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Evaluation of the MICROSEQ 500 16S rDNA-based gene sequencing for the diagnosis of culture-negative bacterial meningitis

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SUMMARY

Gene amplification using 16S rDNA primers has been proposed as a strategy for the diagnosis of bacterial meningitis. The aim of this study was to evaluate the performance of the MicroSeq 500 16S ribosomal DNA test (Applied Biosystems) from patients with suspected bacterial meningitis and CSF negative-culture in comparison to traditional methods.

Twelve purulent culture-negative CSF samples were collected between January 2005 and January 2007. For DNA extraction, 500 µl of CSF samples were treated using the QIAamp mini kit (QIAGEN). The extracted DNA was examined amplifying 500 bp at the 5' end of 16S rRNA gene using MicroSeq500 16S rDNA Bacterial Identification PCR kit and the sequencing reactions were performed with the MicroSeq500 16S rDNA Bacterial Identification Sequencing kit (Applied Biosystems). The sequences were compared with those available in GenBank.

For the culture-negative CSF samples the MicroSeq 500 16S rDNA yielded a positive result in 9 cases (75.0%): three samples were identified as *Streptococcus. pneumoniae*, three as *Neisseria meningitidis*, and the remaining 3 as *Haemophilus influenzae, Abiotrophia defectiva* and *Porphyromonas gingivalis*.

The MicroSeq 500 16S ribosomal DNA test may improve the microbiological diagnosis of bacterial meningitis, especially when spinal fluid samples are obtained after the administration of antimicrobial therapy.

KEY WORDS: Bacterial meningitis, Cerebrospinal fluid, MicroSeq 500, Negative culture, Sequencing

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INTRODUCTION

Acute meningitis is an inflammation of the meninges with an incidence of about 1-6 per 100,000 inhabitants in industrialised countries.

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Bacterial meningitis is a medical emergency; the disease, if untreated, may get worse over a few days and sometimes presents a fulminant course after some hours (Davison *et al.*, 2003; Perrocheau *et al.*, 2004; Salmaso *et al.*, 1997; Schuchat *et al.*, 1997; Tunkel *et al.*, 2004; Van de Beek *et al.*, 2004).

Neisseria meningitidis, *Streptococcus pneumoniae* and *Haemophilus influenzae* type b are the microrganisms most frequently involved, accounting for more than 80% of all cases in community-acquired forms. Gram negative bacteria, particularly *Escherichia coli*, other Streptococci,

Listeria monocytogenes, Staphylococci and any other bacteria may also be involved (Heallh Protection, 2004; Tunkel *et al*., 2004).
Samples of CSF collected from the patients should be processed as soon as possible, in order to maintain at best the vitality of the microrganisms (Tunkel *et al*., 2004). The yield of standard culture methods for bacteria depends on the concentration of the causative agents in the spinal fluid, with rates of isolation ranging from 25% (for samples containing $\leq 10^3$ colony-forming units/mL) to 97% (with $>10^5$ colony-forming units/mL). Culture results are worse for patients who have already received antimicrobial therapy, with isolation rates below 20% (Gray *et al.*, 1992; Van de Beek *et al.*, 2004; Wilson, 2002).
Immunological methods for the detection of bacterial antigens, such as latex agglutination, direct immunofluorescence and EIA/ELISA, are not recommended in the microbiological routine because of their low specificity and sensitivity, the latter being in the range 0-25% for culture-negative patients (Das *et al.*, 2003; Mein *et al*., 1999; Tarafdar *et al.*, 2004).
Recently developed molecular methods could allow an early and accurate etiological diagnosis of meningitis**.** Being independent of bacterial growth, these methods appear particularly promising for the analysis of CSF samples obtained after the administration of antibiotics.
PCR tests are available as diagnostic procedures for specific organisms (such as *S. pneumoniae, N. meningitidis*, *H. influenzae*) (Bosshard *et al*., 2004; Kotilainen *et al.*, 1998; Poppert *et al.*, 2005). The use of broad-range bacterial PCR could help to improve our knowledge of the etiological spectrum of bacterial meningitis, allowing the detection of bacteria infrequently cultivated, or not yet recognized as causative agents of meningitis (Boudewijns *et al.*, 2006; Clarridge, 2004; Greisen *et al.*, 1994; Poxton, 2005).
Amplification using 16S rDNA primers has already seen various applications, particularly in culture-negative bacterial diseases. Recently it has been proposed as a strategy for the diagnosis of culture-negative bacterial meningitis and applied in daily microbiological practice (Deutch *et al.*, 2006; Dicuonzo *et al.*, 1999; Margall *et al*., 2002; Meybeck *et al*., 2006; Pandit *et al.*, 2005; Sacchi *et al*., 2002; Saravolatz *et al.*, 2003; Schuurman *et al*., 2004; Van de beek *et al*., 2004; Xu *et al.*, 2004; Xu *et al*., 2005).
Commercial tests are available for the identification of bacterial species based on the nucleic acid sequences of 16S rRNA (Schuchat *et al.*, 1997). The MicroSeq 500 16S ribosomal DNA (rDNA)-based bacterial identification system (Applied Biosystems Division) has been marketed for rapid and accurate identification of bacterial pathogens: the first 500-bp of the 16S rRNA gene of the bacterial strain are amplified, sequenced and analysed using the database provided by the system or, alternatively, public databases such as GenBank, NCBI (Fontana *et al.*, 2005; Woo *et al.*, 2003).
This study was conducted retrospectively on stored samples of CSF belonging to patients with a discharge clinical diagnosis of culture-negative acute meningitis to evaluate the performance of the MicroSeq 500 16S ribosomal DNA system in comparison with traditional methods.

MATERIALS AND METHODS

Clinical specimens
Between January 2005 and January 2007 the Unit of Microbiology and Virology of Bergamo received 89 samples of cerebrospinal fluid belonging to patients with purulent meningitis, affected by bacterial meningitis. The study included all the culture-negative samples for which, after standard microscopic and cultural procedures, frozen CSF remained available for PCR (12 samples).
Five culture-positive CSF samples (2 *N. meningitidis*, 2 *S. pneumoniae* and 1 *S. pyogenes*) from the same period were analysed as positive controls. Five frozen CSF samples from patients for whom meningitis had been ruled out were analysed as negative controls.
All the CSF samples were obtained by lumbar puncture under aseptic conditions for microbiological examinations, cell count, glucose and protein levels. A separate specimen (500 µL) was kept at -80°C until PCR processing.

Traditional methods
All specimens were immediately sent to the laboratory. CSF were cytocentrifuged at 500 rpm for 5 min (Cytospin 3, Thermo Shandon) and then coloured by Gram staining, and cultured direct-

ly on conventional culture media: chocolate agar, 5% sheep blood agar, colistin-nalidixic acid (CNA) 5% horse blood agar, MacConkey agar and in enriched broth (Haemoline, BioMerieux) incubated for 4 days (Health Protection, 2004).

DNA extraction
QIAamp mini kit (QIAGEN) was used for DNA extraction (500 µl CSF) for all samples in duplicate. The total DNA was eluted in 50 µl of AE buffer by a modification of the procedure described by manufacturer (QIAGEN) and stored at -80°C until measurement in the PCR. Because some CSF extracted did not amplify we diluted 1:3 assuming the presence of inhibitors so it was possible amplify the DNA.

PCR amplification and sequencing
15 µl were examined by amplification 500 bp to the 5' end of 16S rRNA gene using MicroSeq500 16S rDNA Bacterial Identification PCR kit (Applied Biosystems). The bacterial 16S rDNA was amplified in a GeneAmp 9600 thermocycler (Applied Biosystems). Amplicon detection was verified by 2% agarose gel electrophoresis previous purification with YM-100 Microconcentrators (Celera Diagnostics).
The sequencing reactions were performed with MicroSeq500 16S rDNA Bacterial Identification Sequencing kit, cycle reactions were purified using Centri-Sep Spin Columns (Applied Biosystems) and the cycle sequencing products were analysed on 3130 Genetic Analyzer (Applied Biosystems) according to the manufacturer's instructions.

Data analysis
Raw data were analysed with the Sequencing Analysis software v 5.2 (Applied Biosystems). Sequences from both strands were aligned by using NCBI BLAST 2 Sequence (http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi). The resulting consensus sequence, obtained from the double-stranded of the alignment, was aligned with sequences stored in GenBank (BLAST http://www.ncbi.nlm.nih.gov/BLAST). 16S rDNA sequences identified in the present study are deposed in GenBank under accession numbers AY485600 (*Streptococcus pneumoniae*), AB035456 (*Porphyromonas gingivalis*), AY879307 (*Abiotrophia defectiva*), AY613538 (*Haemophilus influenzae*) and AY735374 (*Neisseria meningitidis*).

Criteria for identification
For designation to species or genus level we used the identification criteria formulated by Drancourt *et al.* (2004). The species level was defined as a 16S rRNA sequence similarity of ≥99% with the prototype strain sequence in GenBank, the genus level was defined as a similarity of ≥97% with the prototype strain sequence in GenBank, while a score lower than 97% was not considered acceptable (Drancourt *et al.,* 2000; Drancourt *et al*., 2005).

Determination of analytical sensitivity
Serial 10-fold dilutions of control strains: *S. aureus* ATCC 6538 and *P. aeruginosa* ATCC 9027 (ATCC-EZ-CFU™) effected in double according to the manufacturer's instructions were prepared in NaCl 0.85% medium. The dilutions were extracted and analysed and for confirmation the colonies were counted after plating dilutions on chocolate agar.

RESULTS

The five positive control CSF samples were all MicroSeq 500 16S rDNA positive corresponding with identification obtained by traditional methods. The five negative control CSF samples were all PCR-negative.
The analytical sensitivity of the method, performed on 10-fold serial dilutions of *S. aureus* and *P. aeruginosa* and using extraction protocols QIAamp mini kit, was for these microrganisms mean 1.7 x 10^3 and 1.1 x 10^3 CFU/ml respectively confirmed by counting on agar dilution after incubation at 37° C for 24 hours.
The demographic characteristics and the data regarding microbiological, biochemical findings and antimicrobial therapy are summarized in table 1.
Twelve patients with negative culture were characterised from the following features: age mean 39 years (range 1-78), 9 males (75.0%) and 3 females (25.0%).
The MicroSeq 500 16S rDNA yielded a positive result in 9/12 CSF samples (75.0%): *S. pneumoniae* was identified in 3 samples *N. meningitidis* in another 3 and *H. influenzae, A. defectiva* and *P. gingivalis* respectively in the remainder (Figure 1).
The Gram stain examination was positive in 5 pa-

TABLE 1 - *Microbiological and biochemical findings of negative-culture CSF samples.*

N°	*Hospital Unit*	*Age*	*Direct microscopy*	*Culture*	*WBC/μL*	*% PMNC*	*Glucose (mg/dL)*	*Protein (mg/dL)*	*Therapy prior lumbar puncture*	*16S rDNA*
1	Infectious Diseases	52	DCGP	Negative	4630	95	39	264	Yes	*S. pneumoniae*
2	Infectious Diseases	44	AM	Negative	2940	50	17	267	Yes	Negative
3	Infectious Diseases	26	AM	Negative	2883	70	109	197	Yes	Negative
4	Infectious Diseases	43	AM	Negative	7500	89	73	281	No	*H. influenzae*
5	Infectious Diseases	45	AM	Negative	823	90	0	350	No	*A. defectiva*
6	Infectious Diseases	78	DCGP	Negative	322	80	8	236	Yes	*S. pneumoniae*
7	Pediatrics	5	AM	Negative	2010	60	52	61	Yes	Negative
8	Pediatrics	1	DCGN	Negative	3640	55	0	154	Yes	*N. meningitidis*
9	Pediatrics	9	DCGN	Negative	2700	99	4	746	Yes	*N. meningitidis*
10	Vimercate Hospital	42	DCGN	Negative	16000	95	2	270	Yes	*N. meningitidis*
11	Vimercate Hospital	53	AM	Negative	15000	90	0	449	Yes	*S. pneumoniae*
12	Zingonia Hospital	68	AM	Negative	450	87	23	500	No	*P. gingivalis*

Legend: Hospital Unit (patients 1-9 AO Ospedali Riuniti of Bergamo); AM = absence of microrganisms; DCGP = diplo- cocci Gram positive; DCGN = diplococci Gram negative; WBC = white blood cells; PMNC = polymorphonuclear cells.

FIGURE 1 - *Result of Micro Seq 500 16S rDNA in negative-culture CSF samples. Legend: MW = DNA molecular weight marker (MW VI Boehringer Mannheim).*

tients with community-acquired meningitis (41.7%) and the microscopic identification was then confirmed by MicroSeq 500 16S rDNA identifying 2 cases of *S. pneumoniae* and 3 of *N. meningitidis*.

The patients presented the following CSF abnormalities: white blood cell (WBC)/μL mean 4908, range 322-16000; polymorphonuclear cells percentage (PMNC) mean 80, range 55-90; glucose concentration mean 27 mg/dL, range 0-109, and protein concentration mean 315 mg/dL, range 61-746.

From a clinical point of view the patients were classified as follows:

- 9 patients (75.0%) with community-acquired meningitis;
- 1 patient (n° 4) with post-traumatic meningitis due to a cranial trauma and cerebral commotion;
- 1 patient (n° 5) with nosocomial meningitis, because of spinal anaesthesia for an hiatal hernia operation, performed one week before the appearance of the meningeal symptoms;
- 1 patient (n° 12) with a cerebral abscess, investigated by CT scan after some weeks with fever and vertigo, showing a cystic lesion with compression of the occipital horn in the left anterior ventricle.

Six of these twelve patients (50.0%) had already received antimicrobial therapy prior to lumbar puncture. Bacteria were not detectable in 3 CSF samples (25.0%) by either traditional and molecular methods; these patients had already started antimicrobial therapy prior to lumbar puncture.

DISCUSSION

Bacterial meningitis is a life-threatening disease, however, it is proven that early diagnosis improves clinical outcomes (Tunkel *et al.*, 2004).

Recent molecular methods make it possible to achieve a fast and reliable diagnosis. Widespread use of 16S rRNA gene sequencing for the identification of bacteria offers the opportunity to recognise new etiological agents of clinically relevant infections. On the other hand, these methods are expensive, require highly skilled and ad hoc trained technicians, and available public sequence data bases are not reviewed.

Our results show that the molecular findings could improve the results obtained by conventional methods, especially in culture-negative samples.

The sensitivity of MicroSeq 500 16S ribosomal DNA in combination with the DNA extraction kit, QIAamp mini kit (QIAGEN) was 1.7 x 10^3 CFU/ml for *S. aureus* and 1.1 x 10^3 CFU/ml for *P. aeruginosa,* which could be sufficient for etiological diagnosis in the majority of bacterial meningitis cases (Poppert *et al.*, 2005).

MicroSeq 500 16S ribosomal DNA, demanding a whole working day altogether, is not only useful for the detection of bacteria in CSF, but can be used for the detection of bacteria in many other clinical samples from sterile anatomic sites, particularly in cases of fastidious growing pathogens. Sequencing methods may be limited by the unavailability of consensus on standardised extraction procedures. Another problem with sequencing is mixed infections, although rare they account for up to 1% of cases of meningitis associated with trauma, tumours or infections such as acute paranasal sinusitis extending directly to the meninges. In such instances, sequencing is usually inconclusive (Tunkel *et al.*, 2004).

In our study, the MicroSeq 500 16S ribosomal DNA method allowed an etiological diagnosis in nine culture-negative patients (three with *S. pneumonae*, three with *N. meningitidis* and three with other microrganisms), confirming earlier findings by other Authors (Deutch *et al.*, 2006; Dicuonzo *et al.*, 1999; Margall *et al*., 2002; Pandit *et al.*, 2005; Saravolatz *et al*., 2003; Schuurman *et al.*, 2004; Xu *et al.*, 2005).

P. gingivalis was found in the CSF in patient 12, whereas conventional methods remained negative, as anaerobic culture is not included in the standard procedure for detecting bacterial meningitis in most microbiological laboratories. In the literature, another case of severe brain abscess has recently been reported in which *P. gingivalis* was detected in the cerebrospinal fluid (Schlegel *et al*., 1999). A case of iatrogenic meningitis due to *A. defectiva* following myelography has also been published (Iida *et al*., 2004).

Gram stain examination, culture and molecular method were negative in 3 CSF samples: all these patients had received antimicrobial before lumbar puncture.

In conclusion, the MicroSeq 500 16S ribosomal DNA test, when used in addition to the traditional

microscopical and cultural methods, can improve our ability to make an etiological diagnosis of bacterial meningitis, especially in patients who have received antibiotics prior to lumbar puncture. It has to be remembered that this molecular test cannot replace culture as a necessary step for the susceptibility tests.

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