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Case Report

# Direct 16S rRNA gene sequencing of polymicrobial culture-negative samples with analysis of mixed chromatograms

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Two cases involving polymicrobial culture-negative samples were investigated by 16S rRNA gene sequencing, with analysis of mixed chromatograms. *Fusobacterium necrophorum*, *Prevotella intermedia* and *Streptococcus constellatus* were identified from pleural fluid in a patient with Lemierre's syndrome and *Neisseria meningitidis* and *Escherichia coli* were identified from a petechia in a patient with meningococcal disease.

## Introduction

Identification of bacterial species from culture material by 16S rRNA gene sequencing is now widely used in many clinical microbiology laboratories (Clarridge, 2004; Petti, 2007; Kommedal *et al.*, 2008). Culture-negative samples containing bacteria are also being identified by use of 16S rRNA gene sequencing directly on clinical samples (Clarridge, 2004; Petti, 2007; Petti *et al.*, 2008; Kommedal *et al.*, 2008). If there is more than one bacterial species in these samples, sequencing will often result in mixed chromatograms, which will complicate the exact species identification. To solve this problem, the products from the primary PCR-mediated amplification of the 16S rRNA gene can be separated by cloning or using gradient gel electrophoresis (Garciá-Martínez *et al.*, 1999). This procedure is subsequently followed by sequencing. However, these methods are very time-consuming and labour-intensive and not suitable for routine clinical microbiology (Xu *et al.*, 2003; Zhou & Hickford, 2008). To overcome this procedure, we used a new software-based method, which relies on a mathematical model/algorithm that is dependent upon both correct reading of the chromatograms (base calling) and a subsequent matching procedure (search) (Kommedal *et al.*, 2008). Different fluorescent peaks at the same position in the chromatogram are relatively displaced, and the reading algorithm is able to distinguish and sort out the ambiguous signals at each position. The matching procedure is step-by-step comparing overlapping pieces successively to a database. Identification is based on an empirically set cut-off at $\geqslant$99.3 %, and up to three different bacterial species can be identified from a chromatogram with this method (Kommedal *et al.*, 2008). Recently the results from a study including a collection of 264 clinical samples demonstrated the clinical applicability of the method (Kommedal *et al.*, 2009). The study included an analysis of 66 mixed chromatograms. The method proved particularly useful when applied on samples from patients that had received antibiotics prior to sampling (Kommedal *et al.*, 2009).

We report two cases involving direct 16S rRNA gene sequencing of polymicrobial culture-negative samples with analysis of mixed chromatograms and subsequent identification of up to three different bacterial species.

## Case reports

### Case 1

A 48-year-old male was admitted to hospital because of acute laryngitis and dysphagia. Treatment with penicillin and metronidazole was initiated, and symptoms gradually improved. After 2 weeks of treatment, there was a sudden and rapid aggravation of symptoms, with painful swelling of the neck and elevation of the neutrophilic leukocyte count and C-reactive protein concentration. A CT scan showed accumulation of air in the neck and mediastinum and an empyema in the right lung. Antibiotic treatment was supplemented with meropenem. As Lemierre's syndrome was suspected, the patient was transferred to a hospital with thoracic surgery specialists. On arrival, the patient had a fever (38.9 °C) but was stable. The white blood cell count showed neutrophilic leukocytosis ($22.3\times10^9$ l$^{-1}$) and a C-reactive protein concentration of 369 mg l$^{-1}$ (reference interval $<$10 mg l$^{-1}$). A new CT scan revealed the development of abscesses in the retropharyngeal and parapharyngeal space and also in the mediastinum. Pleural fluid and pus from the empyema and abscesses were obtained for culture during surgery and drainage of the mediastinum. Gram staining of the material did not reveal any bacteria or fungi. All samples, including blood cultures, were culture-negative after 5 days of aerobic and anaerobic incubation.

As a consequence, 16S rRNA gene sequencing of the pleural fluid was performed, using the MicroSeq 500 system

according to the manufacturer's instructions (Perkin-Elmer, Applied Biosystems Division). Sequencing was without result, as no consensus sequence could be obtained. The chromatogram was mixed, with two or more fluorescent peaks in several positions, suggesting that the sample contained multiple bacterial species. Subsequent analysis of the mixed chromatogram with the RipSeq computer program (iSentio, Bergen, Norway) as described by Kommedal *et al.* (2008) identified three bacterial species (default options, except for the signal cut-off on the *y*-axis, which was set to 150): *Fusobacterium necrophorum* (100 %), *Prevotella intermedia* (99.55 %) and *Streptococcus constellatus* (99.33 %). Antibiotic monotherapy with penicillin was continued. The patient recovered slowly and was discharged for ambulatory care after 5 weeks of treatment.

## Case 2

A 15-year-old boy with a 4-day history of a flu-like illness was examined in his home by his general practitioner. The general practitioner observed a petechia flanking the right eye, and meningococcal disease was suspected. A high dose of intramuscular penicillin was immediately administered, and the boy was admitted to hospital. On admission, he had a fever (39.1 °C), was hypotensive (mean arterial pressure 65 mmHg), anuric, not fully conscious and had disseminated petechiae/ecchymosis on most of the body. Blood test results showed signs of disseminated intravascular coagulation and metabolic acidosis. Antibiotic treatment was supplemented with ceftriaxone. The white blood cell count was initially $8.9 \times 10^9$ $l^{-1}$, and the C-reactive protein concentration was 52 mg $l^{-1}$. The following day, the white blood cell count showed neutrophilic leukocytosis of $33.9 \times 10^9$ $l^{-1}$, and the C-reactive protein concentration had increased to 230 mg $l^{-1}$. Although there were no obvious signs of meningitis, a lumbar puncture was performed. Cerebrospinal fluid showed a leukocyte and erythrocyte count of 25 and $12 \times 10^6$ $l^{-1}$, respectively. No bacteria were seen. Cerebrospinal fluid glucose and protein concentrations were normal. A biopsy from a petechia was also performed. Blood cultures, spinal fluid and the petechia were all culture-negative.

In this case, 16S rRNA gene sequencing was performed on the petechia, using the MicroSeq 500 system. The chromatogram had a mixed appearance with two fluorescent peaks in several positions, suggesting that the sample contained at least two bacterial species. For this sample, a consensus sequence (490 bp sequence) was obtained and compared to the MicroSeq 500 library, with a resulting 90 % match with *Escherichia coli*. The sequence was also compared to GenBank using the BLAST search engine. Again, the best match was an *E. coli* with a 90 % match (GenBank accession no. CP001509.1). Analysis of the mixed chromatogram with the RipSeq computer program (default options) identified two bacterial species (Fig. 1):

+ISENTIORIPSEQ
Analysis results

Forward file: 090123_5149f_A01.ab1

**Primer set 1:** 16S (4-531) primers - iSentio

**Solution set 1:** 16S rRNA Human Pathogens – iSentio

| X | Score | Genus | Species | Strain | Accession |
|---|---|---|---|---|---|
| | 100.00 | Escherichia | coli | CFT073 | AE014075 |
| | 100.00 | Escherichia | coli | HS | CP000802 |
| | 100.00 | Escherichia | coli | BL21(DE3) | CP001509 |
| X | 100.00 | Escherichia | coli | T10 | AY804014 |
| | 100.00 | Escherichia | coli | UTI89 | CP000243 |
| | 100.00 | Neisseria | meningitidis | CMCC(B)29205 | DQ201337 |
| X | 100.00 | Neisseria | meningitidis | H276 | AF310580 |
| X | 100.00 | Shigella | flexneri | 8401 | NC_008258 |
| X | 100.00 | Shigella | boydii | Sb227 | CP000036 |
| X | 100.00 | Shigella | dysenteriae | Sd197 | NC_007606 |
| | 99.77 | Escherichia | coli | ATCC 8739 | CP000946 |
| | 99.77 | Neisseria | meningitidis | alpha14 | AM889136 |
| | 99.77 | Neisseria | meningitidis | CMCC(B)29040 | DQ201332 |
| | 99.77 | Shigella | sonnei | Ss046 | NC_007384 |
| | 99.54 | Escherichia | coli | 536 | CP000247 |
| | 99.31 | Escherichia | albertii | 9194 | AY696664 |
| | 99.31 | Escherichia | albertii | Albert 19982 T | NR_025569 |
| | 99.31 | Shigella | boydii | 3553-77 | AY696680 |
| | 99.07 | Neisseria | gonorrhoeae | NCTC 83785 | X07714 |
| | 98.84 | Neisseria | perflava | U15 | AJ239295 |
| | 97.92 | Citrobacter | youngae | GTC 1314 | AB273741 |
| | 97.22 | Citrobacter | sedlakii | CDC 4696-86 | AF025364 |

**Fig. 1.** Case 2: results from the RipSeq program using the default options. Identification with an empirically set cut-off at ⩾99.3 % of two bacterial species from a mixed chromatogram (ab1-file). Bacterial species below the cut-off are shown in grey shading.

*E. coli* (100 %) and *Neisseria meningitidis* (100 %). Of note, the program was not able to discriminate between *E. coli* and *Shigella boydii*/*Shigella dysenteriae*/*Shigella flexneri*, which is a well-known problem, as these species are so closely related. The eluate from the primary PCR performed on the petechia was sent to a reference laboratory for further analysis (Statens Serum Institut, Copenhagen, Denmark). *N. meningitidis*, belonging to serogroup C, was identified using specific primers. Also, a meningococcal antibody test showed a significant increase in antibody titre, confirming the meningococcal disease diagnosis. The boy recovered without sequelae.

## Discussion

The three different bacteria that were identified in our Case 1 all belong to the commensal flora of the oral cavity and

pharynx, with *F. necrophorum* as the most likely and prominent cause of Lemierre's syndrome. The identification of *E. coli* as the most prominent bacterial species in Case 2 was unexpected, and there is no reasonable explanation, apart from contamination from the skin or in the laboratory. In contrast, the finding and confirmation of *N. meningitidis* from the petechia is in complete accordance with the case presentation.

We have, with our two culture-negative polymicrobial cases, shown that it is possible to analyse mixed chromatograms and identify bacterial species with a plausible involvement in the pathogenesis of the infection. The RipSeq program is also suggested as a useful tool in polymicrobial infections, where misleading culture results are expected because of prior antibiotic therapy which may have affected the involved bacteria, permitting only some to be cultured (Kommedal *et al.*, 2009). Although the presented cases are not proof of the general applicability of the RipSeq program, the findings in our specific cases demonstrate the possible potential of mixed chromatogram analysis.

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