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Special Article

RAPID IDENTIFICATION OF NON-SPORING ANAEROBES USING NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY AND AN IDENTIFICATION STRATEGY

***S Menon, R Bharadwaj, AS Chowdhary, DV Kaundinya, DA Palande**

Abstract

Purpose: The non-sporing anaerobes cause a wide spectrum of infections. They are difficult to culture and their identification is tedious and time-consuming. Rapid identification of anaerobes is highly desirable. Towards this end, the potential of nuclear magnetic resonance (NMR) spectroscopy for providing a fingerprint within the proton spectrum of six genera belonging to anaerobes reflecting their characteristic metabolites has been investigated. **Methods:** NMR analysis was carried out using Mercury plus Varian 300 MHz (7.05 T) NMR spectrophotometer on six different anaerobes. These included *Bacteroides fragilis, Prevotella melaninogenica, Prevotella denticola, Fusobacterium necrophorum, Peptococcus niger* and *Peptostreptococcus* spp. After the NMR analysis (256/512 scans), the different peaks were noted. The eight pus specimens, which yielded pure culture of anaerobe, also were analysed similarly. **Results:** The major resonances of multiplex of amino acids/lipid at 0.9 ppm along with lactate/lipid at 1.3 ppm, acetate at 1.92 ppm and multiplex of lysine at 3.0 ppm remained constant to label the organism as an anaerobe. There was a difference found in the MR spectra of different genera and species. A simple algorithm was developed for the identification of the six different anaerobes studied. The MR spectra of the pure culture of the organism matched the MR spectra of pus from which the organism was isolated. **Conclusions:** MR-based identification was of value in the identification of anaerobes. However, a larger database of the peaks produced by anaerobes needs to be created for identification of all genera and species. It could then have the potential of diagnosing an anaerobic infection *in vivo* and thus expedite management of deep-seated abscesses.

Key words: *Anaerobes, nuclear magnetic resonance, rapid technique*

The non-sporing anaerobes are commonly implicated as significant pathogens in human infections.[1] Conventionally, identification of anaerobes is based on morphology, antibiotic sensitivity and biochemical reactions. This is time-consuming and results may vary considerably, depending on the age of culture and nutritional composition of the growth medium. To overcome this disadvantage, end product analysis by gas-liquid chromatography (GLC) was established.[2] This is usually done either from culture media following isolation of the organisms or from the pus specimens directly. However, there are limitations to the direct extrapolation of *in vitro* pus analysis data.[3,4] Keeping this in mind, in the present study, the usefulness of a new technique i.e. nuclear magnetic resonance (NMR) was evaluated for detection and identification of anaerobes. There are reports of using this technique for identification of aerobes and facultative anaerobes,[5,6] but very few reports have appeared describing the spectra of anaerobes.[7,8]

Bacteria contain a number of macromolecules that could potentially contribute to their MR spectra.[9] The principle of the technique is that protons in these molecules, when subjected to an intense magnetic field, have the possibility of orienting themselves with the field (lower energy state) or against the field (higher energy state). The nuclei are predominantly in the lower energy state. However, when this system is subjected to electromagnetic energy in the form of radio frequency irradiation, the protons orient themselves in the higher energy state. As the microenvironments of the various protons often differ, varying amounts of radio frequency energy (resonance energy) must be applied to bring all the protons to the higher energy state. The results are presented on a plot of the resonance frequency (in parts per million - ppm) versus signal intensity.[5] Characteristic signals in the spectra are contributed to the cell wall constituents or metabolites of the microorganism. Since this varies in different organisms, the spectra obtained could contribute to the identification of the organism. The microenvironment of the organism is affected by the end products of bacterial fermentation. Anaerobic metabolism is not as efficient as that of facultative organisms, hence, intermediate products are left that can serve as markers of an organism's identity.[2] These are reflected in the spectra obtained after NMR, so the technique could be a major tool for identification of anaerobes.

Materials and Methods

MR spectra of pure cultures of anaerobes were recorded. The pure culture of anaerobes tested for MR spectra included seven clinical isolates from deep-seated abscesses and an NCTC *Bacteroides fragilis* 2553 obtained from Christian Medical

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College, Vellore that served as a control organism. The clinical isolates studied were *Bacteroides fragilis* (three), *Prevotella melaninogenica* (three) and *Prevotella denticola* (one).

Eight pus specimens collected from brain abscesses, which yielded pure culture of anaerobe, also were analysed. The pus specimens were included to assess if the spectra of direct specimen match with the spectra of pure culture of the respective organism. The total spectral analysis was then used for building an algorithm.

Anaerobes - isolation and identification

Pus specimens were either inoculated at the bedside or transported in Robertson's cooked meat medium. They were then inoculated on blood agar, neomycin blood agar and Bacteroides bile esculin agar. All the anaerobic culture plates were incubated for 48-72 h in a Gas-Pak system (Dynox/Dynamicro, Mumbai, India). All the colonies were tested for aerotolerance. Only strict anaerobes were further identified. Preliminary grouping of anaerobes was done using kanamycin - 1 mg, colistin - 10 μg, vancomycin - 5 μg and sodium polyanethol sulfonate - 1 mg. (Becton-Dickinson, USA). Identification of anaerobes was further done by conventional methods, which included Gram stain, carbohydrate fermentation, bile resistance, indole and lipase production.[10]

MR spectra of pure cultures

A single colony of each organism was subcultured on Brucella blood agar (Hi-Media, Mumbai, India) and incubated at 37 °C for 48 h anaerobically. Several colonies from this plate were then suspended in a 0.9% sodium chloride and adjusted to turbidity equivalent to 0.5 McFarland standard. For each isolate, a lawn culture was made on six Brucella blood agar plates by streaking them with a sterile cotton swab that had been immersed in the suspension of bacteria. These plates again were incubated at 37 °C for 72 h anaerobically. The growth from all the plates was scraped and directly put into deuterium oxide (D_2O; Armar Chemicals, Switzerland). This growth was now matched to McFarland No. 10. The bacterial suspension (0.4 mL) was transferred to an NMR tube and subjected to NMR analysis.

MR spectra of pus specimens

Pus from eight patients, which yielded a single anaerobe in pure culture, were also subjected for NMR analysis. Pus (100 μL) was loaded in 5-mm NMR tube and D_2O was added to make the volume up to 0.4 mL and then subjected to NMR analysis.

NMR analysis was carried out using Mercury plus Varian 300 MHz (7.05 T) NMR spectrophotometer. After the NMR analysis (256/512 scans), the different peaks obtained were noted. Referencing was done with the water peak. The spectral regions of 0.5-4.5 ppm were compared. The spectra, each containing one or more characteristic peaks, were recorded. The interpretation of peaks was done according to the published available literature.[8,11]

Results

NMR spectra of the eight different anaerobes and eight pus specimens, which yielded anaerobes in a pure culture, were studied The majority of the protons found in organic and biological molecules resonate within a narrow spectral window: 0-10 ppm. The lower energy side of the spectrum (larger ppm value) contains signals for protons that require lesser amounts of energy for resonance as compared with the ones on the higher energy side of the spectrum. The important resonances were seen between 0.5 and 4.5 ppm and the other part of the window hardly showed any detectable peaks. Hence, the spectral regions of 0.5-4.5 ppm were compared for each of the six genera studied. On reviewing the spectra, it was observed that the major resonances remained constant within the genera with a narrow range of variability amongst different species. Identical results were obtained with pure cultures and pus from which pure cultures had been obtained.

Reproducibility of the spectra was tested for two important pathogens viz *Bacteroides fragilis* and *Prevotella melaninogenica*. The tests on the pure cultures were repeated twice to assess reproducibility of the technique. The reproducibility of spectra within these strains was 100% with a mild variation in some peaks (±0.01-0.03; Table). The consistency of results within the same species also demonstrates the reproducibility of the technique.

All anaerobes were characterized by presence of multiplex of amino acids/lipid at 0.9 ppm along with lactate/lipid at 1.3 ppm, acetate at 1.92 ppm and multiplex of lysine at 3.0 ppm. Presence of succinate at 2.4 ppm was observed with 13 (81.25%) out of 16 anaerobes tested. There were unidentified multiple peaks at 3-3.5 ppm, which were characteristically seen with *Bacteroides fragilis*. The singlet of glycine was observed in 11 (68.75%) out of 16 anaerobes studied.

Spectra of *Bacteroides fragilis* (local isolates) was found to be matching with that of NCTC *Bacteroides fragilis* 2553, which also matched with the spectra of pus specimen that yielded *Bacteroides fragilis* (Fig. 1). *Prevotella melaninogenica* from pus revealed an additional peak at 1.4 and 2.8 ppm, which was not seen in pure cultures. In case of *Prevotella denticola*, an additional peak at 1.4, 1.6 and 2.4 ppm was noticed with pus while the same were absent from pure culture spectra (Table).

Thus, on the basis of the representative spectra, a simple algorithm (Fig. 2) was developed to distinguish the six genera belonging to non-sporing anaerobes from each other. The presence or absence of significant peaks was used as an identification criterion.

Table: Spectral patterns of the anaerobes studied (Spectral regions of 0.5 to 4.5 ppm)

Organism	Resonances at ppm										
NCTC *B. fragilis* 2553	0.9-1.0 (m)		1.3d*	1.4	1.6	1.9		2.4	3.0	3-3.5(m)	3.5
Bacteroides fragilis local 1	0.9-1.0 (m)		1.3d	1.4	1.6	1.89		2.37	3.0	3-3.5(m)	3.5
Bacteroides fragilis local 2	0.9-1.0 (m)		1.29d	1.4	1.6	1.89		2.37	3.0	3-3.5(m)	3.5
Bacteroides fragilis 3	0.9-1.0 (m)		1.29d	1.4	1.6	1.89		2.37	3.0	3-3.5(m)	3.5
Bacteroides fragilis (pus)	0.9-1.0 (m)		1.29d	1.4	1.6	1.89		2.37	3.0	3-3.5(m)	3.5
Bacteroides fragilis (pus)	0.9-1.0 (m)		1.3d	1.4	1.6	1.92		2.4	3.0	3-3.5(m)	3.5
Bacteroides fragilis (pus)	0.9-1.0 (m)		1.3d	1.4	1.6	1.92		2.4	3.0	3-3.5(m)	3.5
Prevotella melaninogenica local 1	0.9 (m)	1.04	1.27d			1.92		2.3	3.0		
Prevotella melaninogenica local 2	0.9 (m)	1.04	1.27d			1.92		2.3	3.0		
Prevotella melaninogenica local 3	0.9 (m)	1.04	1.27d			1.92		2.3	3.0		
Prevotella melaninogenica (pus)	0.9 (m)	1.04	1.3d	1.4		1.92		2.4	2.8 3.0		
Prevotella denticola local 1	0.9 (m)		1.3d			1.92			3.0		3.5
Prevotella denticola (pus)	0.9 (m)		1.3d	1.4	1.6	1.92		2.4	3.0		3.5
Fusobacterium necrophorum (pus)	0.9 (m)		1.3d	1.4	1.6	1.92			3.0		3.5
Peptococcus niger (pus)	0.9 (m)		1.3d	1.4	1.6	1.92	2.1	2.4	3.0		3.5
Peptostreptococcus spp. (pus)	0.9 (m)		1.3d	1.4	1.6	1.92			3.0	3.1	

m - denotes presence of multiple peaks, d - denotes presence of duplex

Figure 1: Showing comparison of spectra of a. NCTC *Bacteroides fragilis* 2553; b. *Bacteroides fragilis* (Pure Culture); c. The pus specimen which yielded pure growth of *Bacteroides fragilis*

Discussion

Identification of anaerobic bacteria is a challenge to the microbiologist. It is time-consuming and tedious, so often not attempted. Reduction of the time required for identification of these anaerobes is highly desirable for rapid clinical diagnosis and appropriate therapy. Several rapid identification methods are available.[12-14] GLC has been

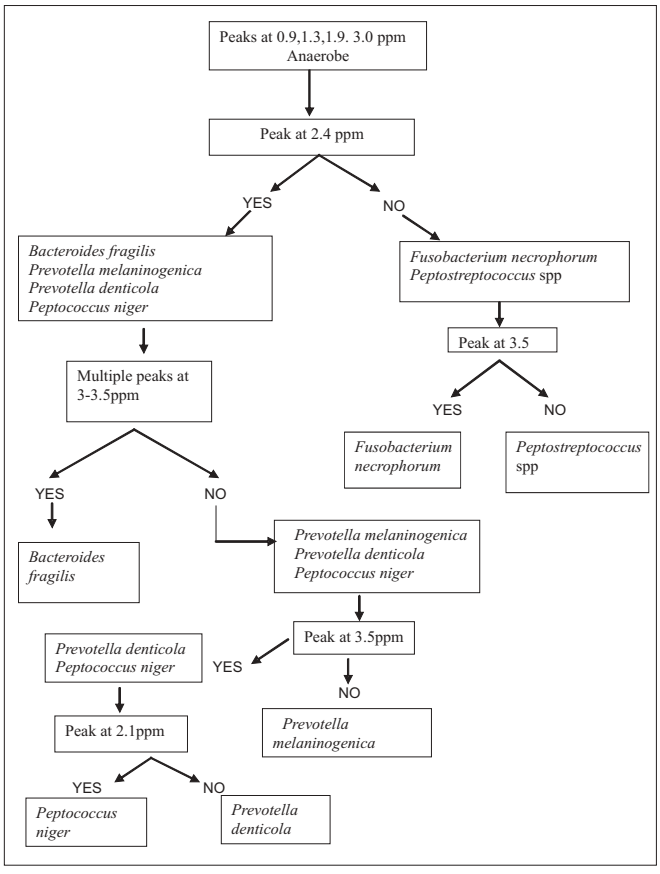

Figure 2: Algorithm for identification of six genera belonging to non-sporing anaerobes

used for rapid detection of anaerobes. However, all these techniques have their own limitations.

In contrast to current diagnostic methods in microbiological laboratories, NMR is an emerging technique, which has been used for the identification of microorganisms. Many laboratories have used this technique to study organisms in pure culture[5,6] or directly from clinical specimens.[7,8] Delpassand *et al*.[5] used this technique for rapid identification of *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Enterococcus faecalis*. Bourne *et al*.[6] used this technique for identification of *Enterococcus*, *Streptococcus* and *Staphylococcus*. Himmelreich *et al*.[15] used this technique for rapid identification of *Candida* species. There are scanty reports describing the spectra of anaerobes using this technique.[7,8,16] In the present study, this technique was evaluated for its usefulness in the rapid identification of anaerobes from pure cultures as well as from the clinical specimens with anaerobic infections.

The major resonances obtained from six genera of anaerobes after subjecting them to NMR were analysed. The analysis showed that anaerobes have major resonances of multiplex of amino acids/lipid at 0.9 ppm along with lactate/lipid at 1.3 ppm, acetate at 1.92 ppm and multiplex of lysine at 3.0 ppm. Presence of peak of succinate at 2.4 ppm was seen with 81.25% of anaerobes, whereas the peak of glycine was observed in 11 (68.75%) out of 16 anaerobes studied (Table). Lia *et al*[8] and Garg *et al*[16] in their studies on anaerobic brain abscesses, where the pus specimens also were subjected to NMR and supplemented with the bacterial information showed resonances of amino acids, lactate, acetate with or without succinate and the cultures from this group grew obligate anaerobes.

The amino acid signals at 0.9 ppm are assigned to valine, leucine and isoleucine.[17] These are considered to represent either the accumulated end products of proteolysis caused by the enzymes secreted by microorganisms or the polymorphonuclear leucocytes in pus or both. These are usually detected in any pyogenic infection whether aerobe or anaerobe.[11] Increase in lactate and acetate presumably originate from the enhanced glycolysis and fermentation of the infecting microorganisms.[18] Succinate is known to be one of the end products of propionic acid fermentation and mixed acid fermentation in various anaerobic bacteria.[19,20] In the present study, 13 (81.25%) anaerobes showed presence of succinate. Lysine was seen to be associated with all the anaerobes tested. Lai *et al*.[8] also have described this signal in their study, but the exact significance of this has not been identified.

There were seven unassigned peaks (1.04, 1.4, 1.6, 1.7, 2.1, 3.1 and 3-3.5 ppm) in the present study. Kim *et al*.[18] also reported similar finding. The peak position in their study was 2.2, 2.9, 3.2, 3.4 and 3.8 ppm. The diagnostic significance of these unassigned peaks remains unclear. However, the present study noticed the peculiar association of the peak at 1.04 ppm with *Prevotella melaninogenica*.

This study noticed that the spectral pattern of the pure culture of the anaerobes matched the spectra of pus showing the same isolate (Fig. 1). Garg *et al*[7] found the spectra of *Bacteroides fragilis* and *Peptostreptococcus* spp. from the pure cultures as well from the pus specimens to be similar. The probable reasons being obligate, anaerobes are particular to their metabolism and follow similar metabolic pathways for growth and energy under *in vivo* as well as in culture conditions.[7]

In case of *Prevotella melaninogenica* and *Prevotella denticola*, there were additional peaks obtained in pus not seen in the pure cultures. However, the major peaks obtained in the culture were also reflected in the pus (Table). This could be due to variations in the number of organisms in pus.

There are a few *in-vivo* MR studies of brain abscesses where the pus specimens also were subjected to NMR and supplemented with the bacterial information.[8,16] Garg *et al*[16] used *in-vivo* MR spectroscopy for etiological characterization of brain abscesses and concluded that it

is possible to differentiate anaerobic brain abscesses from aerobic or sterile brain abscesses on the basis of metabolite patterns obtained. Lai *et al*.,[8] also had similar finding. The ability of MR spectroscopy to identify the microorganisms from the deep-seated abscesses without draining the pus (*in vivo*) has generated great deal of research interest.[8,16] However, results of NMR on mixed bacterial infections still need to be evaluated.

The spectral pattern with the genus showed consistency and varied mildly from species to species. The spectra of local isolates *Bacteroides fragilis* also matched with the control strain of NCTC *Bacteroides fragilis* (Fig. 1).

Our results showed that the spectral peaks characteristic of the *Bacteroides fragilis* were 0.9 (amino-acid/lipid), 1.3 (lactate/lipid), 1.4, 1.92 (acetate), 2.4 ppm (succinate) and 3.0 ppm (lysine) along with the presence of multiple unidentified peaks between 3.0 and 3.5 ppm. *Prevotella melaninogenica* showed the similar pattern as that of *Bacteroides fragilis* with the absence of multiple unidentified peaks between 3.0 and 3.5 ppm. *Prevotella denticola* had an additional peak of 3.5 ppm that helped us to differentiate it from *Prevotella melaninogenica*. There was a conspicuous absence of succinate at 2.4 ppm with *Fusobacterium necrophorum*. Garg *et al*[7] described the spectra for *Bacteroides fragilis* as 0.9, 1.3, 1.47, 1.92, 2.4 and 3.5 ppm, which were found to be matching with the present study with three more extra peaks i.e. 5.23, 4.64 and 8.64 ppm that were not present in this study.

An attempt was also made to analyse the spectra of Gram-positive anaerobic cocci. *Peptostreptococcus* species showed conspicuous absence of peak of succinate while the same was present with *Peptococcus niger*. The important peaks of *Peptostreptococcus* species were 0.9, 1.3, 1.9 and 3.0 ppm, whereas *Peptococcus niger* showed peaks at 0.9, 1.3, 1.9, 3.0, 2.1, 2.4 and 3.5 ppm. Garg *et al*[7] described the spectra for *Peptostreptococcus* species as 0.9, 1.3, 1.47, 1.92, 2.4, 3.5, 4.64 and 5.23 ppm while in this study the spectra were 0.9, 1.3, 1.4, 1.6, 1.7, 1.92, 3.0 and 3.1 ppm. This suggests that the major spectra remained the same in both the studies. Larger studies may be required to reach definitive conclusion.

The consistency in the spectral peaks obtained within the genus and species helped us in developing an algorithm for the identification of six common clinical anaerobic isolates (Fig. 2) A similar algorithm has been developed for the identification of aerobes by Delpassand *et al*.[5] using the spectral results obtained by NMR. No such algorithm for anaerobes has yet been described.

The present study was a preliminary study and an algorithm for only six clinically important anaerobes has been developed. However, more data need to be generated on the spectral pattern of the other common anaerobes causing infection. With an adequate database, the technique has the potential of identifying the causative agent *in vivo* so that appropriate antibiotics can be started without any loss of time.

Thus, our study highlights the potential of the NMR technique in the rapid identification of anaerobic infections. The limiting factors, as on date is the cost of the technique and the paucity of a large database of spectra for more accurate interpretation of the NMR spectral patterns.

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