Cardiobacterium valvarum infective endocarditis and phenotypic/molecular characterization of 11 *Cardiobacterium* species strains

Ming Chen,^{1,2} Michael Kemp,^{1,3} Niels E. Bruun,⁴ Jette M. Bangsborg,⁵ Niels Højlyng,⁶ Annemarie Hesselbjerg,¹ Rimtas Dargis¹ and Jens Jørgen Christensen^{1,7}

Correspondence

Jens Jørgen Christensen
jejc@regionsjaelland.dk

¹Department of Microbiological Surveillance and Research, Statens Serum Institut, Copenhagen, Denmark

²Department of Clinical Microbiology, Rigshospitalet, Copenhagen, Denmark

³Department of Clinical Microbiology, Odense University Hospital, Odense, Denmark

⁴Department of Cardiology, Gentofte University Hospital, Gentofte, Denmark

⁵Department of Clinical Microbiology, Herlev University Hospital, Herlev, Denmark

⁶Infectious Diseases Unit, Medical Department, Roskilde University Hospital, Roskilde, Denmark

⁷Department of Clinical Microbiology, Slagelse Hospital, Slagelse, Denmark

Cardiobacterium valvarum is a newly recognized human pathogen related to infective endocarditis. *Cardiobacterium* species are, however, only rarely the aetiology of infective endocarditis. An infective endocarditis case is presented and, additionally, phenotypic and phylogenetic comparison of a further 10 collection strains, representing the two species within the genus, was performed. *C. valvarum* was isolated from the blood and DNA was present in valvular tissue (partial 16S rRNA gene analysis) from a 64-year-old man with infective endocarditis of the mitral valve, rupture of chordae and prolapse of pulmonary valves in addition to a fluttering excrescence. A mechanical mitral valve and neochordae were inserted successfully. Phenotypically, the two species within the genus *Cardiobacterium* resemble each other greatly. When using the Vitek 2 *Neisseria*–*Haemophilus* identification card, the reaction for phenylphosphonate was positive for all *Cardiobacterium hominis* strains, but negative for all *C. valvarum* strains, thereby separating the two species. The two species made up two separate clusters by phylogenetic examination using 16S rRNA gene sequence analysis.

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INTRODUCTION

The genus *Cardiobacterium* was created in 1964, including one species, *Cardiobacterium hominis*, a fastidious, pleomorphic, Gram-negative bacillus (Slotnick & Dougherty, 1964). The genus *Cardiobacterium* is one of the members of the HACEK group (Das *et al.*, 1997; Lang & Morris, 2002; Slotnick & Dougherty, 1964), a group of Gram-negative bacteria that includes *Haemophilus* spp., *Aggregatibacter actinomycetemcomitans*, *C. hominis*, *Eikenella corrodens* and *Kingella kingae*. Before 2004, *C. hominis* was the only *Cardiobacterium* species known. *Cardiobacterium valvarum* was first identified as a novel species by Han *et al.* (2004) and is a rare aetiological agent of infective endocarditis. Until now, only seven cases of *C. valvarum* infective endocarditis have been reported in the literature (Vaněrková *et al.*, 2010; Geissdörfer *et al.*, 2007; Bothelo *et al.*, 2006; Han, 2005; Han & Falsen, 2005; Han *et al.*, 2004; Hoffman

et al., 2010). In this study, we report a case of infective endocarditis of the mitral valve, rupture of chordae and prolapse of P1 and P2 in addition to a fluttering excrescence caused by *C. valvarum* and an analysis of 11 strains of *Cardiobacterium* species, four of *C. hominis* and seven of *C. valvarum*.

CASE REPORT

A 64-year-old man was admitted to the local hospital due to increasing dyspnoea and severe mitral insufficiency. He worked as a music teacher. He had a 1-year history of moderate mitral valve prolapse and mitral insufficiency diagnosed by echocardiography, and was an outpatient at the cardiology department of the hospital. During the previous year, he had become tired easily and experienced slight dyspnoea when bicycling uphill.

Eight weeks prior to admission, the patient received a tooth implantation on which occasion he did not receive antibiotic prophylaxis. For the previous several weeks, the patient had experienced febrile episodes, profuse sweating and night shiver episodes. A new episode occurred the night before admission, in addition to low blood pressure and fever of 39.9 °C. On admission, he was afebrile with a blood pressure of 130/60 mmHg. Auscultation revealed a grade 2 of 6 systolic heart murmur. A light oedema of both ankles was noted. Laboratory examinations showed haemoglobin of 65 g l⁻¹, leukocyte count of 9.4 × 10⁹ l⁻¹, C-reactive protein of 79 mg l⁻¹, thrombocyte count of 88 × 10⁹ l⁻¹, basic phosphatase of 127 U l⁻¹ (normal range 35–105), bilirubin of 36 µmol l⁻¹ (normal range 5–25) and lactate dehydrogenase of 271 U l⁻¹ (normal range 105–205). Ultrasound examination of the abdomen was normal. Six blood culture bottles (Bactec Plus aerobic/F bottles) were taken and empirical therapy with intravenous penicillin 5 million units four times daily and gentamicin 120 mg twice daily was initiated.

Four out of the six blood culture bottles taken on initial admission yielded growth of Gram-negative rods after 3 days of incubation. Penicillin was changed to intravenous cefuroxime (1.5 g three times a day), and the gentamicin treatment was continued. Echocardiography revealed severe mitral insufficiency with mitral valve prolapse, rupture of chordae and prolapse of P1 and P2 in addition to a fluttering excrescence. Three days after admission, the patient was moved to the cardiology department at a highly specialized hospital for further treatment.

Ten days after admission, the Gram-negative rods were eventually identified as *C. valvarum*. Cefuroxime treatment was changed to ceftriaxone 1 g twice daily.

The patient was operated on 22 days after admission. The mitral valve was replaced with a mechanical valve and neo-chordae were inserted. While culture was negative, bacterial DNA from *C. valvarum* was found on the mitral valve by partial 16S rRNA gene sequence analysis. The patient received 6 weeks of antibiotic treatment of which 2 weeks was post-operative. The patient was discharged clinically well 78 days after admission without paraclinical signs of infection.

METHODS

Bacterial culture and strains. The case strain and 10 other strains examined are listed in Table 1. The case strain was isolated from blood samples.

Phenotypic characterization. Strains were cultured on 5 % and 10 % horse-blood agar and chocolate agar plates. Incubation was at 37 °C in ambient air with and without a supplement of 5 % CO₂. Growth at 22 °C was read for 6 days. Anaerobic growth, on a prereduced medium, was read after incubation for 6 days in an anaerobic jar (70 % N₂, 20 % H₂ and 10 % CO₂). Motility, cellular morphology and Gram-stained smears of all bacterial strains were examined by light microscopy. Phenotypic characterization of strains was mostly conducted using conventional methods as recommended

by the Danish Reference Group for Identification of Clinically Important Bacteria (Bruun *et al.*, 1999). β-Galactosidase production was detected by the method of Kilian & Bülow (1976) with 0.2 % o-nitrophenyl-β-galactoside. All strains were also tested with an API 20NE kit and the Vitek 2 *Neisseria-Haemophilus* identification card and read according to the manufacturer's instructions. Antimicrobial susceptibility testing was performed with Neo-Sensitabs (Rosco) on Danish Blood Agar using breakpoints according to the Clinical and Laboratory Standards Institute for *Haemophilus* species (www.rosco.dk) and for tigecycline using Etest. The case strain was also examined by Etest for susceptibility to selected treatment-relevant antibiotics.

Partial 16S rRNA gene sequencing. Partial 16S rRNA gene sequencing of bacterial strains followed by BLAST examination was performed using two amplification primers, BSF8 and BSR1407, producing a 1399 bp fragment; these fragments were sequenced both ways (Christensen *et al.*, 2005). For partial 16S rRNA sequencing of bacterial DNA in valve tissue PCR was done on DNA extracted by a QIAamp DNA Mini kit (Qiagen) using the primers BSF8 and BSR534, which produce a 526 bp product (Christensen *et al.*, 2005). All fragments were sequenced both ways. The edited sequences were compared to deposited sequences in the NCBI 'bacteria' database (BLAST examination) and evaluated for the best and second best taxon matches taking into consideration the percentage identity (number of identical bases between the query and the subject sequence in the database), Max scores (indication of alignment concordance) and E-values (indication of statistical significance of a given alignment). Partial 16S rRNA sequencing of bacterial DNA in valve tissue was done as previously described (Koch *et al.*, 2010).

RESULTS

Phenotypic characterization

Growth characteristics. The phenotypic features of the 11 strains were compared. When incubated in ambient air supplemented with 5 % CO₂ on 5 % and 10 % horse blood agar as well as on chocolate agar, all strains grew with colonies <1 mm after 24 h incubation and with grey colonies sized 1–2 mm after 48 h incubation, except for four strains [CCUG 12990, CCUG 31208, CCUG 48245^T (*C. valvarum*) and CCUG 31207 (*C. hominis*)], which had not grown after 24 h, but grew with small colonies sized <1 mm after 48 h. Colonies were similar, being small, round, opaque, smooth, grey and glistening. For all strains there was slight α-haemolysis on blood agar plates. There was no difference in colony morphology between *C. valvarum* and *C. hominis*, and no difference in morphology on 5 % and 10 % blood agar and chocolate agar plates. Strains were Gram-negative rods that varied in size, typically being pleomorphic and some had pointed ends. All strains were facultatively anaerobic and non-motile. Some strains had an acidulous smell.

Biochemical reactions. All strains were oxidase-positive, catalase-negative and nitrite reductase-negative, except for one *C. hominis* strain (CCUG 31207) which was nitrite reductase-positive. All strains produced acid from glucose (no gas), maltose, mannitol, mannose, sorbitol and sucrose, but not from lactose or galactose. Eight out of eleven strains were indole-positive. Ten out of eleven

Table 1. Characteristics of the 11 *Cardiobacterium* strains examined and the results of near full-length (1399 bp) 16S rRNA gene sequence analysis

Strain designation(s)*	Source and disease/year	Species designation of strain/best taxon match	Max score	Identities of base composition	No. of identical taxon match	Difference to second best taxon match		Second best match
						Max score	Base composition	
Case strain (SSI: AB2924)	Blood, endocarditis/2006	<i>C. valvarum</i>	2470	1345/1350 (99 %)	5	240	1314/1365 (96 %)	<i>C. hominis</i>
(SSI: AB2089)	Blood, endocarditis/1983	<i>C. hominis</i>	2435	1367/1381 (98 %)	1	200	1312/1364 (96 %)	<i>C. valvarum</i>
CCUG (EF) 2711 ^T , ATCC 15826, NCTC 10426 (SSI: AB2098)	Blood, endocarditis/1965	<i>C. hominis</i>	2441	1367/1376 (99 %)	1	200	1312/1359 (96 %)	<i>C. valvarum</i>
NCTC 10666 (SSI: AB 1616)	Blood, endocarditis/1966	<i>C. hominis</i>	2448	1367/1381 (98 %)	1	298	1312/1364 (96 %)	<i>C. valvarum</i>
CCUG 31207 (SSI: AB2167, Kilian C22)	Human tooth plaque/1985	<i>C. hominis</i>	2423	1320/1320 (100 %)	2	236	1280/1326 (96 %)	<i>C. valvarum</i>
CCUG (EF) 13150 (SSI: AB2102)	Human subgingival pocket periodontitis/1982	<i>C. valvarum</i>	2432	1366/1380 (98 %)	5	226	1322/1381 (95 %)	<i>C. hominis</i>
CCUG 12990	Human subgingival pocket, amelogenesis imperfecta/1982	<i>C. valvarum</i>	2752	1490/1490 (100 %)	5	406	1381/1435 (96 %)	<i>C. hominis</i>
CCUG (EF) 13094	Human subgingival pocket/1982	<i>C. valvarum</i>	2481	1345/1346 (99 %)	5	212	1328/1378 (96 %)	<i>C. hominis</i>
CCUG 31208 (SSI: AB2168, Kilian C50)	Human tooth plaque/1985	<i>C. valvarum</i>	2756	1492/1492 (100 %)	5	394	1383/1434 (96 %)	<i>C. hominis</i>
CCUG 48245 ^T , MDA 3079, DSM 17211, ATCC BAA-694, NCTC 13294, CIP 108480	Blood, endocarditis, ruptured cerebral aneurysm/2001	<i>C. valvarum</i>	2758	1493/1493 (100 %)	5	412	1383/1437 (96 %)	<i>C. hominis</i>
CCUG 53031	Blood, aortic valve replacement/2006	<i>C. valvarum</i>	2217	1200/1200 (100 %)	5	245	1161/1208 (96 %)	<i>C. hominis</i>

*ATCC, American Type Culture Collection, Manassas, VA, USA; CCUG, Culture Collection of the University of Göteborg, Sweden; NCTC, National Collection of Type Cultures, Colindale, England; SSI, Statens Serum Institute, Copenhagen, Denmark (SSI strain with internal SSI number).

strains were sucrose-positive. All strains were tested with an API 20NE kit, but there were no positive reactions. Table 2 shows the positive and variable biochemical reactions of the 11 strains in the Vitek 2 *Neisseria-Haemophilus* identification card wells. Eleven wells containing the following substances were read as negative for all strains: arginine arylamidase, glycogen, β -galactopyranoside indoxyl, maltotriose, L-proline arylamidase, ornithine decarboxylase, L-lysine-arylamidase, D-galactose, phosphorylcholine, urease, D-malate and D-xylose. Three reactions were positive for all strains and seven reactions were variable among both species (see Table 2). One reaction, the well containing phenylphosphonate, was positive for all *C. hominis* strains, but negative for all *C. valvarum* strains, thereby being useful in separating the two species. Results obtained by automatic reading were as follows: two strains were

identified as *Kingella kingae* (CCUG 53031 and AB2924, acceptable identifications), one strain (CCUG 48245) could not be identified, and all other strains were identified as *C. hominis* (acceptable–excellent identifications).

Antimicrobial susceptibility. All 11 strains were sensitive to penicillin, ampicillin, mecillinam, cefuroxime, ceftazidime, piperacillin/tazobactam, moxifloxacin, gentamicin, ciprofloxacin, colistin and tigecycline (by Etest with MIC range 0.125–1 $\mu\text{g ml}^{-1}$). All strains were sensitive to erythromycin, except for two strains [CCUG 53031 (*C. valvarum*) and SSI: AB2089 (*C. hominis*)] which were resistant and intermediate-susceptible, respectively. The case strain was also shown by Etest to be susceptible to penicillin (MIC 0.094 $\mu\text{g ml}^{-1}$), ampicillin (MIC 0.025 $\mu\text{g ml}^{-1}$), moxifloxacin (MIC 0.032 $\mu\text{g ml}^{-1}$), ciprofloxacin

Table 2. Positive and variable test results obtained when using the Vitek 2 *Neisseria*–*Haemophilus* identification card on 11 *Cardiobacterium* strains

Substrates with negative test results for all strains are given in Methods.

Substrate in well	Tests positive or variable among strains	
	<i>C. valvarum</i> (n=7)	<i>C. hominis</i> (n=4)
Test result different for the two species		
Phenylphosphonate	0/7	4/4
Positive or predominantly positive test results for both species		
Leucine arylamidase	7/7	4/4
Ellman	7/7	4/4
D-Glucose	7/7	4/4
Phenylalanine arylamidase	6/7	4/4
Maltose	7/7	4/4
Saccharose/sucrose	6/7	4/4
Negative or predominantly negative test results for both species		
L-Glutamine	0/7	1/4
α -Arabinosidase	1/7	0/4
Pyruvate	2/7	0/4
D-Ribose 2	0/7	2/4
Variable test results within and among the two species		
γ -Glutamyltransferase	4/7	4/4
D-Mannose	4/7	4/4
L-Pyrrolidonyl arylamidase	6/7	1/4
Phosphatase	6/7	0/4
Tyrosine arylamidase	1/7	1/4
Ala-Phe-Pro-arylamidase	3/7	3/4
N-Acetyl-D-glucosamine	2/7	3/4

(MIC 0.004 $\mu\text{g ml}^{-1}$) and meropenem (MIC 0.004 $\mu\text{g ml}^{-1}$).

16S rRNA gene sequencing and phylogenetic analysis

Table 1 shows the results obtained by 16S rRNA gene sequencing and subsequent BLAST examination of the bacterial strains. Seven and four strains were identified as *C. valvarum* and *C. hominis*, respectively. The phylogenetic trees (Fig. 1a, b) show that strains within the two species were very similar and that the two species were well separated. *C. valvarum* strains had sequence similarities ranging between 98 and 100 %, Max scores between 2217 and 2758, and Max score differences to the next best taxon match, which for all strains was *C. hominis*, ranging from 212 to 412. *C. hominis* strains had sequence similarities ranging between 98 and 100 %, Max scores between 2423 and 2435, and Max score differences to the next best taxon match, which for all strains was *C. valvarum*, ranging from 200 to 298.

Partial 16S rRNA gene sequence examination of the surgically removed valve showed *C. valvarum* as best match with a Max score of 857 and number of identities being 469 out of 471, with *C. hominis* as second best match (score 719 bits, 449 of 476 identities).

DISCUSSION

Infective endocarditis is most commonly caused by *Staphylococcus aureus*, coagulase-negative staphylococci and streptococci (McDonald, 2009). Up to 3 % of cases of infective endocarditis are attributable to HACEK organisms (Das *et al.*, 1997). Bacteria in this group have in common a culture requirement for an enhanced CO₂ atmosphere and the ability to infect human heart valves. *C. valvarum* is also a new member of the HACEK group and was first reported by Han *et al.* (2004). The first reported strain was grown from the blood of a 37-year-old man who had insidious endocarditis with a sudden rupture of a cerebral aneurysm. Characterization of the organism through phylogenetic and phenotypic analyses revealed a novel species of *Cardiobacterium*, *Cardiobacterium valvarum*.

At least seven previous cases of infective endocarditis caused by *C. valvarum* have been reported worldwide (Vaněrková *et al.*, 2010; Geissdörfer *et al.*, 2007; Bothelo *et al.*, 2006; Han & Falsen 2005; Han *et al.*, 2004; Hoffman *et al.*, 2010). In six reported cases, blood cultures yielded growth of *C. valvarum* strains and in one case bacteriological diagnosis was made by 16S rRNA gene amplification, sequencing and BLAST examination of valvular tissue alone; in two cases, bacteriological diagnosis was obtained by a positive blood culture as well as by molecular examination of valvular tissue, as in the case described here. In five of the previously described cases, the aortic valves were involved (in one case in addition to the tricuspid valves), and in one case, details were not given. Of the five cases of aortic involvement, one was fatal (infection in a prosthetic valve) and valve replacement was performed in three cases. The ruptured intracranial aneurysm described by Han *et al.* (2004) illustrates the propensity of *C. valvarum* to cause an embolism, a feature it has in common with *C. hominis* as well as its tendency to cause exceptionally low grade and insidious infections (Das *et al.*, 1997).

The present case adds to the description of the infective potential of *C. valvarum* as it was the mitral valve that was harmed and rupture of chordae occurred. Valve replacement was needed and neochordae were inserted. The patient described in the present report had a 1-year history of a moderate mitral valve prolapse and mitral insufficiency diagnosed by echocardiography and had received a tooth implantation 8 weeks prior to admittance. Most reported *C. valvarum* infective endocarditis cases were related with periodontal diseases. Four of the strains were isolated from the oral cavity of patients with periodontitis, subgingival pockets or tooth plaque, further supporting

Fig. 1. (a) Phylogenetic tree determined on the basis of the sequences of the 16S rRNA gene obtained by the unrooted neighbour-joining method in the MEGA (version 4.0) program package. The scale bar indicates the evolutionary distance between the sequences determined by calculation of the per cent sequence divergence. It clearly divides the two species *C. hominis* and *C. valvarum* into two groups. (b) Minimal evolution algorithm (suppressed) obtained by using the MEGA (version 4.0) program and based on the 16S rRNA gene. It clearly divides the two species *C. hominis* and *C. valvarum* into two groups.

that the most probable way of the bacterium entering the bloodstream is via the upper respiratory tract.

C. valvarum has been described as a fastidious Gram-negative bacillus that grows better on sheep blood agar than on chocolate agar. In contrast to *C. hominis*, *C. valvarum* should grow more slowly, be non-haemolytic on sheep blood agar, and not utilize sucrose, maltose or mannitol (Han *et al.*, 2004). However, phenotypic tests may not always allow the two *Cardiobacterium* species to be distinguished and different phenotypic methodologies may give different results as seen with regard to results obtained for acid production from sucrose, maltose and mannitol. In such cases, genotypic identification is a good help and should be performed. Phenotypic characterization analysis shows that *C. valvarum* and *C. hominis* have many reactions in common. When using conventional methods, the only difference we found between these two *Cardiobacterium* species was acid production from raffinose, which for *C. hominis* strains was positive. However, no acid was produced from raffinose by *C. hominis* in the report by Han & Falsen (2005). It is difficult to distinguish *C. valvarum* and *C. hominis* from their phenotypic characterization. No reactions were positive among reactions included in the API 20NE kit. Automatic reading when using the Vitek 2 *Neisseria*–*Haemophilus* Identification Card did not separate the two species as the database only includes *C. hominis*. However, results from the well containing phenylphosphonate seem useful as a test separating the two species. One research group (Geissdörfer *et al.*, 2007) used the API NH identification system, which is commonly used for identification of *Haemophilus* species and *Neisseria* species of clinical importance. The outcome of the reactions was well defined, but resulted in a misidentification as *Haemophilus parainfluenzae*. Therefore, they concluded that API NH may be used for determination of key reactions in the case of fastidious Gram-negative rods such as *C. valvarum*, but for final identification, molecular tools, e.g. 16S rRNA gene sequence analysis, are indispensable. The present data show that *C. valvarum* and *C. hominis* are susceptible to many antibiotics. These data are consistent with previous data also showing sensitivity to most used β -lactams. The patient described here was treated successfully with cefuroxime and ceftriaxone.

Conventional methods for microbial identification require the recognition of differences in morphology, growth, enzymic activity and metabolism to define genera and species. As seen above, *C. hominis* and *C. valvarum* have many similarities and few different reactions. Full-length and partial 16S rRNA gene sequencing methods have emerged as useful tools for identifying phenotypically aberrant and relatively non-aberrant micro-organisms and have made major contributions to taxonomy within the last decades. When performing short (526 bp) and near full-length (1399 bp) 16S rRNA gene sequence analysis all examined strains convincingly were allocated to either *C. hominis* or *C. valvarum*. These results are in accordance

with the results reported in the individual case reports. Phylogenetic trees based on 16S rRNA gene sequence analysis clearly divided the examined strains into the two species. One group (Petti *et al.*, 2005) examined three blood-culture strains received for further identification, of which one turned out to be *C. valvarum*, where the initial phenotypic identifications were erroneous. The authors conclude that this may have misled clinicians, and potentially impacted patient care; 16S rRNA gene sequencing is a more objective identification tool, unaffected by phenotypic variation or technologist bias, and has the potential to reduce laboratory errors. In two of the case reports, the presence of *C. valvarum* 16S rRNA gene sequences was also demonstrated in removed valvular tissue, as in our case.

In conclusion, we describe what is believed to be the eighth case of infective endocarditis due to *C. valvarum*, and the first case affecting the mitral valves and with ruptured chordae. Clinical microbiologists and physicians should be aware that *C. valvarum* is a potential agent of infective endocarditis when HACEK strains are isolated from blood cultures. Our data show that *C. valvarum* and *C. hominis* strains have high phenotypic resemblance with few differentiating reactions, which makes us speculate that some of the early *C. hominis* strains identified by phenotypic methods might be *C. valvarum*. 16S rRNA gene sequence analysis has proved to be a very useful and reliable tool for identifying strains and confirming their presence in valvular tissue.

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