animals, Justus-Liebig-Universität Giessen, Germany, in 2015. The organism was obtained in high numbers with *Staphylococcus epidermidis*, and *Staphylococcus haemolyticum*, which both appeared in low numbers. In addition, the isolate M214-96-1 was obtained from a vaginal swab of a dog submitted for diagnostic procedures at the SRUC Veterinary Services, Inverness, UK. Both isolates P5197-15 and M214-96-1 were identified as *Arcanobacterium* spp. by 16S rRNA gene sequencing (GenBank accession no. LT745899 and AJ234062, respectively). *A. canis* DSM 25104^{T} was used as the positive control.

2.2. Phenotypic characterization

Both isolates, P5197-15 and M214-96-1, were characterized by cultural and biochemical investigation following Christie–Atkins–Munch-Peterson (CAMP)-like hemolytic reactions with *Rhodococcus equi*. Isolates features were evaluated as previously described for *A. canis* and other bacteria of the genus *Arcanobacterium* [15–18]. Deviating from the studies above, cultivation was performed for 24 h at 37 °C under anaerobic conditions using an AnaeroGen™ bag (Oxoid Ltd., Basingstoke, UK). Comprehensive biochemical characteristics were determined in triplicate using the API-Coryne test (BioMerieux Deutschland GmbH, Nürtingen, Germany) by the manufacturer's instructions. In addition, MALDI-TOF MS analysis was performed using the extraction procedure described by the previous study [19].

2.3. Extraction of DNA from cultures

The genomic DNA of the present and control isolates was isolated using the DNeasy blood and tissue kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's instructions. DNA concentrations and template purities were determined using the NanoDrop®2000c spectrophotometer (Thermo Fisher Scientific GmbH, Dreieich, Germany).

2.4. SYBR Green real-time PCR

Real-time PCR and data analysis were performed using the LightCycler®96 system (Roche Diagnostic GmbH, Mannheim, Germany). Four genomic targets of each isolate, including the 16S rRNA gene and the genes of *rpoB, gap*, and *tuf*, were amplified by real-time PCR. The PCR mixture for each gene amplification had a total volume of 30 µL and consisted of the components shown in Table 1. All runs included a negative control reaction without target DNA and one positive control reaction with A. canis DSM 25104T template DNA. The fluorescence of all reaction mixtures was measured at the end of the elongation step of each amplification cycle. The PCR cycle number at which the fluorescence curve of a sample intersected with the threshold line (designated as the Cq value) was recorded. All real-time PCR reactions were evaluated using the LightCycler®96 application software. Amplification products were analyzed with melting temperature (Tm) calling analysis. Subsequently, PCR products were purified and sequenced by Sanger sequencing (Eurofins Genomics Germany GmbH, Ebersberg, Germany). The partial sequences of four different genomic targets of both *A. canis* isolate P5197-15, and M214-96-1 were aligned and analyzed using the clusterW approach of MegAlign version 15. Additionally, sequences of the 16S rRNA gene, as well as of genes *gap*, *tuf*, and *rpoB*, were compared to reference sequences of other *Arcanobcterium* and *Actinomyces* isolates available at the GenBank database using the BLAST algorithm provided by the National Center for Biotechnology Information (NCBI).

Table 1. Sequences of oligonucleotide primers and thermal cycling settings.

Oligonucleotide primers	Sequences	Program*	Primers References
16 rRNA UNI-L	5´-AGAGTTTGATCATGGCTCAG-3´	1	[20]
16 rRNA UNI-R	5´-GTGTGACGGGCGGTGTGTAC-3´		
rpoB-F	5´-CGWATGAACATYGGBCAGGT-3´	2	[21]
rpoB-R	5´-TCCATYTCRCCRAARCGCTG-3´		
gap-F	5´-TCGAAGTTGTTGCAGTTAACGA-3´	3	[22]
gap-R	5´-CCATTCGTTGTCGTACCAAG-3´		
tuf-F	5´-GGACGGTAGTTGGAGAAGAATGG-3´	4	[18]
tuf-R	5´-CCAGGTTGATAACGCTCCAGAAGA-3´		

*Thermal cycling conditions:

1: 10 µL PCR gradient water, 15 µL 2× FastStart Essential DNA Green Master (Roche Diagnostic GmbH, Mannheim, Germany), 1 µL of each primer (0.4 µM), and 3 µL of DNA template. Preincubation 1x (10 min at 95 °C), amplification x30 (30 s at 95 °C, 60 s at 58 °C, 60 s at 72 °C), final elongation x1 (5 min at 72 °C), melting x1 (10 s at 95 °C, 60 s at 65 °C, 1 s at 97 °C).

2: 9.5 µL PCR gradient water, 0.5 µL DMSO, 15 µL 2× FastStart Essential DNA Green Master (Roche Diagnostic GmbH, Mannheim, Germany), 1 µL of each primer (0.4 µM), and 3 µL of DNA template. pre-incubation 1x (10 min at 95 °C), amplification x40 (30 s at 95 °C, 60 s at 50 °C, 60 s at 72 °C), final elongation x1 (2 min at 72 °C), melting x1 (10 s at 95 °C, 60 s at 65 °C, 1 s at 97 °C).

3: 9 µL PCR gradient water, 0.5 µL DMSO, 0.5 µL (25 mM) $MgCl_2$, 15 µL 2× FastStart Essential DNA Green Master (Roche Diagnostic GmbH, Mannheim, Germany), 1 µL of each primer (0.4 µM), and 3 µL of DNA template. Pre-incubation 1x (10 min at 95 °C), amplification x40 (30 s at 95 °C, 60 s at 42 °C, 60 s at 72 °C), final elongation x1 (2 min at 72 °C), melting x1 (10 s at 95 °C, 60 s at 65 °C, 1 s at 97 °C).

4: 8 µL PCR gradient water, 2 µL (25 mM) $MgCl_2$, 15 µL 2× FastStart Essential DNA Green Master (Roche Diagnostic GmbH, Mannheim, Germany), 1 µL of each primer (0.4 µM), and 3 µL of DNA template. Pre-incubation 1x (10 min at 95 °C), amplification x30 (30 s at 95 °C, 60 s at 58 °C, 60 s at 72 °C), final elongation x1 (2 min at 72 °C), melting x1 (10 s at 95 °C, 60 s at 65 °C, 1 s at 97 °C).

3. Results

3.1. Phenotypic results

According to the phenotypic tests (Table 2), both isolates investigated in this study could be reliably identified as *A. canis*. The phenotypic characteristics appeared nearly equivalent to those previously presented for *A. canis* DSM 25104^T.

Table 2. The phenotypical characteristics of *A. canis* be evaluated in this study.

Phenotypical properties	***A. canis* P5197-15**	***A. canis* M214-96-1**	***A. canis* DSM 25104^T*[12]**
Hemolysis on sheep blood agar	+	+	+
CAMP-like reaction with			
Rhodococcus equi	+	+	+
Reverse CAMP reaction	-	-	-
Nitrate reduction	+	-	-
Pyrazinamidase	+	-	-
Pyrrolidonyl arylamidase	+	-	+
Alkaline phosphatase	+	+	+
β-Glucuronidase (β-GUR)	(+)	-	+
β-Galactosidase (β-GAL)	+	+	+
α-Glucosidase (α-GLU)	+	+	+
N-acetyl-βGlucosaminidase (β-NAG)	+	+	+
Esculin (β-Glucosidase)	-	-	-
Urease	+	-	-
Hydrolisis of Gelatine	+	-	-

Acid formation from:			
D-Glucose	+	+	+
D-Ribose	+	+	+
D-Xylose	-	-	-
D-Mannitol	-	-	-
D-Maltose	+	+	+
D-Lactose	+	+	+
D-Saccharose	+	+	+
Glycogen	+	+	+
Catalase	-	-	-

Notes: +: positive reaction; (+): two results showed positive reaction; −: negative reaction by API-Coryne test (BioMerieux Deutschland GmbH, Nürtingen, Germany) with three repetitions.

3.2. MALDI-TOF MS results

With log (score) values between 2.14 and 2.54, the studied isolates could be characterized as *A. canis* according to MALDI-TOF MS analysis. They matched type isolate *A. canis* DSM 25104^T to the species level.

3.3. Melting curves analysis

The melting temperature profile of all amplified target genes of isolates P5197-15 and M214-96-1 was specific and similar to those of *A. canis* DSM 25104^T (Figure 1). Tm for 16S rRNA, *rpoB*, *gap*, and *tuf* gene amplifications averaged 89.14°C ± 0.04, 86.16°C ± 0.05, 87.89°C ± 0.17, 87.87°C ± 0.04, and 89.73°C ± 0.04, respectively. The weak melting peaks produced from the negative control were due to nonspecific signals or the formation of primer dimers (Figure 1 (a), (c), (d)) with more than 10°C differences of Tm calling compared to the positive control.

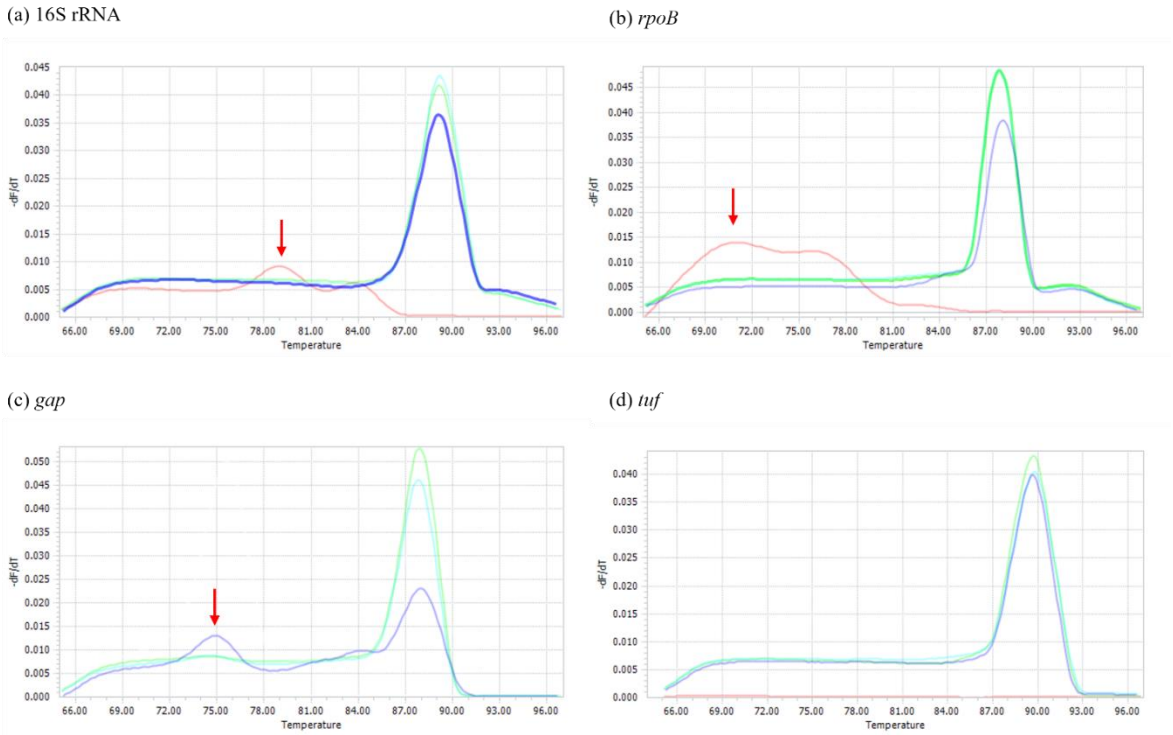

Figure 1. Melting curves: (a) 16S rRNA gene; (b) *rpoB*; (c) *gap*; (d) *tuf* gene amplifications; red arrow showing the non-specific signals or formation of primer dimers.

3.4. Sequencing results

The species classification based on 16S rRNA gene sequencing indicated a sequence identity of at least 99.9% between *A. canis* isolates P5197-15 and M214-96-1 and *A. canis* DSM 25104^{T}. In addition, for the other target genes *rpoB*, *gap*, and *tuf*, the concordance for all molecular targets was 99.5%, 98.9%, and 99.4%, respectively. Figures 2 and 3 depict typical dendrograms of the sequencing data of the 16S rRNA gene and the genes *rpoB*, *gap*, and *tuf*. The alignment of sequences is also shown in Figure 2 and Figure 3.

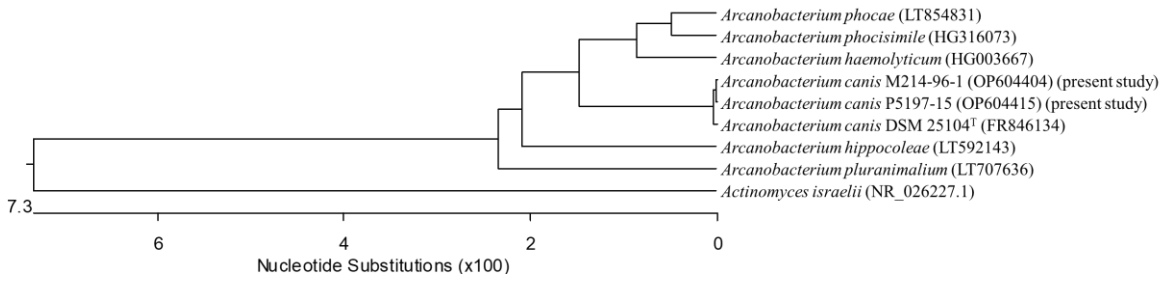

Figure 2. Clustering analysis of the 16S rRNA gene sequences of *A. canis* and other key species of the genera *Arcanobacterium* and *Actinomyces* retrieved from NCBI GenBank. The designation in the parentheses indicates the NCBI accession number.

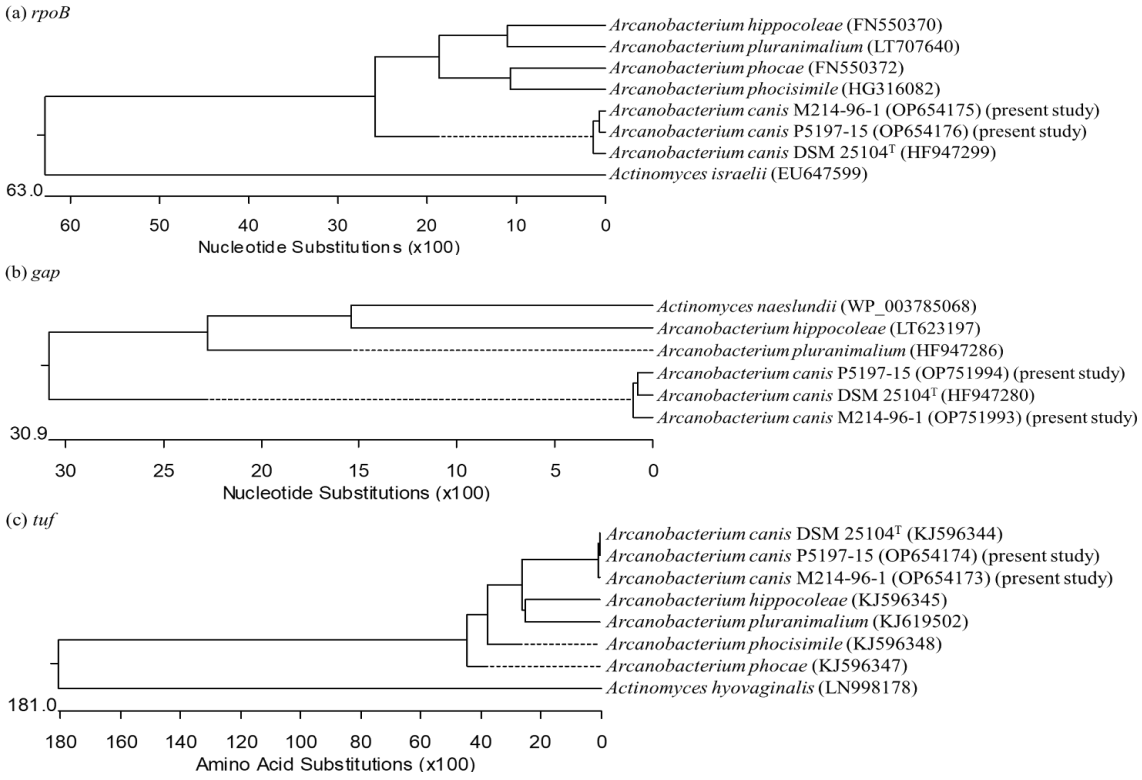

Figure 3. Clustering analysis of the genes: (a) *rpoB*; (b) *gap*; (c) *tuf* of both *A. canis* and other key *Arcanobacterium* and *Actinomyces* species retrieved from NCBI GenBank. The designation in the parentheses indicates the NCBI accession number.

4. Discussion

Because there is a limited number of documented *A. canis* isolates (only five isolates have been officially reported) by Hijazin et al. [12] and Osama et al. [13], isolating this species poses a more significant challenge. Nevertheless, the conventional procedure for identifying and characterizing *A. canis* necessitates a combination of both phenotypic and genotypic tests. Advances in molecular methods, such as real-time PCR followed by sequencing, have improved efficiency and allowed for a more thorough characterization of bacterial species [23–25].

In this study, we focused on two specific isolates of *A. canis*, P5197-15 and M214-96-1, obtained from companion animals. The primary objective was to characterize these isolates using phenotypic and genotypic methods to establish their identity [26–30] as *A. canis*. The initial step involved phenotypic characterization, which assesses the isolates' observable physical and biochemical traits. This process was carried out to determine whether the isolates exhibited key characteristics consistent with *A. canis*. We relied on several criteria, including biochemical properties and CAMP-like hemolytic reactions. These criteria were compared to established reference data (Table 2). Based on the results of the phenotypic

investigation, we determined that both isolates, P5197-15 and M214-96-1, could be reliably identified as *A. canis*. It indicates that the observed phenotypic characteristics of these isolates closely matched those previously documented for *A. canis* DSM 25104^T, a reference isolate [12]. These phenotypic characteristics are crucial in ensuring accurate species classification since each species of *Arcanobacterium* shows specific phenotype characteristics [18, 26, 31, 32]. The results of this phenotypic research strongly indicated that both isolates were true of the *A. canis* species, as their features closely resembled those of a well-established reference isolate. This phenotypic identification was used for further genotypic investigations to confirm their classification as *A. canis*.

Furthermore, this work utilized MALDI-TOF MS (Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry) analysis as a supplementary technique to confirm the classification of the bacterial isolates P5197-15 and M214-96-1 as members of the *A. canis* species. MALDI-TOF MS is a powerful analytical technique that rapidly identifies microorganisms based on their mass spectra, which are unique molecular fingerprints [33, 34]. The analysis generated log (score) values for the two bacterial isolates, which ranged from 2.14 to 2.54. These log (score) values indicate the degree of similarity between the mass spectra of the isolates and the reference spectra in the MALDI-TOF MS database. The key significance of these log (score) values lies in their ability to confirm the identity of the bacterial isolates. In this context, log (score) values between 2.0 and 3.0 are typically considered reliable for species-level identification. Therefore, the log (score) values between 2.14 and 2.54 obtained in this study fall within this range, signifying a strong match between the mass spectra of P5197-15 and M214-96-1 and those of known *A. canis* isolates stored in the MALDI-TOF MS database. The log (score) values effectively classify these isolates as *A. canis*, providing more confidence in phenotypic identification. This result reinforces the conclusion drawn from the phenotypic characterization that P5197-15 and M214-96-1 indeed belong to the species *A. canis*. MALDI-TOF MS is known for its accuracy and speed in identifying microorganisms [35, 36]. It is a valuable tool in clinical and research settings because it can rapidly confirm the identity of bacteria, allowing for quick and reliable species classification [37–39]. The MALDI-TOF MS analysis, with log (score) values falling within the accepted range for species-level identification, served as an independent method to further validate that the bacterial isolates P5197-15 and M214-96-1 were indeed representatives of the species *A. canis.* This analytical technique adds more confidence to the overall identification process, making the species classification more robust and reliable.

The genotypic characterization of the two *A. canis* isolates, P5197-15 and M214-96-1, involved a real-time PCR assay to analyze specific target genes related to the

Arcanobacterium genus. To genetically characterize the two isolates, a protocol real-time PCR was developed. This real-time PCR protocol was designed to amplify target genes associated with the *Arcanobacterium* genus specifically. Real-time PCR is a precise and efficient method for quantifying DNA and monitoring the progress of the amplification process in real-time [40]. Before conducting the real-time PCR, we determined the DNA yield of the isolates P5197-15 and M214-96-1. This measurement revealed that both isolates had sufficient DNA concentrations, with 28.2 ng/µL for P5197-15 and 27.5 ng/µL for M214-96-1. These concentrations ensured enough genetic material was available as a template for the real-time PCR reactions. To confirm the specificity of the real-time PCR assay, we examined the melting curves generated during the PCR process. Identical melting curves, particularly concerning the position of the melting peak of each PCR product, indicated that the amplification was specific to the target genes [41, 42]. This specificity is crucial to ensure that the PCR accurately amplifies only the genes of interest. We determined the melting temperature (Tm) profiles for the amplified target genes, including the 16S rRNA gene, *rpoB, gap*, and *tuf*. These profiles showed that the Tm values for each of these genes were very close. For instance, the average Tm for the 16S rRNA gene was 89.14°C ± 0.04. Such closely matched Tm values suggest that the investigated isolates, P5197-15 and M214-96-1, were genetically similar to *A. canis* DSM 25104^T at the species level.

Sequencing and subsequent genomic analyses were performed to further confirm the isolates' identity. The results showed that P5197-15 and M214-96-1 shared a high sequence similarity with *A. canis* DSM 25104^T. Specifically, they exhibited at least 99.9% similarity in the 16S rRNA gene sequence and strong concordance (98.7% to 99.5%) with other molecular targets such as *rpoB, gap*, and *tuf*. These molecular targets have been confirmed as powerful genotyping tools for the genus *Arcanobacterium* [31]. The high similarity results in this study further confirmed their identification as *A. canis* species. Figures 2 and 3 in the study depict dendrograms based on sequencing data of the 16S rRNA gene and the genes *rpoB, gap*, and *tuf*. While the 16S rRNA gene analysis initially allowed for assignment to the genus *Arcanobacterium*, genomic profiling of other characteristic genes (*rpoB, gap*, and *tuf*) provided further evidence that both isolates represented the *A. canis* species. This comprehensive genetic analysis solidified the conclusion that real-time PCR was a valuable tool for the molecular characterization of *A. canis*.

Notably, the study highlights that it is the first to describe a real-time PCR workflow specifically tailored for amplifying genomic targets suitable for characterizing *A. canis*. This innovative approach enhances our understanding of the genetic makeup of this bacterial species and contributes to more accurate identification and characterization in research and

diagnostic settings [43]. In summary, the genotypic characterization of these *A. canis* isolates involved. The newly established real-time PCR protocol was followed by sequence analysis of the amplified specific targets, DNA yield determination, amplicon specificity confirmation, and analysis of melting temperature profiles and sequence similarity. These comprehensive genetic analyses verified the identity of the isolates as representatives of the *A. canis* species. They underscored the utility of the established real-time PCR protocol for molecular characterization.

Real-time monitoring of the amplification process and evaluating melting curves obtained from various PCR products allow direct selection of potential *Arcanobacterium* representatives and evaluation of the amplification specificity of characteristic genomic targets. Five isolates of *A. canis* have been reported to cause possible infection in companion animals [12,13]. Therefore, this study might help to gain more insight into the pathological significance of this species in companion animals. In addition, real-time PCR offers more capacity for processing large samples, which may benefit larger-scale diagnostics or field studies on the occurrence of *A. canis* in various animal species. We suggest that the established real-time PCR protocol proved to be a suitable tool for selecting potential *Arcanobacterium* candidates and, at the same time, generating templates applicable for sequencing and further characterization of unknown isolates at the species level.

5. Conclusions

In summary, this research endeavors to tackle the obstacles related to the isolation and characterization of *A. canis* isolates, a species that has a scarcity of recorded isolates. The research employs a combination of phenotypic and genotypic tests, demonstrating the utility of real-time PCR followed by sequencing for improved efficiency and thorough characterization of *A. canis* isolates. The phenotypic investigation based on biochemical properties and hemolytic reactions successfully identified the two tested isolates (P5197-15 and M214-96-1) as *A. canis*, with characteristics similar to previously reported isolates. Additionally, MALDI-TOF MS analysis confirmed their classification within the *A. canis* species. Genotypic characterization through real-time PCR targeting specific *Arcanobacterium* genes provided further evidence of the isolates' identity. Sequencing and genomic analyses revealed high similarity to the *A. canis* type isolate, confirming their status as representatives of this species. Notably, the study introduces a real-time PCR workflow for amplifying genomic targets specifically for *A. canis*, which has not been described previously. Real-time PCR offers advantages regarding rapid, sensitive, and specific laboratory confirmation of microorganisms. This established protocol allows for real-time monitoring of the amplification process, evaluation of melting curves, and direct selection of potential *Arcanobacterium* representatives, accelerating the identification of isolates eligible for species-level characterization. The findings of this study contribute to our understanding of the pathological significance of *A. canis* in companion animals, as only a limited number

of isolates have been associated with infections in these animals. Furthermore, the real-time PCR protocol's capacity for processing large samples may be valuable for broader diagnostics or field studies on the occurrence of *A. canis* across different animal species. In summary, this research presents a useful molecular diagnostic tool for identifying and characterizing *A. canis* isolates, shedding light on the species' potential implications in companion animals and enabling broader-scale investigations.

Supplementary Materials: This study's datasets are available in the NCBI GenBank repository (OM663752, OM673022, OP316951, OP316953, OP316952). All derived data supporting the study's results are accessible upon request from the corresponding author, Dr. med. vet. Amir Abdulmawjood.

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