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Philip J. Migliore, M.D.
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Dear Dr. Migliore:

We have submitted a manuscript entitled "Rapid Identification of Common Human Pathogens Using High Resolution H_1-Nuclear Magnetic Resonance Spectroscopy" to the Journal of Clinical Microbiology for publication. We would like to thank the Moran Foundation for financial support for this project.

Sincerely,

Charles Stager

Charles E. Stager, Ph.D.

Rapid Identification of Common Human Pathogens Using High Resolution Proton Magnetic Resonance Spectroscopy.

Running Title : Bacteria and Nuclear Magnetic Resonance Spectroscopy

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ABSTRACT

Routine procedures for recovery of bacteria from clinical specimens involve culturing the latter on various non-selective and selective agar media. The bacteria are then identified by means of biochemical and immunological test procedures. Reduction of the time required to identify the bacteria is highly desirable for rapid clinical diagnosis. Towards this end the potential of proton nuclear magnetic resonance (NMR) spectroscopy for providing a "fingerprint" within the proton spectrum of five bacterial genera, reflecting their characteristic cell wall constituents, has been investigated.Establishing a database of high resolution proton NMR spectra of a large number of bacterial species is a prerequisite for attaining this objective. A small database has been established for the five most common human pathogens : *Escherichia coli, Klebsiella pneumoniae*, *Pseudomonas aeruginosa, Staphylococcus aureus* and *Enterococcus faecalis*. Based on the presence of characteristic resonances in their spectra, a simple algorithm has been developed to differentiate and identify these microorganisms.

INTRODUCTION

A rapid, specific and sensitive method for accurately identifying microorganisms that cause serious infections would help physicians verify their presumptive clinical diagnosis and lead to prompt initiation of a tailored antibiotic regimen. This is conventionally carried out by culturing the bacterium *in vitro* and subjecting it to the usual identification protocols. The pathogenicity of bacteria and their susceptibility to antimicrobial agents may be ascribed in part to the molecular composition of the cell wall,cytoplasm, periplasm and membranes that separate these compartments (4). Such components would include lipopolysaccharides (9,11), membranes and cytoplasmic proteins (1,2,5,7), peptidoglycans (6,8), teichoic acids (3) and several different types of phospholipids (10).

Nuclear magnetic resonance (NMR) spectroscopy is an unusually powerful technique for studying these individual components especially when they are isotopically enriched. In the present study, we have used high field (9.4 T) proton NMR to distinguish bacterial cell types which differ in their molecular composition. Due to differences in composition of their cell wall and cytoplasmic constituents, individual bacterial species exhibit spectra with characteristic "marker" resonances. Accordingly, *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Staphylococcus aureus and Enterococcus faecalis* can be differentiated by their proton NMR spectra. A limited database of the H-1 NMR spectra of ten strains of each of the microorganisms was established.

MATERIALS AND METHODS

Ten strains each of *E. coli, K. pneumoniae, P.aeruginosa, S.aureus* and *E. faecalis* were obtained from clinical specimens at Ben Taub General Hospital in Houston, TX. A single colony was subcultured to a sheep blood agar (SBA) plate (Becton Dickinson Microbiology Systems, Cockeysville, MD) and incubated at 35 C for 18 h. Several colonies were suspended in 0.9% sodium chloride and adjusted to a turbidity equivalent to a 0.5 McFarland standard. The microorganisms were then prepared for NMR spectroscopy by the following method. A SBA plate was streaked for confluent growth with a sterile dacron tipped swab that had been immersed into the suspension of bacteria. The SBA plate was inverted and incubated at 35 C for 18 h. Growth from the plate was harvested with a sterile dacron tipped swab into a plastic centrifuge tube containing 10 ml of sterile distilled water. The specimen was centrifuged at 2000 x g for 15 min to sediment the bacteria. The supernatent was aspirated and an equal volume of deuterium oxide (Isotec, Inc., Miamisburg, OH) was added to the sediment and a total volume of 0.4 ml of bacterial suspension was transferred to a NMR tube (5 mm internal diameter; Norell, Inc., Mayslanding, N.J.) and capped.

NMR Spectroscopy. Spectra (8 K data points) were acquired using a Bruker AM-400 wide bore NMR spectrometer at a field strength of 9.4 T (400 MHz for ^{1}H). The residual water signal was suppressed by presaturation for 2 sec prior to the data acquisition. A total of 256 scans were collected per microorganism. The time interval between loading the sample in the spectrometer and the printout of the spectrum was about 20 min. The spectra were referenced with an internal standard using sodium tetradeutero-trimethylsilyl-propionate ($\delta = 0$ ppm). In addition to the Bruker DISNMR program, off-line data processing was carried out on a Sun 3/60 workstation using the NMRi software developed by New Methods Research (Syracuse, N.Y.)

RESULTS

NMR spectra were acquired on 10 strains each of *E. coli, K. pneumoniae, P. aeruginosa, S. aureus and E. faecalis*, which had been processed by the method previously described. Typical spectra of each of the five genera studied and obtained under conditions of water presaturation are shown in Fig. 1. These spectra each contain one or more characteristic resonances or group of resonances. Based on representative spectra, an algorithm (Fig. 2) was developed to distinguish these microorganisms from one another. The presence or absence of significant peaks in their ^{1}H spectrum was used as a decision criterion. The signal intensities in the spectra were classified relative to that of the most intense peak (excluding that of residual water) as high (70-100%), medium (60-30%) or low (30-10%). The first decision step in the algorithm (Fig. 2) is the presence or absence of high intensity resonances in the region 0.8 ppm to 1.1 ppm to distinguish *E. coli* from the other micro-organisms. The presence of several medium intensity resonances near to each other with a broad high intensity envelope at 1.00 ppm appear to be charcteristic of *E. coli.* The positions of these signals are characteristic for the methyl groups in valine, leucine and isoleucine as well as for the terminal methyl groups in long chain fatty acids. The second decision making criterion in the algorithm is the absence of a high intensity singlet peak at 3.26 ppm which distinguishes *E. faecalis* from the other organisms which exhibit this resonance in their NMR spectra. This signal can be ascribed to O-methyl as well as N-methyl groups. As a confirmation ,the ^{13}C NMR spectrum of *S. aureus* in D2O exhibited several signals in the region 56.0 ppm $\pm$ 2 ppm which are characteristic for O-methy carbons. The presence of a medium intensity peak in the spectrum of the *S. aureus* strains at 3.00 ppm distinguishes it from *K. pneumoniae* and *P. aeruginosa*. The presence of medium intensity peaks within the region 2.00 to 2.35 ppm was characteristic for *P. aeruginosa* whereas *K. pneumoniae* did not exhibit this resonance. Fifteen spectra (three strains each of the five genera) were blind coded by one author and were then given to three other authors for assignment using the algorithm. All organisms were corresctly identified by this process.

DISCUSSION

Bacteria contain a number of different macromolecular and multi-molecular ensembles that could potentially contribute to their respective ^1H spectra (4). The phospholipids, which serve as the basic framework for the cell's cytoplasmic membrane (gram -positive and gram -negative organisms) and outer membrane (gram negative organisms), have perhaps the highest molar concentration of chemically similar moieties : fatty acyl, CH_2, and CH_3 groups; but most bacterial cell membranes contain high proportions of membrane proteins that significantly restrict lipid motions thereby reducing their contribution to the isotropic ^1H spectrum. Many of the cell proteins are typically large molecules embedded in or completely spanning a membrane bilayer; their restricted motions and relatively low molar concentrations make them difficult to study by ^1H NMR.

Most gram -positive bacteria have a relatively thick (20-80 nm) continuous cell wall, which is largely composed of peptidoglycan. When thick cell walls are found, other biopolymers such as teichoic acids, polysaccharides and peptidoglycolipids are covalently coupled to the peptidoglycan, forming a rather rigid lattice of moieties with restricted motions and low spectral intensities. By contrast in gram-negative bacteria, the peptidoglycan layer is thin (5-10 nm), perhaps allowing significant motional freedom and detectable spectral intensity. A lipoprotein layer is present in the gram-negative cell wall, thereby stabilizing the outer membrane and cross linking it to diaminopimelic acids on the peptidoglycan layer. Embedded in the outer leaflet of the outer membrane is lipopolysaccharide. Its lipid component (lipid A) anchors the molecule in the membrane, while the polysaccharide component is exposed to the outer surface of the bacteria and probably experiences considerable molecular motion and probably contributes significant intensity to the ^1H spectrum (10,11).

The present report does not address the assignment of particular molecular species in bacterial cells to salient spectral peaks. These assignments are the focus of continuing studies. A much larger database with a variety of genera and species would have to be examined to establish the general utility of high field NMR spectroscopy for identification of bacteria. With the

accumulation of a larger data bank of spectra, it may well be possible to conduct the algorithm matching by the use of computer programs. One disadvantage of NMR spectroscopy is the relatively large amount of sample required and could limit its use for routine application. A possible application would be for reference identification of unusual genera that are not included in the database of commercial systems. Another area for investigation would be the definitive identification of a particular strain of bacteria involved in a nosocomial outbreak. In preliminary studies we have identified *E. coli* and *P. aeruginosa* directly from blood culture vials by NMR spectroscopy. The erythrocytes were lysed with Triton-X 100 and the bacteria were harvested for NMR spectroscopy by centrifugation (unpublished data).

In summary,this is a preliminary report demonstrating that NMR spectroscopy can rapidly identify five genera of bacteria that commonly cause infections in humans and points out other potential applications for this technique. Work is in progress to expand the existing database by including other genera and species.

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Fig 1. ^{1}H-NMR spectra (400 MHz) of the five genera : *E. coli* (a), *K. pneumoniae* (b), *P. aeruginosa* (c), *S. aureus* (d), and *E. faecalis* (e).

4.0
3.0
2.0
1.0
PPM

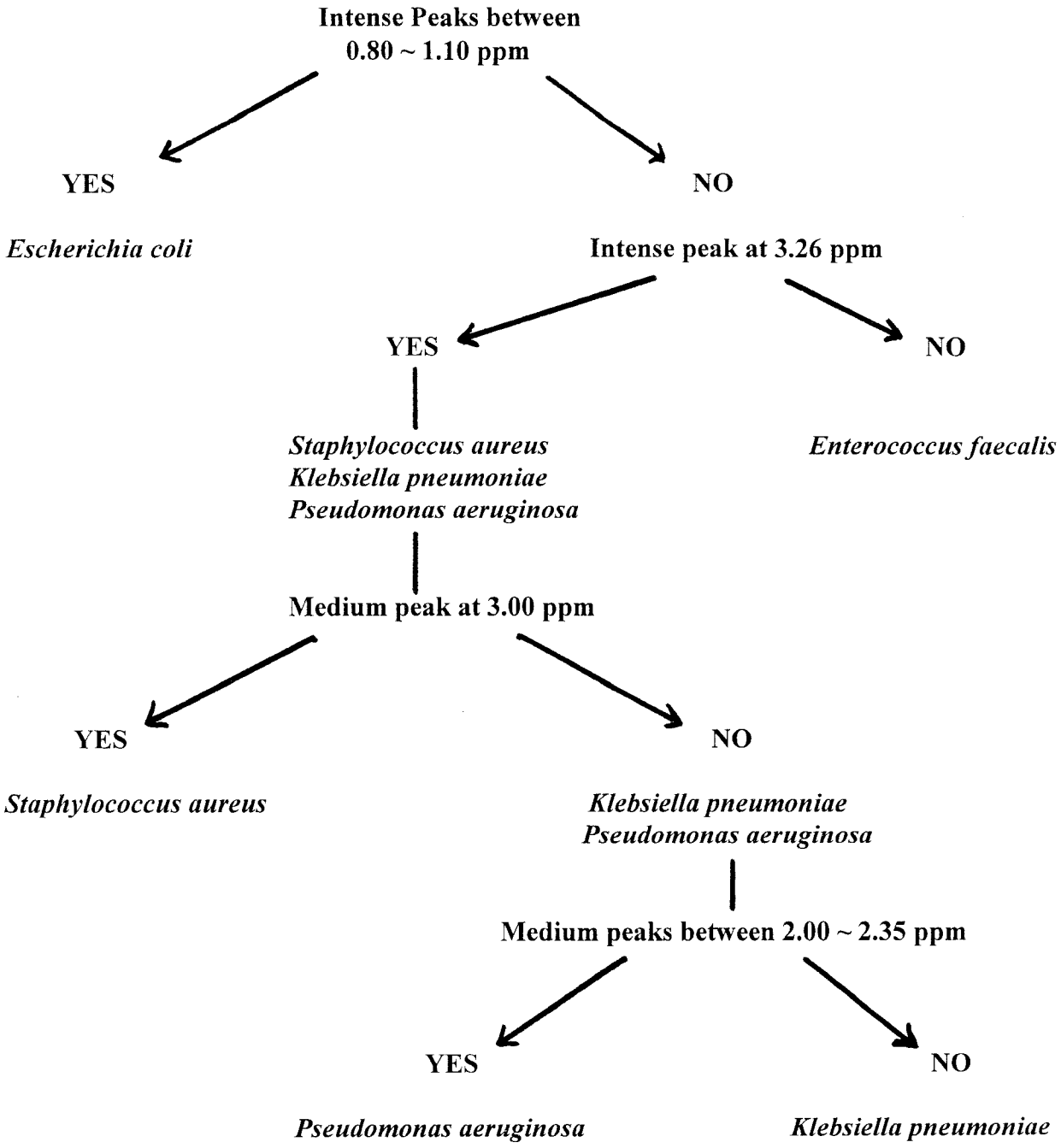
4.0
3.0
2.0
1.0

4.0
3.0
2.0
1.0
PPM

4.0
3.0
2.0
1.0
.4

4.0
3.0
2.0
1.0

Fig.2 Algorithm for identification of common human pathogens as compiled for the ^{1}H NMR spectral database.


Intense Peaks between
0.80 ~ 1.10 ppm
YES
NO
Escherichia coli
Intense peak at 3.26 ppm
YES
NO
Staphylococcus aureus
Klebsiella pneumoniae
Pseudomonas aeruginosa
Enterococcus faecalis
Medium peak at 3.00 ppm
YES
NO
Staphylococcus aureus
Klebsiella pneumoniae
Pseudomonas aeruginosa
Medium peaks between 2.00 ~ 2.35 ppm
YES
NO
Pseudomonas aeruginosa
Klebsiella pneumoniae