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Helcococcus ovis, an Emerging Pathogen in Bovine Valvular Endocarditis[▽]

Peter Kutzer,[1]* Christoph Schulze,[1] Andreas Engelhardt,[1] Lothar H. Wieler,[2] and Marcel Nordhoff[2]

Landeslabor Brandenburg, 15236 Frankfurt (Oder), Germany,[1] and Institut für Mikrobiologie und Tierseuchen, Freie Universität Berlin, 10115 Berlin, Germany[2]

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The initial isolation of *Helcococcus ovis* from a valvular thrombus prompted us to investigate the prevalence of this bacterium in bovine valvular endocarditis. Specimens from 55 affected hearts were examined by culture using Columbia blood agar and cross streaking the inoculated plate with a *Staphylococcus aureus* strain. As confirmed by 16S rRNA gene sequencing, *H. ovis* was isolated with an unexpectedly high frequency of 33%, predominantly as heavy growth and pure culture. The majority of *H. ovis* isolates showed distinct satellitism around *S. aureus* and pyridoxal dependency, resembling "nutritionally variant streptococci" (now assigned to the genera *Abiotrophia* and *Granulicatella*). Using the API rapid ID 32 Strep, API ZYM, and Rosco Diatabs systems, incongruent results were obtained for alkaline phosphatase, β-galactosidase, β-glucuronidase, and leucine aminopeptidase activities. Based on the satellitism/pyridoxal dependency; hemolysis on blood agar; the API rapid ID 32 Strep results for arginine dihydrolase, α-galactosidase, β-galactosidase, β-glucuronidase, and pyroglutamic acid arylamidase activities; hippurate hydrolysis; and acidification of sucrose, a scheme for the identification of *H. ovis* and its differentiation from other members of the *Helcococcus* genus and the pyridoxal-dependent species *Abiotrophia defectiva*, *Granulicatella adiacens*, and *Granulicatella elegans* is proposed. By establishing specific fluorescence in situ hybridization, large *H. ovis* aggregates were specifically detected within the fibrinous exudate of the valvular thrombi. Our results demonstrate for the first time that *H. ovis* represents an emerging pathogen in bovine valvular endocarditis that is frequently isolated if appropriate culture conditions are used.

Helcococcus ovis, belonging to the family *Peptostreptococcaceae*, is a catalase-negative, facultatively anaerobic, gram-positive coccus first described by Collins et al. in 1999 (4). Initially recovered from lung, liver, and spleen of a sheep together with *Arcanobacterium pyogenes* and from a case of subclinical ovine mastitis together with a *Staphylococcus* species, its clinical significance was unclear (4). Later on, *H. ovis* was isolated from equine and bovine pulmonary abscesses and two cases of bovine valvular endocarditis, indicating that *H. ovis* might be etiologically involved in infections of different mammalian hosts and organ systems (10, 12, 15, 18).

To the best of our knowledge, so far only four *H. ovis* strains have been characterized phenotypically (4, 15, 18). On blood agar, colonies were described as pinpoint, nonpigmented, and nonhemolytic at 24 h but slightly alpha-hemolytic after 72 h of incubation. Initially growth restricted to the periphery of a *Staphylococcus* species was observed for the *H. ovis* strain (CCUG 39041) from sheep and the equine isolate. However, after repeated subculture on blood agar, the strains lost their dependency on the *Staphylococcus* species (4, 18). Biochemical tests were performed using the API rapid ID 32 Strep and API ZYM kits (bioMérieux) (4), the API 20 Strep kit (bioMérieux) (18), or without further specification (15), leading to varying results for alkaline phosphatase, leucine aminopeptidase, and pyrrolidonyl arylamidase activities.

During routine bacteriology of a bovine heart showing valvular endocarditis, we isolated *H. ovis* in pure culture, pointing toward an etiological significance of this bacterium in that particular case. We therefore initiated the present study, investigating the prevalence of *H. ovis* in this important disease by examining a considerable number of bovine endocarditis samples.

MATERIALS AND METHODS

Collection of specimens. In a 1.5-year period, 54 samples from bovine hearts with valvular endocarditis were collected during meat inspection at two abattoirs located in the Federal State of Brandenburg, Germany. The specimens were refrigerated immediately at 4°C and transported to the laboratory on the same day. One additional specimen was obtained during a necropsy carried out in the pathology department of the Landeslabor Brandenburg, Frankfurt (Oder), Germany.

Pathological examination. All heart samples were examined macroscopically, and affected parts were determined. From six hearts with positive *H. ovis* culture results, representative tissue sections were fixed in 10% (vol/vol) neutral buffered formalin, embedded in paraffin wax, cut at 4-μm thickness, and stained with hematoxylin and eosin for histological investigation. In addition, Gram staining was used to characterize bacterial aggregates present in the slides.

Bacteriology. Primary culture was carried out by transferring valvular plaque material on Columbia agar containing 5% sheep blood (CBA) (Oxoid, Wesel, Germany) and overlaying the inoculated area with a single streak of *Staphylococcus aureus*. CBA was incubated at 36°C in ambient air for 48 h. Isolates showing satellitism were examined phenotypically and by 16S rRNA gene sequencing to verify *H. ovis*. All other isolates were identified by conventional bacteriological methods described elsewhere (20). For storage, *H. ovis* colony material was suspended in a cryovial containing 1.8 ml brain heart infusion broth and 0.2 ml sterile glycerol and kept at −80°C.

Phenotypic characteristics. The *H. ovis* isolates were retrieved from storage, grown on Columbia blood pyridoxal agar (CBPA) (Columbia agar base [Merck, Darmstadt, Germany] supplemented with 10% [vol/vol] defibrinated sheep blood and 0.002% [wt/vol] pyridoxal HCl [Sigma-Aldrich, Deisenhofen, Germany]) at 36°C in an atmosphere of 6% CO_2 for 48 h, and subcultured once for 24 h using the same conditions. The isolates were examined and compared to the *H. ovis* strains CCUG 37441^T and CCUG 39041 for the following characteristics: Gram stain reaction, catalase reaction, vancomycin resistance, anaerobic growth on CBPA, satellitism, pyridoxal dependency, and various biochemical reactions. For testing satellitism and pyridoxal dependency, a saline suspension equivalent to a

* Corresponding author. Mailing address: Landeslabor Brandenburg, Gerhard-Neumann-Str. 2/3, 15236 Frankfurt (Oder), Germany. Phone: 49 335/52 17 118. Fax: 49 335/52 17 120. E-mail: peter.kutzer@llb.brandenburg.de.

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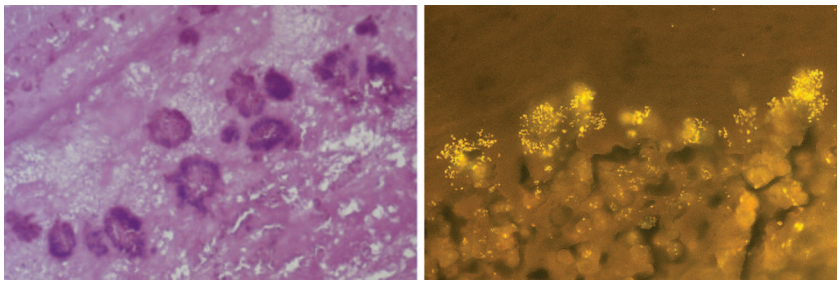

FIG. 1. Gram staining (left) (magnification, ×100) and FISH (right) (magnification, ×400) showing multifocal aggregates within the fibrinous exudate of an *H. ovis* culture-positive valvular thrombus.

turbidity of a 0.5 McFarland standard was plated onto CBA. Thereafter, an *S. aureus* streak was placed on one half and a pyridoxal HCl (0.01% [wt/vol] aqueous solution)-impregnated 6-mm paper disk on the other half of the agar plate. Incubation was performed at 36°C in an atmosphere of 6% CO_2 for 24 and 48 h. The strains *Abiotrophia defectiva* DSM 9849^T, *Granulicatella adiacens* DSM 9848^T, and *Granulicatella elegans* DSM 11693^T served as positive controls and the strain *Helcococcus kunzii* DSM 10548^T as a negative control.

Biochemical tests were performed in duplicate using the API rapid ID 32 Strep and API ZYM kits according to the manufacturer's instructions (bioMérieux, Nürtingen, Germany). In addition, alkaline phosphatase, arginine dihydrolase, α-galactosidase, β-galactosidase, β-glucosidase, β-glucuronidase, leucine aminopeptidase, and pyrrolidonyl arylamidase activities; hippurate hydrolysis; and acid production from maltose and sucrose were examined using Rosco Diatabs (A/S Rosco, Taastrup, Denmark). Diatabs tests for alkaline phosphatase activity and hippurate hydrolysis were analyzed at 4 h and the remaining reactions after overnight incubation.

16S rRNA gene sequencing analysis. DNA extraction from *H. ovis* isolates was performed using the Qiagen tissue kit according to the bacterial support protocol. For PCR amplification, approximately 1,490 bp of the 16S rRNA gene was amplified using forward primer Seq-fw (5′-TGGCTCAGGACGAACGCT-3′, positions 20 to 37 in the *Escherichia coli* 16S rRNA gene) and Seq-rev (5′-CTT CGGGTATTGCCAACTC-3′, positions 1416 to 1434 in the *Escherichia coli* 16S rRNA gene). Amplification of the 16S rRNA gene was conducted in a final volume of 50 μl containing 2 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates, 10 pmol of each primer, and 2.5 U *Taq* polymerase. Samples were preheated at 94°C for 5 min, followed by amplification at 94°C for 30 s, 56°C for 30 s, and 72°C for 60 s. A total of 30 cycles were carried out, with a final elongation step at 72°C for 10 min. Successful amplification was verified by agarose gel electrophoresis.

Sequencing of the 16S rRNA gene was conducted commercially (AGOWA, Berlin, Germany), resulting in at least 1,290 bp for each strain. Phylogenetic analyses were performed by comparative 16S rRNA gene sequence analysis using the Lasergene software package (DNASTAR Inc., Madison, WI).

Fluorescence in situ hybridization (FISH). The *H. ovis*-specific probe HOVIS (5′-ATAGTATAGTTTCTTCGGAAAC-3′), corresponding to positions 91 to 104 in *E. coli* 16S rRNA, was designed after comparative analysis of the 16Sr RNA gene sequences obtained for the *H. ovis* endocarditis isolates and the sequences of *H. ovis* CCUG 37441^T, *H. kunzii* DSM 10548^T, and *Helcococcus sueciensis* CCUG 47334^T as deposited in the GenBank database. The specificity of the probe HOVIS was checked against all 16S rRNA gene sequence entries available in GenBank and the Ribosomal Database Project II using the software tool "Probe Match," which is part of the Ribosomal Database Project environment (11).

The oligonucleotide probe HOVIS used for the FISH technique was synthesized commercially (Thermo Hybaid, Ulm, Germany) and 5′ labeled with Cy3 (indocarbocyanine). Representative tissue samples were prepared as described for histological investigations and deparaffanized in xylene prior to FISH. To test the specificity of the probe and to adjust stringency, fixed control strains were used at formamide concentrations of 10, 20, 30, and 40% (vol/vol).

FISH was performed at 48°C in a humid chamber for 18 h using 20 μl hybridization buffer (30% [vol/vol] deionized formamide, 0.9 M NaCl, 20 mM Tris-HCl [pH 7.4], 0.01% [wt/vol] sodium dodecyl sulfate) and 100 ng of HOVIS for each section. After incubation, sections were washed with distilled water at 48°C for 10 min and mounted with ProLong antifade reagent (Molecular Probes, Leiden, The Netherlands). Fluorescence microscopy was performed using a Leica DMBL microscope (Leica, Wetzlar, Germany).

To assess specific hybridization conditions, the following *Helcococcus* strains as well as strains of unrelated bacterial species were included in FISH experiments to serve as positive and negative controls: *H. ovis* CCUG 37441^T, *H. ovis* CCUG 39041, *H. kunzii* DSM 10548^T, *H. sueciensis* CCUG 47334^T, *A. pyogenes* IMT 8803, *Clostridium perfringens* ATCC 13124^T, *Enterococcus dispar* DSM 6630^T, *Enterococcus faecium* ATCC 6057, *S. aureus* ATCC 25923, and *Streptococcus* sp. strain ATCC 9932.

RESULTS

Pathology and bacteriology. All 55 hearts investigated showed typical lesions of chronic valvular endocarditis. The valvular thrombi were of variable size and composed of a luminal zone containing unorganized fibrinous exudate. Deeper parts of the thrombi were organized by granulation tissue maturing into avascular, collagen-rich scar tissue. The type and quantity of affected valves were determined as follows: tricuspic, 39; tricuspic and bicuspic with myocard abscesses, 1; tricuspic and pulmonic, 1; pulmonic, 6; bicuspic, 6; aortic, 1; and atrioventricular but not further identifiable, 1. In *H. ovis* culture-positive thrombi, histology revealed large aggregates of gram-positive cocci within the luminal fibrinous exudate (Fig. 1).

From all valvular plaque specimens, bacteria were recovered as heavy growth, mainly in pure culture or accompanied by just a few colonies of unspecific flora, e.g., coliform bacteria, enterococci, and/or coagulase-negative staphylococci. Mixed infections were found in only three cases (Table 1). *H. ovis* was

TABLE 1. Bacteriological findings in 55 cases of bovine valvular endocarditis

Predominant bacterial species	Coisolate(s)	No. of cases
A. pyogenes	None	33
	S. aureus	1
H. ovis	None	16
	Pseudomonas aeruginosa, *Enterococcus faecalis*	1
	Streptococcus dysgalactiae subsp. *dysgalactiae*	1
S. dysgalactiae subsp. *dysgalactiae*	None	1
Streptococcus sp.	None	1
Member of the family *Neisseriaceae*	None	1

TABLE 2. API rapid ID 32 Strep results for 18 *H. ovis* endocarditis isolates compared to results obtained for *H. ovis* control strains

Phenotypic characteristic[a]	Test result for *H. ovis* strain:		Aggregated test result[b] (no. of positive isolates) for *H. ovis* endocarditis isolates
	CCUG 37441^T	CCUG 39041	
Arginine dihydrolase	−	−	− (0)
β-Glucosidase	−	−	− (0)
β-Galactosidase[c]	+	+	+ (18)
β-Glucuronidase	+	+	V (11)
α-Galactosidase	−	−	− (0)
Alkaline phosphatase	−	−	− (0)
Ribose	−	−	− (0)
Mannitol	−	−	− (0)
Sorbitol	−	−	− (0)
Lactose	−	−	V (4)
Trehalose	−	−	V (5)
Raffinose	−	−	− (0)
Acetoin	−	−	− (0)
Alanyl-phenylalanyl-proline arylamidase	−	−	− (0)
β-Galactosidase[d]	−	−	− (1)
Pyroglutamic acid arylamidase	−	−	− (0)
N-Acetyl-β-Glucosaminidase	−	−	− (0)
Glycyl-tryptophan arylamidase	−	−	− (0)
Hippurate hydrolysis	−	−	− (0)
Glycogen	−	−	− (0)
Pullulan	−	−	V (11)
Maltose	+	+	+ (18)
Melibiose	−	−	− (0)
Melezitose	−	−	− (0)
Sucrose	−	−	− (0)
L-Arabinose	−	−	− (0)
D-Arabitol	−	−	− (0)
Methyl-β-D-glucopyranoside	−	−	− (0)
Tagatose	−	−	− (1)
β-Mannosidase	−	−	− (0)
Cyclodextrin	−	+	+ (16)
Urease	−	−	− (0)

[a] In order of the arrangement in the kit.
[b] +, V, and −, ≥85%, 16 to 84%, and ≤15% of strains were positive, respectively.
[c] With resorufin-β-D-galactopyranoside as the substrate.
[d] With 2-naphtyl-β-D-galactopyranoside as the substrate.

TABLE 3. API ZYM results for 18 *H. ovis* endocarditis isolates compared to the results obtained for *H. ovis* control strains

Phenotypic characteristic[a]	Test result for *H. ovis* strain:		Aggregated test result[b] (no. of positive isolates) for *H. ovis* endocarditis isolates
	CCUG 37441^T	CCUG 39041	
Alkaline phosphatase	+	+	+ (18)
Esterase (C4)	+	+	+ (18)
Esterase lipase (C8)	+	+	+ (18)
Lipase (C14)	−	−	− (0)
Leucine arylamidase	+	+	+ (18)
Valine arylamidase[c]	+	+	+ (16)
Cystine arylamidase	+	+	+ (18)
Trypsin	−	−	− (0)
α-Chymotrypsin	−	−	− (0)
Acid phosphatase	+	+	+ (18)
Naphtol-AS-BI-phosphohydrolase	+	+	+ (18)
α-Galactosidase	−	−	− (0)
β-Galactosidase	−	−	− (0)
β-Glucuronidase	−	−	− (0)
α-Glucosidase[c]	+	+	V (14)
β-Glucosidase	−	−	− (0)
N-Acetyl-β-glucosaminidase	−	−	− (0)
α-Mannosidase	−	−	− (0)
α-Fucosidase	−	−	− (0)

[a] In order of the arrangement in the kit.
[b] +, V, and −, ≥85%, 16 to 84%, and ≤15% of strains were positive, respectively.
[c] Frequently weak reaction.

obtained from 17 cows and 1 bull originating from 17 different farms. The mean age of the animals was 47 months (range, 20 to 75 months).

In primary culture, the *H. ovis* isolates demonstrated distinct satellitism around the *S. aureus* streak. For only one isolate, a slight growth outside the *S. aureus* hemolysis zone could be observed. Colony polymorphism, ranging from pinpoint, grayish colonies to tiny, translucent colonies, was common and remained stable after subculture on CBPA. All *H. ovis* isolates were nonhemolytic on CBA and CBPA after 48 h of incubation.

Phenotypic characteristics. All *H. ovis* strains were gram positive to gram labile, with cocci arranged in clusters and pairs. They were catalase negative and vancomycin sensitive and grew facultatively or anaerobically. After subculture and storage, 14 *H. ovis* endocarditis isolates (78%) showed satellitism and pyridoxal dependency, whereas the *H. ovis* type strain (CCUG 37441^T) did not. *H. ovis* strain CCUG 39041 yielded an ambiguous result, characterized by marked growth restricted to the zones of *S. aureus* hemolysis and pyridoxal disc diffusion at 24 h but sparse growth even outside these zones after 48 h of incubation.

Details of the biochemical characterization are given in Tables 2 to 4. The results for some enzyme activities differed depending on the test system used. Alkaline phosphatase activity was always positive in the API ZYM system but negative in the API rapid ID 32 Strep (color shift to very pale yellow) and the Rosco Diatabs systems. β-Galactosidase activity was continuously demonstrated for the substrate resorufin-β-D-galactopyranoside (API rapid ID 32 Strep) but not for 2-naphtyl-β-D-galactopyranoside (API rapid ID 32 Strep and API ZYM). Using the Rosco *o*-nitrophenyl-β-D-galactopyranoside Diatabs, predominantly weak positive reactions were observed. All strains except one that were positive for β-glucuronidase in the API rapid ID 32 Strep yielded positive results with the respective Rosco Diatabs, but all strains were negative in the API ZYM. All strains displayed strong leucine aminopeptidase activity in the API ZYM, but four endocarditis isolates and *H. ovis* CCUG 39041 were negative using the Rosco Diatabs, exhibiting a yellow or yellow-orange color.

16S rRNA sequence analysis. All *H. ovis* endocarditis isolates shared an almost-identical 16S rRNA gene sequence, with at least 99.6% sequence identity. By comparing their 16S rRNA gene sequences to all available entries in GenBank, every strain could be assigned to the species *H. ovis*, showing at least 99.7% sequence identity with *H. ovis* CCUG 37441^T.

FISH. Using 30% (vol/vol) formamide, only *H. ovis* was detected by FISH with the HOVIS probe, whereas no signals

TABLE 4. Rosco Diatabs results for 18 *H. ovis* endocarditis isolates compared to the results obtained for *H. ovis* control strains

Phenotypic characteristic	Test result for *H. ovis* strain:		Aggregated test result[a] (no. of positive isolates) for *H. ovis* endocarditis isolates
	CCUG 37441^T	CCUG 39041	
Alkaline phosphatase	−	−	− (0)
Arginine dihydrolase	−	−	− (0)
α-Galactosidase	−	−	− (0)
β-Galactosidase[b]	+	+	+ (16)
β-Glucosidase	−	−	− (0)
β-Glucuronidase	+	+	V (10)
Leucine aminopeptidase	+	−	V (14)
Pyrrolidonyl arylamidase	−	−	− (0)
Hippurate hydrolysis	−	−	− (0)
Maltose	+	+	+ (18)
Sucrose	−	−	− (0)

[a] +, V, and −, ≥85%, 16 to 84%, and ≤15% of strains were positive, respectively.
[b] Predominantly weak reaction.

were obtained from the negative control strains. When FISH was applied to the tissue sections, *H. ovis* was demonstrated in large amounts, predominantly as multifocal aggregates within the fibrinous exudate in luminal parts of the valvular thrombi (Fig. 1).

DISCUSSION

Valvular endocarditis is an important cause of illness and death in adult cattle, predominantly cows, with an incidence ranging from 0.12% at slaughter to up to 9% in necropsy cases (13, 21). *A. pyogenes* is known to be the most important bacterial agent in bovine endocarditis (13, 14, 16, 19, 21) and was also found in the majority of cases (62%) in this study. Remarkably, as confirmed by 16S rRNA gene sequence analysis, *H. ovis* (33%) represented the second most common isolate, recovered mainly in pure culture.

Histologically, the *H. ovis*-induced valvular lesions were characterized by large bacterial aggregates, thus corresponding to the results of Post et al. (15). In addition, we applied FISH in the postmortem examination and specifically detected *H. ovis* in the superficial parts of the lesions. Due to the fastidious character of some bacteria capable of causing endocarditis, culture often remains difficult. Therefore, FISH has already been proven to be an important diagnostic tool for the detection of human endocarditis pathogens (8), and here we also demonstrate its usefulness in bovine endocarditis.

Satellitism and pyridoxal dependency were found to be major characteristics of *H. ovis* endocarditis isolates, resembling the case for the so-called "nutritionally variant streptococci" (7), which are now designated *A. defectiva*, *G. adiacens*, and *G. elegans* (5, 9, 17). As reported for *Abiotrophia* and *Granulicatella* species (1, 6), *H. ovis* may also adapt to growth without a *Staphylococcus* strain or pyridoxal supplement (4, 18), which might complicate the phenotypic identification. In the present study, the first two *H. ovis* isolates obtained demonstrated satellitism in primary culture. Prior to storage, they were transferred three or four times on CBA with an *S. aureus* streak. After recovery, growth on CBA did not require *S. aureus* or pyridoxal. Therefore, all other isolates were subcultured only once on CBPA before storage at −80°C, but despite this measure, two of them also lost satellitism and pyridoxal dependency.

Examination of biochemical reactions using different test systems revealed incongruent results for previously defined key characteristics, i.e., alkaline phosphatase, β-glucuronidase, and leucine aminopeptidase activities (4), and for β-galactosidase activity. This might be due to the various substrates contained in the systems and indicates the need for precise method specification when presenting biochemical data. If commercially available test kits are used, the manufacturer's instructions should be strictly followed to achieve comparable results. In contrast to the work of Collins et al. (4), production of esterase C4 and esterase lipase C8 were consistently observed using the API ZYM. Moreover, the large majority of *H. ovis* strains displayed weak valine arylamidase activity.

Based on evaluation of the phenotypic characteristics examined during this work, an API rapid ID 32 Strep-based scheme for the identification of *H. ovis* and its differentiation from other *Helcococcus* species and the pyridoxal-dependent species *A. defectiva*, *G. adiacens*, and *G. elegans* is proposed (Table 5). The predicted results for *H. kunzii*, *H. sueciensis*, *A. defectiva*,

TABLE 5. Phenotypic characteristics, including API rapid ID 32 Strep-based tests, potentially useful in the identification of *H. ovis* and its discrimination from other *Helcococcus* species and the pyridoxal-dependent species *A. defectiva*, *G. adiacens*, and *G. elegans*

Phenotypic characteristic	Result for species[a] (source)					
	H. ovis (animal)	*H. kunzii* (human)	*H. sueciensis* (human)	*A. defectiva* (human)	*G. adiacens* (human)	*G. elegans* (human)
Satellitism/pyridoxal dependency	+	−	−	+	+	+
Hemolysis on sheep blood agar	γ	γ	γ	α	α	α
Arginine dihydrolase	−	−	−	−	−	+
α-Galactosidase	−	−	−	+	−	−
β-Galactosidase[b]	+	+	+	+	−	−
β-Galactosidase[c]	−	−	+	+	−	−
β-Glucuronidase	V	−	−	−	+	−
Hippurate hydrolysis	−	−	−	−	−	+
Pyroglutamic acid arylamidase	−	+	−	+	+	+
Sucrose	−	−	−	+	+	+

[a] +, V, and −, ≥85%, 16 to 84%, and ≤15% of strains were positive, respectively.
[b] With resorufin-β-D-galactopyranoside as the substrate.
[c] With 2-naphtyl-β-D-galactopyranoside as the substrate.

G. adiacens, and *G. elegans* (1–3, 5, 9, 17) were verified for the API rapid ID 32 Strep kit using the type strains of the respective species. In contrast to previous findings (1), β-galactosidase activity was negative for the type strain of *G. adiacens* (DSM 9848^T).

The high culture prevalence and FISH results presented here demonstrate that *H. ovis* is an emerging pathogen in bovine valvular endocarditis. For the future, attention should be paid to this bacterium in veterinary microbiology, ensuring appropriate culture conditions crucial for its isolation, particularly coincubation of specimens with a *Staphylococcus* strain and use of pyridoxal-supplemented media.

Although one report assumes the skin to be the source of *H. ovis* (18), there is no valid information concerning the natural habitat. Thus, it remains to be investigated how *H. ovis* enters the bloodstream and how this pathogen circumvents serum complement activity.

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