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Correct Species Identification of Streptococcus Using Multi Locus Sequence Analysis for Disproving Relapse of Two Episodes of Infective Endocarditis

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Abstract: When repetitive infective endocarditis episodes occur in the same patient, accurate species identification is essential for differentiating relapse and a new episode. We report a case, which has had four episodes of IE within 2 years: episode 1 due to Enterococcus faecalis, episode 2 due to Mitis group Streptococcus, Episodes 3 and 4 due to Salivarius group Streptococcus. Detailed molecular examinations using Multi Locus Sequence Analysis (MLSA) convincingly documented that the two strains from episodes 3 and 4 were different. Thus, it was not a relapse, but a new infection and, importantly, not the result of antibiotic treatment failure.

Keywords: Infective endocarditis, intergenic spacer region (ITS) sequencing, multi locus sequence analysis (MLSA), non-hemolytic streptococci, partial oral endocarditis treatment (POET).

INTRODUCTION

Infective endocarditis (IE) is a serious condition with high morbidity and mortality. About 50% of the patients transferred to tertiary centres undergo surgery and the mortality during admission is high [1]. Detection of the microbiological etiology is of paramount importance both regarding the treatment of IE and also regarding locating the point of entry for the bacteria.

A repetitive episode of IE occurs in 2-31 % of cases [1]. Differentiation between relapse (with the same bacterial species) and a new episode (with a new species) is essential in evaluation of the effect of therapy and subsequent treatment. However, this distinction can be troublesome as some of the bacterial species causing IE may be difficult to separate from each other.

We report a case, in which 4 episodes of IE occurred within 2 years. During the third episode, the patient was enrolled in a randomised study [2] where the antibiotic treatment partially was given orally (POET: Partial Oral Endocarditis Treatment). Detailed molecular examinations, including Multi Locus Sequence Analysis (MLSA) were important for outlining species identifications, which excluded antibiotic treatment failure.

CASE REPORT

A 64-year-old man was admitted to the department of infectious diseases on suspicion of a neurological infection (episode 1). He suffered from diabetes mellitus type 2, hypertension, podagra, and had undergone surgery for a herniated disc and bilateral knee alloplastic. Blood cultures yielded growth of Enterococcus faecalis and non-haemolytic streptococci (NHS). Identification of the NHS strain was inconclusive when pursued with VITEK 2 (bioMérieux, Marcy l'Etoile, France).

Magnetic resonance (MR) - scan showed sequels after ischemic stroke in the cerebellum. Transthoracic (TTE) and transoesophageal (TOE) echocardiography showed aortic valve with vegetations and a mitral valve vegetation of 2 x 2 cm. Antibiotic treatment was given according to Danish guidelines. The treatment given was benzylpenicillin G 5 MIE 4 times a day for six weeks and gentamicin 240 mg a day for two weeks [1]. Urgent surgery was performed with replacement of the aortic valve with a biological prosthesis and repair of the mitral valve. One month after discharge, the patient was seen in the outpatient clinic in good health with no signs of relapse.

Four months later the patient was admitted to the department of orthopedics with an infected knee alloplastic and temperature of 39 ˚C (episode 2). The knee alloplastic operation had been performed 6 years prior to this admission and prophylactic antibiotics had been given, i.e. Cefuroxime 1,5 grams before the operation and 750 mg 6 hours after the operation. Cultures of the arthrocentesis and blood cultures grew Streptococcus sanguinis (Mitis group) identified by VITEK 2. Due to the possibility of having IE, antibiotic treatment was given with benzylpenicillin G 5 MIE 4 times a day for six weeks and gentamicin 240 mg a day for two weeks [1]. The knee prosthesis was explanted. At this point, sequence analysis of Intergenic Spacer Region (ITS) and glutamate dehydrogenase gene (*gdh)* was performed for the

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isolates from the two episodes for definitive species identification [3]. The NHS strain from episode 1 was identified as Streptococcus infantarius subsp. infantarius (Bovis group) and the isolate from episode 2 as Streptococcus mitis (Mitis group). Due to the relation of Bovis group streptococci to gastrointestinal cancers, the patient had a gastroscopy and colonoscopy performed, which both were normal. Leucocyte-scintigraphy excluded any other bone infection.

The patient remained in good health without any febrile episodes for 20 months. Then he developed fever and signs of pneumonia (episode 3). On admission, blood cultures this time revealed Streptococcus salivarius identified with VITEK 2. Therefore this was interpreted as a new infection without any relation to the first two episodes. Infection of the aortic valve prosthesis was suspected as the patient fulfilled one major and 2 minor of the Duke's modified criteria of infective endocarditis [4] and treatment for suspected IE was started with benzylpenicillin G 5 MIE 4 times a day and gentamicin 240 mg a day. After 11 days the patient was enrolled in the POET (Partial Oral Endocarditis Treatment) study, for design, see [2]. He was randomized to oral treatment and received amoxicillin 1g 4 times a day and rifampicin 600 mg twice a day. After six weeks he was fit and discharged.

The patient was seen after one week and after one month as part of the POET trial. There were no clinical signs of infection, blood cultures were without bacteriae and the patient reported being well at both visits and the third visit was planned to take place two months later.

Sixty-nine days after the end of treatment, the patient was admitted with fever (episode 4). He complained of back pain radiating to the left buttock. TOE showed no significant vegetations but once again treatment was started for possible IE. MR-scan excluded spondylodiscitis but showed severe protrusion of the disc between L2/L3 and L4/L5.

Blood cultures showed Streptococcus salivarius/vestibularis (identified by VITEK 2 with low discrimination,). Thus, the antibiotic treatment was changed to intravenous benzylpenicillin. It was important to know, whether this patient had a relapse after oral treatment, or whether he had a new episode with a new bacteria species. Therefore, Multi Locus Sequence Analysis (MLSA) was performed assigning the strain from the episode 3 as S. salivarius, and the strain from episode 4 as S. vestibularis (see below). At the last follow-up transthoracic echocardiography was without signs of IE, blood samples were normal and blood cultures were without growth of bacteria and without any signs of relapse. The patient was doing well.

MICROBIOLOGY

Microbiological Routine Analysis

Blood cultures from episodes 3 and 4 grew Gram positive cocci in chains in four bottles and 12 bottles (BacT/Alert, bioMérieux, Marcy l'Etoile, France) on day 2 and days 1 and 2, respectively. Identification was routinely performed by VITEK 2 and MALDI-TOF MS (VITEK MS with the SARAMIS database, bioMérieux, Marcy l'Etoile, France). Both strains were designated to the Streptococcus salivarius group by VITEK 2 with low discrimination. Only the isolate from episode 4 obtained a MALDI-TOF result as Streptococcus salivarius/vestibularis score 84 (below the 90-cut-off).

Antimicrobial susceptibility testing was performed using The European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints and disk diffusion methodology, supplemented with Etest (bioMérieux, Marcy l'Etoile, France) for penicillin and gentamicin according to manufacturer's instructions. Both strains were susceptible to penicillin. However, the strain from episode 3 was resistant to erythromycin, while the strain from episode 4 was susceptible.

Molecular Methods Used for Characterization of Strains

Partial 16S rRNA Gene Sequencing

MicroSeq 500 system; Perkin-Elmer, Applied Biosystems Division, Foster City, CA) was performed on the isolates from episodes 3 and 4 and sequences were compared to the MicroSeq ID 2.0 500-bp library. The following results were obtained: episode 3 (463 bp-sequence): S. salivarius (ATCC 7073) 99.98%, S. vestibularis 99.72%, S. salivarius subsp. thermophilus (ATCC 19258) 99.54%, and episode 4 (497 bp-sequence): S. vestibularis 100%, S. salivarius (ATCC 7073) 99.56%, S. salivarius subsp. thermophilus (ATCC 19258) 99.13%. Thus, we could not achieve a species identification based on partial 16S rRNA gene sequence analysis.

ITS Sequence Analysis

Species identification based on ITS sequence analysis was performed as described previously [3]. The sequences of the ITS region were compared to sequences deposited in the NCBI database by using the BLAST search engine (www.ncbi.nlm.nih.gov/BLAST) and by taking into consideration the percentage and number of identities, the maximum score, and E values for the best and the next best taxon matches. The best taxon match of the strain from episode 3 was with S. salivarius, with a Maximum score of 414; there were five identical hits. The distance to the next best taxon match (S. thermophilus or S. vestibularis) was only five. The best taxon match of the strain from episode 4 was S. thermophilus or S. vestibularis, with a Maximum score of 414, while the distance to the next best taxon match (S. thermophilus or S. vestibularis) again was only five. The alignment of the ITS region sequences from these two strains showed only one base difference. Therefore we could not identify the two strains to the species level based on the ITS sequence analysis either.

Multi Locus Sequence Analysis (MLSA)

MLSA was conducted as described by Bishop *et al.* (2009) [5]. Seven house-keeping genes, *map, pfl, ppaC, pyk, rpoB, sodA* and *tuf*, were sequenced (GATC-Biotech, Germany) and edited according to the reference sequences obtained from eMLSA (http://viridans.emlsa.net/). The edited sequences were submitted to eMLSA that performed the species assignment according to its position within an

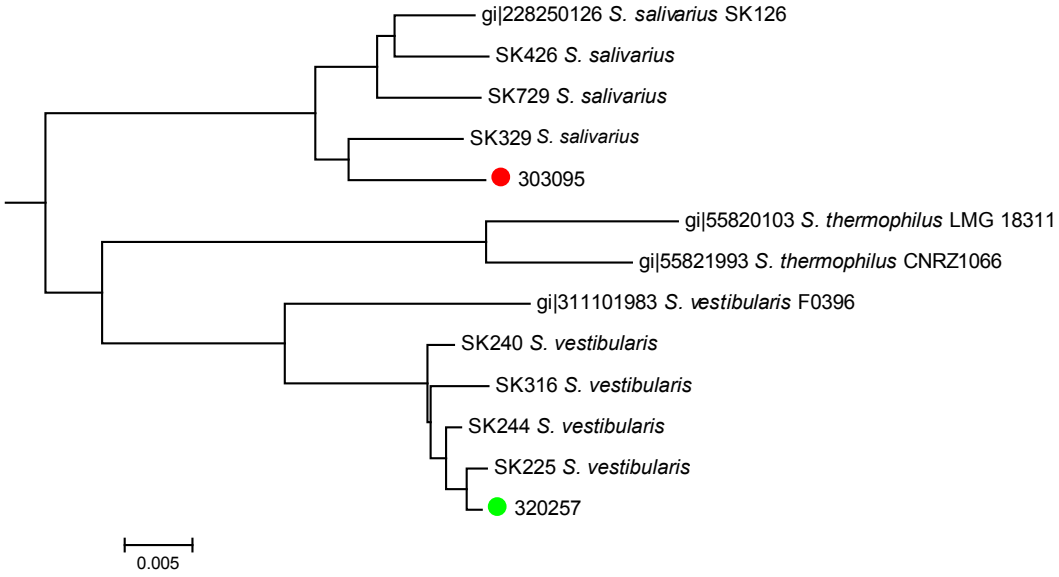

Fig. (1). Phylogenetic tree generated with eMLSA net with reference strains of the salivarius group streptococci. The isolate from the third episode (303095) is assigned to the S. salivarius cluster, while isolate from the fourth episode (320257) is in the S. vestibularis cluster.

assigned species cluster on the reference tree. In our case, eMLSA assigned the isolate from the episode 3 as S. salivarius, while the isolate from episode 4 was assigned as S. vestibularis (Fig. **1**).

Therefore we could conclude that the isolate from episode 3 was S. salivarius and the isolated from episode 4 was S. vestibularis by using MLSA. Neither 16S rRNA gene nor ITS sequence analysis was sufficient for species identification for these two strains.

DISCUSSION

IE relapses are defined as repeat episodes of IE caused by the same microorganism, which suggest failure of antimicrobial therapy. Relapse IE mandate a search for a persistent focus of infection, a longer course of treatment, or even surgery. Precise species identification of the bacterial strains from these IE episodes is essential for clarifying relapse from a new infection. NHS is one of the most common causes of IE which contribute to 20-50% of IE cases [6-8]. Strains belonging to NHS are closely related, which makes the identification to the species level difficult by conventional microbiological methods [9-11]. MALDI-TOF MS is a new revolution for rapid and accurate species identification based on ribosomal proteomics. However for NHS strains, it is reported to be sufficient only to the group level [11]. Many molecular methods based on single gene sequence analysis have the same drawback [11]. MLSA, a method based on sequence analysis of seven house-keeping genes, is a strong molecular tool capable of differentiating between closely related strains [5]. In this case a relapse caused by S. salivarius was suspected, since it was not possible to differentiate the two strains from episodes 3 and 4 by the routine methods (VITEK and MALDI-TOF MS). Though sequence analysis based on both ITS and 16S rRNA gene had shown two different species as best taxon match from the two episodes, the distance to the second best taxon match was too short to be convincing. MLSA convincingly documented that the two strains were different (Fig. **1**). Thus, we confirmed that the patient indeed had a new infection with a new bacterium and not a relapse and that the antibiotic treatment had been adequate. This was very important because of the patient's inclusion in a randomised study addressing antibiotic treatment of IE earlier. Despite a thorough search during every episode we have not been able to locate the portal of entry. The patient has had consultations at our dentists at every admission according to the guidelines. Positron emission tomography – computed tomography (PET-CT) has also not revealed the entry point for the bacteriae.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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