Molecular Identification of *Gemella* Species from Three Patients with Endocarditis

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Received 31 July 1997/Returned for modification 10 October 1997/Accepted 30 December 1997

*Gemella morbillorum* and *Gemella haemolysans* are opportunistic pathogens which cause endocarditis and other severe infections. We report on three patients with endocarditis, one with endocarditis caused by *G. haemolysans* and two with endocarditis caused by *G. morbillorum*. The paucity of reports concerning these bacteria is probably related to the difficulties associated with their identification. For example, one of the strains reported in this study was originally sent to our laboratory with a preliminary characterization as a short “gram-negative” coccobacillus, highlighting the specific problem associated with Gram staining of these bacteria. The usefulness of 16S rRNA gene amplification, partial sequencing, and comparison of the nucleotide sequence to those in databases when standard phenotypic identification schemes are not helpful is emphasized. We also suggest that the use of simple tests, such as testing susceptibility to vancomycin for gram-negative bacteria and colistin for gram-positive bacteria, could prevent misinterpretation of Gram staining in gram-variable bacteria such as *Gemella* spp.

Gemella morbillorum and *Gemella haemolysans* are gram-positive coccoid commensal organisms of the mucous membranes of humans and other warm-blooded animals. However, as “opportunistic pathogens,” gemellae are able to cause severe localized and generalized infections.

The cases of *Gemella* infection reported to date have been predominantly endovascular infections (1, 6, 8–12, 15, 22, 25, 28, 29, 31, 33–35, 39, 40, 43, 45). Among these cases of *Gemella* infection, most are endocarditis, usually associated with previous valvular damage and/or poor dental state. Central nervous system and skeletal infections have also been described (4, 13, 23, 36, 38, 48). In a study of 52 cases of “streptococcal” endocarditis, gemellae represented 6% of the viridans group streptococci and 5% of all isolates (17). In our hospital center, gemellae were the cause of 6% of the cases of endocarditis caused due to nonstaphylococcal gram-positive cocci, were the cause of 13.3% of the cases of endocarditis caused by viridans group streptococci, and represented 2.9% of all bacterial isolates causing 70 cases of endocarditis diagnosed during a 3-year period (unpublished data).

These bacteria are easily decolorized during Gram staining and sometimes appear as elongated cells, explaining why they were first described as *Neisseria* (46). They may also be more involved in clinical disease than is presently recognized, because they can be incorrectly identified as viridans group streptococci or left unidentified (42).

In this paper we report on two patients with *G. morbillorum* endocarditis and one patient with *G. haemolysans* endocarditis. The identification of one *G. morbillorum* strain was achieved with the help of partial 16S RNA gene sequencing, since the bacterial isolate appeared to be gram negative. Partial 16S rRNA gene sequencing was also used as a reference technique to confirm the identities of a further two strains of *Gemella*. The results obtained by electron microscopy and analysis of cell wall fatty acids are reported, and the previous cases of endocarditis caused by *G. haemolysans* and *G. morbillorum* are reviewed.

CASE REPORTS

**Patient 1.** A 63-year-old man who was a heavy smoker with chronic obstructive bronchitis and a very poor dental state was admitted to hospital complaining of intermittent fever, loss of weight, and anorexia over a 1-month period. He had no history of rheumatic disease, although a moderate heart murmur had been discovered 1 year previously, and at that time an echocardiogram had yielded moderate mitral valve regurgitation. On initial examination, the patient had an increased heart murmur, a temperature of 38.5°C, and a pulse of 100 beats/min. An ejection systolic murmur was heard at the apex of the heart. Laboratory investigations showed a hemoglobin concentration of 100 g/liter, an erythrocyte sedimentation rate of 97 mm/h, and a leucocyte count of 8.53 × 10^9/liter (77% neutrophils). A transesophageal echocardiogram demonstrated the presence of vegetation on the mitral valve with moderate mitral valve regurgitation. Six sets of blood samples for cultures were taken on admission and the following day, and the blood samples were inoculated into BACTEC aerobic (NR 6-A+) and anaerobic (NR-7A+) bottles in a BACTEC NR-860 automated instrument (Becton Dickinson Diagnostic Instrument Systems, Sparks, Md.). All yielded slowly growing, gram-positive cocci, subsequently identified as *G. haemolysans*. A kidney echography, carried out for microscopic hematuria, yielded a lesion inside of the left kidney which was compatible with an abscess. The patient’s treatment began the day after his admission, in which treatment with amoxicillin (4 g intravenously at 6-h intervals) and amikacin (5 mg/kg of body weight intravenously at 8-h intervals) was begun. His condition improved rapidly, and after 2 weeks of this regimen, he underwent cardiac surgery in order to remove the motile vegetation. One week after surgery antibiotic therapy was discontinued. After 2 years of follow-up he remains well.

**Patient 2.** A 74-year-old man with a history of Pott’s disease in infancy, chronic alcoholism, and a poor dental state was
admitted to hospital complaining of intermittent fever, sweating, loss of weight, and basithoracic pain over a 3-month period. He had neither a history of rheumatic disease nor a previous heart murmur. On initial examination, the patient had a diastolic heart murmur, a temperature of 38°C, and a pulse of 100 beats/min. Laboratory investigations showed a hemoglobin concentration of 95 g/liter, an erythrocyte sedimentation rate of 63 mm/h, and a leukocyte count of 12.8 × 109/liter (87% neutrophils). A transthoracic echocardiogram demonstrated aortic valve incompetence but failed to show any vegetation. Six sets of blood samples for culture were taken on admission and the following day, and the blood samples were inoculated into BACTEC aerobic (NR 6-A*) and anaerobic (NR-7A*) bottles in a BACTEC NR-860 automated instrument. All yielded slowly growing gram-variable cocci and cocobacilli subsequently identified as *G. morbillorum*. The patient's treatment began the day after his admission and comprised amoxicillin (4 g intravenously at 6-h intervals) and gentamicin (1 mg/kg intravenously at 8-h intervals). Although his condition improved rapidly, it was necessary to transfer him to a cardi-thoracic unit because he was also suffering from significant aortic valve regurgitation and a dilated left ventricle. The aortic valve was successfully replaced with a prosthetic device, and the patient made an uneventful postoperative recovery. Gentamicin was discontinued 1 week later, and amoxicillin was discontinued 3 weeks later. After 1 year of follow-up he remains well.

**Patient 3.** A small, fastidious, gram-negative rod which had been isolated from the blood of a male patient with infectious endocarditis by using BACTEC aerobic (NR 6-A*) and anaerobic (NR-7A*) bottles was sent to our laboratory for identification, because it was thought that it could be a *Bartonella* sp. The patient was a farmer and suffered from a bacuscipid aortic valve. Clinical symptoms included intermittent fever and a weight loss of 12 kg over a period of several months. Attempts to amplify citrate synthase and 16S rRNA genes with specific primers for *Bartonella* (27) were unsuccessful. Standard phenotypic characterization methods for gram-negative rods also failed to provide an identity. The bacterium was identified as *G. morbillorum* by 16 rRNA gene sequencing.

**MATERIALS AND METHODS**

**Phenotypic identification.** Presumptive identification of the *Gemella* organisms was achieved through assessment of colonial morphology, hemolysis on Columbia agar with 5% sheep blood (BioMerieux, Marcy l’Etoile, France), microscopic appearance after Gram staining, and the results of biochemical tests (with the API 20 Strep and the API 20A systems according to the manufacturer's instructions). Growth was also attempted in broth containing 6.5% NaCl. Susceptibility to vancomycin and colistin was assessed with 30-μg vancomycin and 50-μg colistin disks (Sanofi Diagnostic Pasteur, Marnes la Coquette, France) and Mueller-Hinton broth with 5% sheep blood agar (BioMerieux) by the conventional disk diffusion test method (2). The strain was considered to be susceptible to vancomycin and to colistin when inhibition zone diameters of ≥12 and ≥15 mm, respectively, were observed.

**Cellular fatty acids.** Colonies of the three isolates and of the type strains *G. haemolyans* ATCC 10379 and *G. morbillorum* ATCC 27824 were grown on Trypticase soy agar with 5% sheep blood (Becton Dickinson) at 38°C for 48 h. They were then saponified, and cell wall fatty acids were extracted and analyzed by gas chromatography as reported previously (37).

**Processing for electron microscopy.** Bacterial cells were harvested from colonies grown on Columbia agar and for fixation were suspended in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) containing 0.1 M sucrose. The fixed cells were washed overnight with the same buffer and were then fixed for 1 h at room temperature with 1% osmium tetroxide in 0.1 M cacodylate buffer. Dehydration was performed by washing the cells in gradually increased concentrations (25 to 100%) of ethanol. The cells were then embedded in Epon 812. Thin sections were cut from embedded blocks with an LKB Ultratome III microtome and were poststained with a saturated solution of methanol-uranyl acetate and lead citrate in water before examination on a Zeiss JEM 1200 EX electron microscope.

**PCR amplification and sequencing of the PCR product.** DNA extracts, suitable for use as templates in PCRs, were made from 200 μl of a bacterial suspension by using a QIAamp Blood kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. All primer sets used in this study are listed in Table 1. DNA extracts were amplified by a PCR incorporating universal primers d1d and r2p (49) (Eurogentec, Seraing, Belgium). PCR amplifications were performed in 100-μl volumes incorporating 10 μl of extracted DNA, 10 μl of 10× reaction buffer, 100 μM (each) deoxynucleoside triphosphate, 0.2 μM (each) primer, 2 U of Taq polymerase (Perkin-Elmer Cetus, Norwalk, Conn.), and sterile, distilled water. Amplifications were carried out in a Perkin-Elmer 9600 thermal cycler for 35 cycles, with each cycle consisting of denaturation at 95°C for 30 s, primer annealing at 55°C for 30 s, and extension at 72°C for 60 s. The success of the amplification was determined by ethidium bromide staining following the resolution of products by 1% agarose gel electrophoresis. Each experiment included sterile water (no DNA) as a negative control and Escherichia coli DNA as a positive control. The products of 16S rRNA gene amplification were purified for sequencing by using Microspin S-400 HR columns (Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's instructions. Sequencing reactions were prepared by using the Amplitype sequencing kit (Perkin-Elmer), according to the manufacturer's instructions, and one of six 5′-fluorescein-labelled primers was incorporated into the reaction mixture (Table 1). The thermal cycle used in each sequencing reaction was dependent on the base sequence of the primer (Table 1). When the primer annealing temperature was ≥50°C, an initial denaturation step at 95°C for 1 min was followed by 25 cycles, with each cycle comprising denaturation at 95°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 1 min. Amplification was completed by an extension step of 5 min at 72°C to allow complete extension of the amplified products. When the primer annealing temperature was <50°C, an initial denaturation step at 95°C for 1 min was followed by 30 cycles, with each cycle comprising denaturation at 95°C for 30 s, annealing at 50°C for 30 s, and extension at 60°C for 2 min. Ten supplementary cycles of denaturation at 95°C for 10 s and extension at 60°C for 90 s were performed in order to increase the amount of polymerization. Sequencing reactions were carried out on a Perkin-Elmer 9600 thermal cycler, and reaction products were resolved by electrophoresis on a 6% polyacrylamide gel incorporated into an ALF Automatic Sequencer (Pharmacia Biotech).

**Sequence analysis.** Partial 16S rRNA gene sequences derived from each reaction mixture were evaluated in a complete sequence by using PC Gene software (IntelliGenetics Inc.). The complete sequence was then compared with all bacterial sequences available in the GenBank database, by using the multisequence comparison program FASTA (part of the BISANCE software package) (14).

**RESULTS**

**Bacterium from patient 1.** The bacterium isolated from patient 1 was thought to be a strain of *G. haemolyans* on the basis of conventional phenotypic identification. Growth was easily achieved on Columbia agar with incubation at 37°C under 5% CO2, but only poor growth was obtained under anaerobic conditions. Colonies were small and weakly beta-hemolytic after 5 days of incubation. Microscopic examination after Gram staining yielded gram-positive cocci which became
TABLE 2. Results of phenotypic characterization of strains isolated in this study

<table>
<thead>
<tr>
<th>Feature</th>
<th>Patient 1, G. haemolysans</th>
<th>Patient 2, G. morbillorum</th>
<th>Patient 3, G. morbillorum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Voges-Proskauer</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Leucine aminopeptidase</td>
<td>+</td>
<td>+</td>
<td>+ (weak)</td>
</tr>
<tr>
<td>Pyrrolidone arylamidase</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Esculin hydrolysis</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Acid from glucose</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Acid from sucrose</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Acid from maltose</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Acid from mannitol</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Acid from mannoose</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Acid from trehalose</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Growth in 6.5% NaCl</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Vancomycin susceptibility</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

**DISCUSSION**

G. morbillorum was originally proposed as *Diplococcus ru- beolate* (47), and a second member of the genus *Diplo-
coccus morbillorum* was added soon after (41). However, on reappraisal the two *Diplococcus* species were considered to be identical and were unified under the name *D. morbillorum*. Although *D. morbillorum* could be grown under aerobic conditions, it was primarily anaerobic, and on this basis it was transferred to the genus *Peptostreptococcus* (44), only to be reclassified into the genus *Streptococcus* (26). *G. haemolysans* was first described in 1938 as *Neisseria haemolysans* (46) (demonstrating its indeterminate Gram staining characteristic), but the species was reclassified into a new genus, *Gemella* (7), following demonstration of biochemical differences with other *Neisseria* species. The nucleotide sequence of the 16S rRNA gene of *Streptococcus morbillorum* was found to closely resemble that of *G. haemolysans* (32), and on the basis of DNA-DNA hybridization results and G+C content analysis, it was proposed that *S. morbillorum* be transferred to the genus *Gemella* as *G. morbillorum* comb. nov. (30). The phylogenetic relationships of *Gemella* spp. to other gram-positive bacteria are presented in Fig. 1.

*G. morbillorum* and *G. haemolysans* are uncommon causes of infectious endocarditis; a review of the literature reveals only 10 cases caused by *G. morbillorum* (1, 6, 10, 12, 29, 33, 35, 40, 45) and 12 cases caused by *G. haemolysans* (8, 9, 11, 15, 22, 25, 28, 31, 34, 39, 43). Two additional cases of *G. morbillorum* endocarditis have been recorded among 52 apparent failures of endocarditis prophylaxis (17), and 8 cases have been recorded among 364 cases of streptococcal endocarditis (19). The patients described in Table 3 and Table 4 demonstrate that poor dental state and/or dentistry are predisposing factors, as in patients with endocarditis caused by viridans group strepto-
cocci. Previous valvular damage is also a common occurrence. In most cases, the infections were successfully treated with antibiotic therapy, usually benzylpenicillin or amoxicillin asso-
ciated with gentamicin.

*Gemella* spp. possess a typical gram-positive cell wall structure, as confirmed by electron microscopy in this study. How-
ever during Gram staining, cells are easily decolorized and may therefore may appear to be gram variable and even gram negative. It is likely that Gram staining abnormality and morph-
ological polymorphism are responsible for the misidentifi-
cation of *Gemella* spp. and, thus, perhaps for the fact that so few cases of *Gemella* infection are reported. Rapid phenotypic identification systems are unable to identify accurately all strains of these species (3, 20), even though manufacturers have significantly improved their databases over recent years (e.g., the Rapid ID 32 Strep database [21]).

Nevertheless, for two cases of infection described in this report, identification of *G. morbillorum* or *G. haemolysans* as the causative agents was achieved by standard phenotypic identifi-
cation schemes. Identification of the organism causing in-
fec tion in patient 3 was difficult, however, even though a large number of biochemical tests were performed. After its identifi-
cation had been achieved by the PCR-based approach with the 16S rRNA gene and the bacterium had been recognized as being gram positive, re-assessment of phenotypic characteristics revealed a correct identity. The same problem had already occurred with a short, “gram-negative” bacillus, isolated from a patient with infectious arthritis, which had been sent to our laboratory for identification; analysis of its 16S rRNA gene also revealed that this isolate was *G. morbillorum* (unpublished data).

In our laboratory, we now routinely use cell wall fatty acid...
analysis and/or 16S rRNA gene analysis when a bacterium is not easily identified by phenotypic schemes. About 0.05 to 0.1% of all isolates fall into this category. Amplification with universal primers, partial sequencing (about 900 bp) with conserved primers, and sequence comparison take less than 3 days. If the partial 16S rRNA gene sequence obtained shares more than 99% similarity with a specific 16S rRNA gene sequence in the database, the bacterium may be considered to be identi-

![Phylogenetic tree based on 16S rRNA gene sequences available in the GenBank database obtained by the neighbor-joining method. The phylogenetic relationships of *G. haemolysans* and *G. morbillorum* with selected gram-positive bacteria with low G+C contents are shown. The bar represents a 1% difference in nucleotide sequence.](http://jcm.asm.org/)

**FIG. 1.** Phylogenetic tree based on 16S rRNA gene sequences available in the GenBank database obtained by the neighbor-joining method. The phylogenetic relationships of *G. haemolysans* and *G. morbillorum* with selected gram-positive bacteria with low G+C contents are shown. The bar represents a 1% difference in nucleotide sequence.

| TABLE 3. Summary of patients with reported cases of *G. morbillorum* endocarditis and features of our two patients with *G. morbillorum* endocarditis |
|---|---|---|---|---|---|---|
| Patient no./age (yr)/sex | Underlying conditions or source of infection | Infected valve | Therapy | Valve replacement | Outcome | Reference, year |
| 1/60/m | Dental manipulation | NA | Penicillin, rifampin, gentamicin, erythromycin | No | Cure | 12, 1984; 35, 1989 |
| 2/38/m | Anal subcutaneous sphincterotomy, sigmoidoscopy | Mi | Benzylpenicillin, gentamicin | No | Cure | 35, 1989 |
| 3/39/m | Dental manipulation, bicuspid aortic valve | Ao | Benzylpenicillin, gentamicin | Yes | Cure | 35, 1989 |
| 4/42/m | Mi regurgitation | Mi | Benzylpenicillin, streptomycin | No | Cerebral mycotic aneurysm, cure | 10, 1990 |
| 5/19/m | Intravenous drug abuser | Tri | Flucloxacillin, gentamicin | No | Cure (?; reduction in the size of vegetation) | 6, 1992 |
| 6/48/m | Poor dentition, systolic heart murmur, end-stage renal disease | Mi | Vancomycin | No | Wrist arthritis, cure | 40, 1993 |
| 7/64/m | Dental manipulation | Mi, Ao | Benzylpenicillin, gentamicin, erythromycin, rifampin | Yes | Acute appendicitis, cure | 45, 1994 |
| 8/29/f | Hypertrophic obstructive cardiomyopathy, dental manipulation | Na | Benzylpenicillin, gentamicin, erythromycin, rifampin | No | Cure | 29, 1994 |
| 9/74/m | Left colonic resection for rectal neoplasia | Tri | Cefuroxime, amikacin, benzylpenicillin | No | Pulmonary embolism, cure | 1, 1995 |
| 10/75/m | Rheumatic fever in infancy | Mi | Benzylpenicillin, gentamicin, teicoplanin, rifampin, erythromycin | No | Allergic reaction to antimicrobial agents, cure | 33, 1995 |
| 11/74/m | Poor dentition, chronic ethylism | Ao | Amoxicillin, gentamicin | Yes | Cure | Patient 2 |
| 12/NA/m | Bicuspid aortic valve | Ao | NA | NA | NA | Patient 3 |

*Abbreviations: Ao, aortic valve; Mi, mitral valve; Tri, tricuspid valve; NA, not available; m, male; f, female.

*No evidence of vegetation on echocardiogram.*
fied, provided that the genes of closely related species share a significantly lower degree of similarity. However, if this is not the case, complete identification can be achieved in two ways. First, confirmation can be achieved by a few simple additional biochemical tests, as described for patient 3 of this study. This method is quick and cheap, but it requires that a bacterium be easily subcultured. If this is not possible, additional sequencing can be used in order to determine a complete 16S rRNA gene sequence. This second approach is more expensive and time-consuming, but it avoids the need for additional phenotypic or specific antibody tests, both of which may not be routinely available in the diagnostic laboratory and thus would require referral to a reference laboratory. Furthermore, this approach also allows the description of new pathogens (16) which may have been misidentified by standard phenotypic identification schemes. Finally, the use of this technique for identification does not require an experienced microbiologist and gives a universal bacterial identification ability to all laboratories, provided that they are equipped with an automated sequencer. With increasing availability and decreasing costs, such equipment is likely to become a feature of more and more routine laboratories in the years to come.

However, for the present, phenotypic characterization remains the standard approach to bacterial identification, with Gram staining being one of the most important first steps of most routine identification schemes (5). A mistake at this stage can lead to the application of inappropriate tests and therefore unnecessary delays in processing; thus, it is important that all efforts be made to ensure correct interpretation of the Gram staining result. The reference method for assessing cell wall type is electron microscopy, and although it is accurate, the method is expensive, is time-consuming, and requires specialized equipment. As an alternative we have added vancomycin and colistin to our standard antibiotic susceptibility tests. Most gram-positive bacteria are susceptible to vancomycin, whereas most gram-negative bacteria are susceptible to colistin. Such a method has previously been shown to be beneficial for the determination of cell wall type among nonenterobacterial rods (24). The test is easy to perform (Fig. 2) and is inexpensive. It must be noted, however, that this test is not definitive, since a
vancomycin-resistant strain of *G. haemolysans* has been encountered (18).

In conclusion, for bacteria which are easily identified by phenotypic schemes (especially with the help of commercial identification kits or simple additional tests) and which represent more than 99.9% of the bacteria isolated in clinical microbiology laboratories, no additional identification technique is required. However, 16S rRNA gene analysis is becoming more competitive in terms of efficiency and accuracy for fastidious bacteria or those not easily identified by phenotypic schemes. The use of 16S rRNA gene analysis should also lead to an increase in the number of descriptions of new pathogens and in the recovery of unexpected pathogens.

**ACKNOWLEDGMENT**

We thank Richard Birtles for correcting the manuscript.

**REFERENCES**