Molecular Characterisation of an *Arcanobacterium* sp. Isolate from a Buffalo (*Bubalus bubalis*)

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Abstract: The objective of the current investigation was to provide a comprehensive characterisation of an *Arcanobacterium* isolate derived from *Bubalus bubalis*. The confirmation of the species identity of *Arcanobacterium* sp. 15/M226/2/9 in this study was achieved by examining phenotypic characteristics and phylogenetic analysis. These analyses involved determining hemolysis on Columbia sheep blood agar and biochemical parameters using the Api-Coryne test kit, MALDI-TOF MS, and partial sequencing of the universal gene encompassing the 16S ribosomal RNA (rRNA) gene, the 16S-23S rDNA intergenic spacer region (ISR), and the two housekeeping genes *rpoB* and *gap*. Species classification based on sequencing of the 16S rRNA gene and the ISR revealed a sequence identity of 98.7% each between *Arcanobacterium* sp. 15/M226/2/9 and *A. bovis* reference strain C605018/01/1^T. In addition, for the other target genes, *rpoB* and *gap,* the concordance was 96.6% and 98.8%, respectively. The present study showed that 15/M226/2/9 isolated from *B. bubalis* could be a novel species.

Keywords: *Arcanobacterium* sp., buffalo, 16S dRNA, ISR, *rpoB*, *gap*.

INTRODUCTION

Arcanobacterium species are significant in veterinary medicine due to their association with various animal diseases [1,2], ranging from skin infections to more systemic and potentially life-threatening conditions [3]. In particular, identifying and characterising *Arcanobacterium* species isolated from domestic and wild animals are crucial for understanding their epidemiology, pathogenicity, and zoonotic potential. This study focused on the molecular characterisation of an *Arcanobacterium* isolate obtained from a buffalo (*Bubalus bubalis bubalis*), an important livestock species in many parts of the world. The buffalo, serving as a source of milk and meat, plays a pivotal role in the agricultural economy of several countries [4,5]. It is, therefore, of great interest to study the pathogens that affect their health and performance. Investigating *Arcanobacterium* infections in buffalo enables researchers to create improved diagnostic instruments, therapies, and preventive measures to promote animal health, boost livestock productivity, and mitigate economic losses while concurrently protecting public health.

An *Arcanobacterium* isolate, designated strain 15/M226/2/9, derived from a pathologically altered lung from a buffalo, may demonstrate distinct genetic, morphological, or pathogenic characteristics that differentiate it from the current understanding of this bacterial group. The strain may have atypical phenotypic traits, including colony form, growth circumstances, or biochemical activity, which could facilitate its differentiation from other *Arcanobacterium* species. Differentiations in hemolytic activity, metabolic enzyme synthesis, or carbohydrate fermentation profiles may assist in the identification procedure. However, phenotypic characteristics alone are often insufficient to accurately identify bacterial species, especially within genera that include close relatives [6]. Consequently, molecular characterisation, including the

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sequencing of the 16S rRNA gene and additional genetic markers (e.g., 16S-23S rDNA intergenic spacer, *rpoB*, and *gap*), may disclose substantial distinctions from previously identified *Arcanobacterium* species. The universal 16S ribosomal RNA (rRNA) gene, widely regarded as the backbone of bacterial phylogeny and taxonomy [7], was sequenced to establish a preliminary phylogenetic position. In addition, the 16S-23S rDNA intergenic spacer region (ISR) was analysed. The ISR offers higher resolution for bacterial differentiation due to its variability among closely related species [8]. Furthermore, sequences of various housekeeping genes were examined, including the beta subunit of the RNA polymerase encoding gene (*rpoB*) and the glyceraldehyde 3-phosphate dehydrogenase encoding gene (*gap*). These genes, known for their conserved nature across bacterial species, provide additional information regarding the phylogenetic relationships and species delineation [9-11]. The characterisation of this *Arcanobacterium* isolate from a buffalo may disclose numerous critical distinctions from previously identified *Arcanobacterium* species, with substantial ramifications for veterinary diagnostics and animal health management. Determining the distinctions of this isolate from recognised *Arcanobacterium* species could enhance veterinary diagnostic proficiency, optimise animal health management, and tackle broader public health issues.

MATERIAL AND METHODS

Bacterial Strains

Arcanobacterium sp. 15/M226/2/9 was collected from the lung of a 4.5-year-old male buffalo (*B. bubalis bubalis*) post-mortem examined at the APHA Veterinary Investigation Centre, Thirsk, UK. The lungs of this animal showed several histopathological findings, as reported in Table **1**. *Arcanobacterium* sp. 15/M226/2/9 was a collection strain from the Institute of Hygiene and Infectious Diseases of Animals, Justus-Liebig-Universität Giessen, Gießen, Germany. *A. bovis* C605018/01/1^T was used as a reference strain.

Phenotypic Characterisation

Hemolysis on Columbia sheep blood agar was determined for phenotypic characterisation [12]. In addition, an Api-Coryne test kit (BioMerieux Deutschland GmbH, Nürtingen, Germany) was utilised following the manufacturer's instructions. Isolate 15/M226/2/9 was further subjected to MALDI-TOF MS analysis, following the extraction methodology outlined by Bastin *et al*. [13]. The examined bacteria were grown for 48 hours at 37°C in a microaerobic jar using a CampyGen™ bag (Oxoid Ltd., Basingstoke, UK).

Genotypic Characterisation

Genomic DNA from both the current and the reference isolate *A. bovis* was extracted using the DNeasy blood and tissue kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's instructions. The spectrophotometer NanoDrop® 2000c (Thermo Fisher Scientific GmbH, Dreieich, Germany) was used to determine DNA concentrations and purities in all eluates.

The DNA obtained was subjected to real-time PCR to amplify four genomic targets, including the 16S rRNA gene, the 16S-23S rRNA ISR, and the housekeeping genes *rpoB* and *gap*. Reactions were performed on the LightCycler®96 instrument (Roche Diagnostic GmbH, Mannheim, Germany) and evaluated using the corresponding application software. Parameters of the real-time PCR assays are

Table 1: Histopathology of the Lung of a 4.5-Year-Old Male Buffalo

Location	Lesio
Alveolar septae	Severe haemorrhage, edematous expansion
Alveoli	Severe haemorrhage, fibrinous exudates present
Interlobular septae	Edematous expansion, fibrinous exudates present
Pleura	Edematous expansion
Airway epithelium	Automatically separating from underlying structures
Vascular endothelium	Early necrotic
Tunica adventitia	Edema
General	Mononuclear inflammatory coccobacilli bacteria, as well as large numbers of rod-shaped and filamentous bacteria, are present.

Table 2: Phenotypical Characteristics of *Arcanobacterium* sp. Isolate Evaluated in this Study

Phenotypical properties	*Arcanobacterium* sp. 15/M226/2/9	*A. bovis* DSM C605018/01/1^T *
Haemolysis on sheep blood agar	+	+
Nitrate reduction	-	-
Pyrazinamidase	+	+
Pyrrolidonyl arylamidase	+	+
Alkaline phosphatase	-	-
β-Glucuronidase (β-GUR)	+	+
β-Galactosidase (β-GAL)	+	+
α-Glucosidase (α-GLU)	-	-
N-Acetyl-βGlucosaminidase (β-NAG)	-	-
Aesculin (β-Glucosidase)	+	+
Urease	-	-
Gelatin hydrolysis	+	+
Acid formation of:		
D-Glucose	+	+
D-Ribose	+	+
D-Xylose	-	-
D-Mannitol	-	-
D-Maltose	+	-
D-Lactose	+	-
D- Saccharose	-	-
Glycogen	-	-
Catalase	-	+

*Findings by Sammra *et al*. (2020). Positive result: +, negative result: -.

given in Table **3**. The total volume of each PCR mixture was 30 µL. Each experimental trial contained a non-template control without target DNA and positive control with template DNA of *A. bovis* reference strain C605018/01/1^T. Fluorescence was measured at the end of each amplification cycle's elongation phase. The recorded Cq value was the PCR cycle number at which the fluorescence curve of a sample intersected the threshold line. PCR cycles were followed by a melting temperature (Tm) assay to assess the specificity of the PCR products obtained. All reactions were evaluated using the LightCycler®96 application software. Results are presented as the average value accompanied by the standard deviation. For purification and Sanger sequencing, PCR products were sent to Eurofins Genomics Germany GmbH (Ebersberg, Germany). The ClustalW technique of MegAlign version 15 was used to align and analyse the partial sequences of the 16S rRNA gene, the ISR, and the genes *rpoB* and *gap*. Furthermore, these four genomic targets were compared with reference sequences of other *Arcanobaterium* species, *Trueperella pyogenes* and *Trueperella pecoris*, obtained from the GenBank database provided by the National Center for Biotechnology Information (NCBI) using the BLAST algorithm (NCBI).

RESULTS

Phenotypic Test Results

Based on the phenotypic tests (Table **2**), *Arcanobacterium* sp. 15/M226/2/9 mainly showed the same characteristics compared to *A. bovis* reference strain C605018/01/1^T. However, the present study found different results on several phenotypic properties involving acid formation from D-maltose, D-lactose, and catalase. Phenotypic assays frequently involve evaluating the bacterium's capacity to ferment various carbohydrates, leading to acid generation. This investigation demonstrated that strain 15/M226/2/9 exhibited a different capacity to produce acid from

Table 3: Sequences of Oligonucleotide Primers and Thermal Cycling Settings

Oligonucleotide primers	Sequences	Program
16 rRNA UNI-L	5´-AGAGTTTGATCATGGCTCAG-3´	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).
16 rRNA UNI-R	5´-GTGTGACGGGCGGTGTGTAC-3´	
ISR-F	5´-GGTACCTTAGATGTTTCAGTTC-3´	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. Preincubation 1x (10 min at 95°C), amplification x30 (30 s at 95°C, 60 s at 45°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).
ISR-R	5´-TTGTACACACCGCCCGTCA-3'	
rpoB-F	5´-CGWATGAACATYGGBCAGGT-3´	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. preincubation 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).
rpoB-R	5´-TCCATYTCRCCRAARCGCTG-3'	
gap-F2b	5´-TTGACCGACAACAAGACCCT-3'	9.5 µl PCR gradient water, 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. Preincubation 1x (3 min at 94°C), amplification x30 (30 s at 94°C, 40 s at 50°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).
gap-R	5´-CCATTCGTTGTCGTACCAAG-3'	

D-maltose and D-lactose compared to the reference strain *A. bovis*. These discrepancies may signify alterations in metabolic pathways or enzyme activities related to carbohydrate utilisation, implying that strain 15/M226/2/9 may metabolise these sugars differently from the reference strain. In addition, the catalase test result for strain 15/M226/2/9 differed from that of the reference strain *A. bovis*. Catalase is an enzyme that decomposes hydrogen peroxide into water and oxygen, and it is frequently utilised to differentiate various bacterial species. This variance in our result may indicate disparities in the strain's capacity to withstand oxidative stress, potentially influencing its growth in specific settings.

MALDI-TOF MS Results

Based on the results obtained from MALDI-TOF MS analysis, the strain under investigation was close to the *Arcanobacterium pluranimalium* species, with a log (score) of 1.86. A log score of 1.86 indicates a probable association with *A. pluranimalium* at the genus level. However, species-level identification remains inconclusive. The score suggests that although the strain exhibits certain protein profile similarities with *A. pluranimalium*, discrepancies may hinder a definitive match, indicating it could represent a different species or a variation with unique traits. Additional study, including sequencing, is required to ascertain the precise species identity of the strain.

Melting Curves Analysis

Arcanobacterium sp. 15/M226/2/9 exhibited melting curves similar to *A. bovis* strain C605018/01/1^T (Figure **1**), indicating that both strains belonged to the same species. The melting points (Tm) for the amplification of 16S rRNA, 16S-23S rRNA ISR, *rpoB,* and *gap* genes were determined to be 89.10 °C ± 0.04, 85.30 °C ± 0.11, 87.28 °C ± 0.18, and 88.02 °C ± 0.11, respectively. The presence of weak melting peaks observed in the negative control can be attributed to either non-specific signals or the formation of primer dimers (Figure **1a**, **b**, **c**, **d**). These peaks exhibited Tm differences of more than 10 °C compared to the positive control.

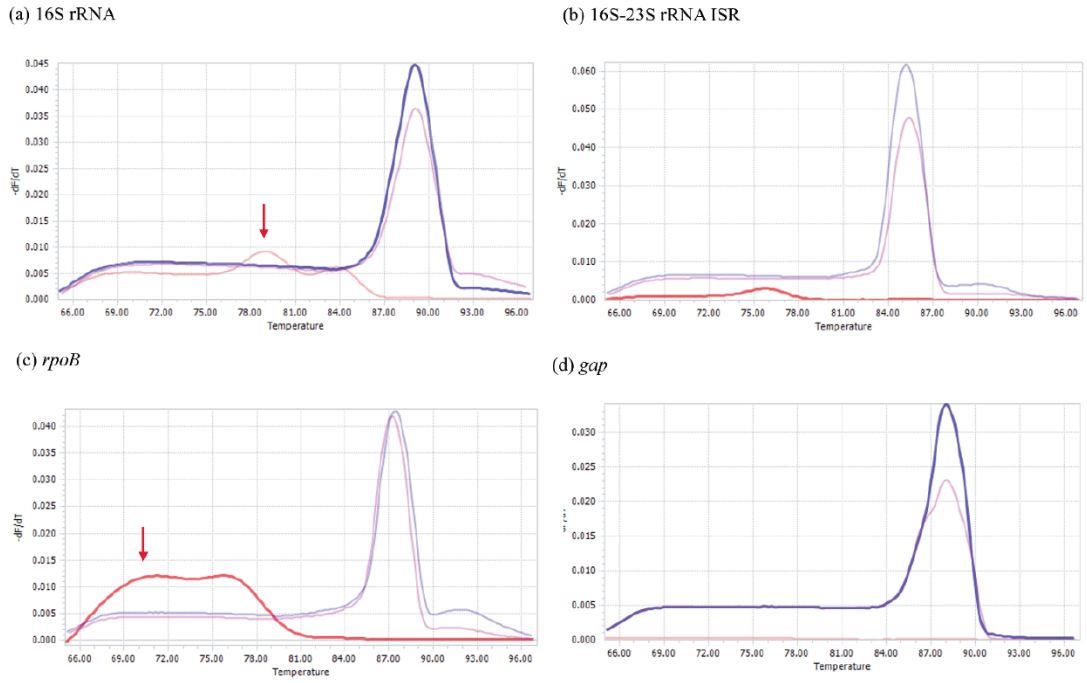

Figure 1: Melting curves of *Arcanobacterium* sp. 15/M226/2/9 and the *A. bovis* isolate for 16S rRNA (**a**), 16S-23S rRNA ISR (**b**), *rpoB* (**c**), and *gap* (**d**), gene amplifications. The melting curves analysis generated Tm of 89.10 °C ± 0.04, 85.30 °C ± 0.11, 87.28 °C ± 0.18, and 88.02 °C ± 0.11 for 16S rRNA, 16S-23S rRNA ISR, *rpoB*, and *gap* gene amplifications, respectively. Red arrow showing non-specific signals or formation of primer dimers.

Sequencing Results

The comparison of *Arcanobacterium* sp. 15/M226/2/9 and *A. bovis* C605018/01/1T using sequence analysis of the 16S rRNA gene and the ISR revealed a sequence identity of 98.7% for each. Furthermore, the concordance rates for the molecular targets *rpoB* and *gap* were 96.6% and 98.8%, respectively. Figures **2** and **3** illustrate dendrograms of the sequencing data for the 16S rRNA gene, the 16S-23S rRNA ISR, and the genes *rpoB* and *gap*.

DISCUSSION

This study examines *Arcanobacterium* sp. 15/M226/2/9, isolated from the lung of a 4.5-year-old male buffalo post-mortem at the APHA Veterinary

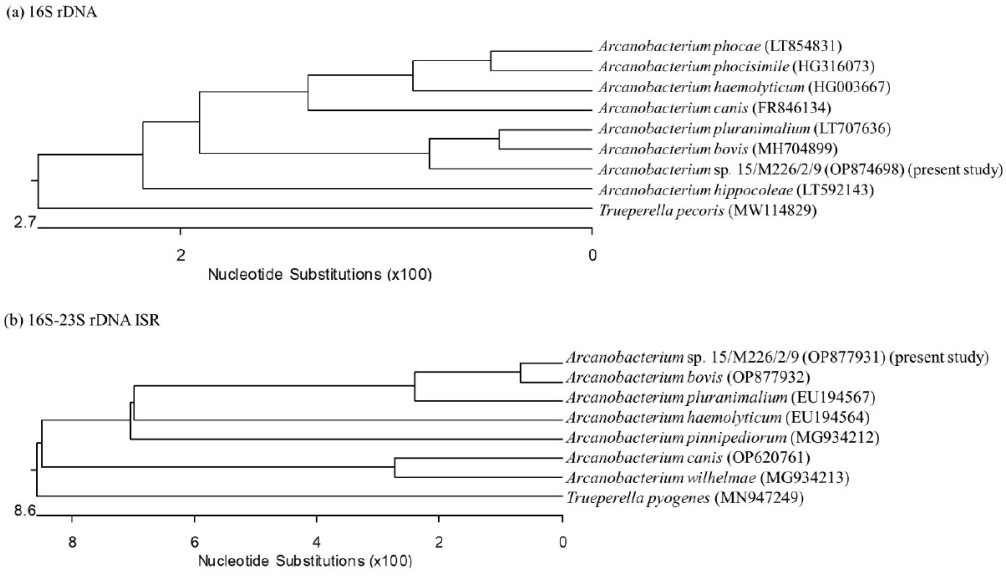

Figure 2: Clustering analysis of the 16S rRNA gene (a) and the 16S-23S rRNA ISR (b) sequences of *Arcanobacterium* sp. 15/M226/2/9 and other key species of the genera *Arcanobacterium* and *Trueperella* retrieved from NCBI GenBank.

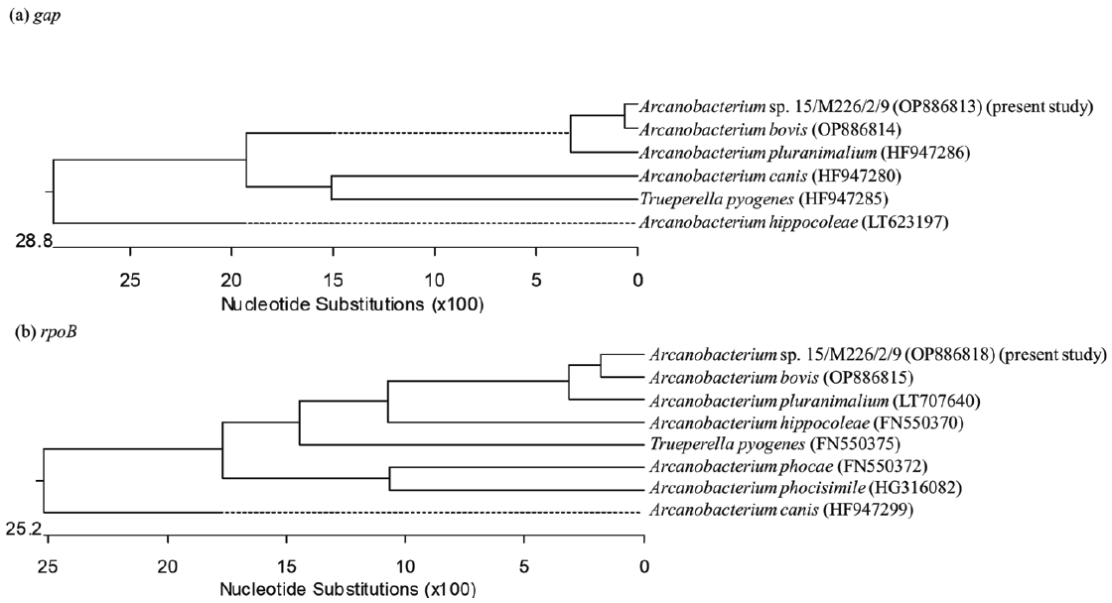

Figure 3: Clustering analysis of the genes *gap* (**a**) and *rpoB* (**b**) of *Arcanobacterium* sp. isolate 15/M226/2/9 and other key *Arcanobacterium* and *Trueperella* species retrieved from NCBI GenBank.

Investigation Centre in Thirsk, UK. This strain demonstrates various unique attributes that underscore its importance in veterinary science, particularly with respiratory infections in buffalo. The strain showed haemolysis on 5% sheep blood agar and had biochemical properties similar to *A. bovis*, with more reactive acid formation from D-maltose and D-lactose but a lack of catalase compared to the original description of this species [12]. The haemolysis results on 5% sheep blood agar indicated that *Arcanobacterium* sp. 15/M226/2/9 has a characteristic linked to pathogenic potential in certain bacterial species. It exhibited comparable biochemical traits to *A. bovis*, although with notable distinctions: increased acid production from D-maltose and D-lactose and a lack of catalase activity relative to the standard characterisation of *A. bovis*. These distinctions imply possible metabolic adaptations or unique enzymatic characteristics, suggesting that *Arcanobacterium* sp. 15/M226/2/9 may be a variation with distinct biochemical properties.

MALDI-TOF MS analysis in this study indicated that the strain is most closely related to *A. pluranimalium* DSM 13483^T with an average log (score) value of 1.86. Nevertheless, this score falls short of the threshold required for accurate species-level identification (log score > 2.0) (14)(15). This indicates that the current MALDI-TOF MS database does not provide the resolution to distinguish between closely related *Arcanobacterium* species. The results underscore the necessity of augmenting the MALDI-TOF MS reference database to enhance the identification and classification of *Arcanobacterium* species, particularly those derived from animals such as buffaloes. Further research is therefore needed, including the isolation of more *Arcanobacterium* strains. Information on specific genus and species characteristics is required to expand and refine the MALDI-TOF MS database to make it a suitable tool for differentiating the diversity of *Arcanobacterium* spp. (16). Based on phenotyping tests, these results indicate that the *Arcanobacterium* sp. 15/M226/2/9 does not belong to *A. bovis.*

A SYBR Green real-time PCR was performed in the present study. Interestingly, while sequencing of the 16S rRNA gene, 16S-23S rRNA ISR, and the housekeeping genes *gap* supported the affiliation of 15/M226/2/9 within the genus *Arcanobacterium*, sequencing of the *rpoB* gene suggested divergence from known species, indicating that the isolate may represent a novel species within the genus (17). The *rpoB* gene encodes the beta subunit of bacterial RNA polymerase. Its sequence is more discriminatory than the 16S rRNA gene in distinguishing different species of bacteria because the divergence levels of the *rpoB* gene sequence are significantly higher than those of the 16S rRNA gene (18). Moreover, the partial *rpoB* gene sequence exhibits the precise reading frame, which leads to the easy verification of sequence accuracy (19). Therefore, the *rpoB* gene is a robust tool for bacterial identification (20), especially for *Arcanobacterium* species (21)(22)(2)(23). Additionally, the *gap* gene, which codes for glyceraldehyde-3-

phosphate dehydrogenase, is highly conserved and could serve as an effective marker in PCR assays for distinguishing between *Arcanobacterium* species (1, 23-25). Molecular identification methods, including 16S rRNA gene sequencing, 16S-23S rRNA ISR analysis, and sequencing of housekeeping genes like *gap*, categorised the strain into the *Arcanobacterium* genus. Sequencing of the *rpoB* gene revealed divergence from established *Arcanobacterium* species, suggesting that strain 15/M226/2/9 may constitute a novel species within the genus. A phylogenetic study utilising the 16S rRNA gene, 16S-23S rRNA ISR, *gap*, and *rpoB* sequences showed that *Arcanobacterium* sp. 15/M226/2/9 differs from *A. bovis*, hence confirming the suggestion that this strain does not belong to the established taxonomy of recognised *Arcanobacterium* species.

The identification of this probable novel *Arcanobacterium* species holds considerable significance for veterinary therapy concerning buffalo. Buffaloes are economically significant animals, and respiratory diseases can severely affect their health and productivity. This discovery broadens the range of recognised bacterial species linked to respiratory illnesses in buffalo. The deficiencies of current diagnostic instruments, such as MALDI-TOF MS, in precisely identifying this strain underscore the necessity for more thorough diagnostic methodologies and enhanced databases. Utilising molecular techniques such as *rpoB* gene sequencing may improve the precision of bacterial identification in clinical contexts.

CONCLUSION

The study of *Arcanobacterium* sp. 15/M226/2/9 indicates the possibility of an unknown species within the *Arcanobacterium* genus and underscores the necessity of enhancing diagnostic techniques and databases for improved buffalo health management. Future research involving whole genome sequencing is essential to verify this strain's taxonomic classification and clarify its involvement in buffalo disease.

AVAILABILITY OF DATA AND MATERIALS

The raw data are available upon request.

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

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