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Applications of ^1H Nuclear Magnetic Resonance Spectroscopy in Clinical Microbiology

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Abstract

Proton nuclear magnetic resonance (^1H NMR) is a spectroscopic technique usually used for structural determination of molecules. In recent years, this technique has been employed for easy and quick recognition of microorganisms, in antimicrobial susceptibility tests and even for the diagnosis of different infectious conditions. Though ^1H NMR shows great potential for expanded applications in microbiological studies, to date applications of proton NMR to microbiological research are not totally standardized. In this chapter, we summarize the state of knowledge about ^1H NMR and its current and potential applications in this field.

Keywords: nuclear magnetic resonance, ^1H NMR, applications, clinical microbiology, microorganisms

1. Introduction

Scientific progress made in the recent years has enabled the development of new techniques that facilitate and improve microbiological study. In this way, nuclear magnetic resonance (NMR) is a spectroscopic technique easy to use and quick to recognize microorganisms and provides sensitivity to antimicrobials. Anyway, to date we have not consensus about the usefulness of these techniques that are not totally standardized. In this chapter, we summarize the state of knowledge about NMR in microbiological studies.

2. Nuclear magnetic resonance (NMR) spectroscopy

NMR is a spectroscopic technique initially developed by Felix Bloch and Edward M. Purcell that relies on the magnetic properties of the atomic nucleus. Since 1946, it has become a powerful and extremely valuable tool for chemists, physicists, biochemists and more recently for the medical practitioners [1–5].

Although the most widespread application of NMR is the structural determination of molecules, the technique offers the advantage of direct mixture analysis, and therefore, NMR has demonstrated a unique potential to be used for metabolic mixture analysis, fastidious bacteria included [6]. In comparison with other techniques employed for mixture analysis, NMR can be used to directly investigate biological samples and cell cultures without requiring significant sample preparation. Moreover, the technique allows the determination of compound ratios in a highly reproducible manner. For these reasons, metabolomics and metabonomics are driving new technological advances in the NMR field. Thus, the combination of sophisticated NMR-based methods for mixture analysis with the power of statistical and chemometric methods makes NMR spectroscopy the technique of choice for complex biological mixture analysis, especially in clinical and biomedical researches [3, 4].

In the biological field, the NMR technique [7] is employed to determine the structure and function of macromolecules. Additionally, NMR allows the determination of metabolic changes in organisms in response to external stimuli, through the identification and quantification of metabolites (metabolomics/metabonomics). Metabolomics is the study of global metabolite profiles of a cell under a given set of conditions; however, the terms metabolomics and metabonomics are used in the literature interchangeably [8]. Jeremy Nicholson was the pioneer of the approach, 'the distinction between the two terms metabolomics/metabonomics is mainly philosophical rather than technical' [9].

Since NMR is the only technique that allows to carry out *in vivo* analysis, it has applications in medical diagnosis, for example, magnetic resonance tomography (MRT).

The use of NMR in metabolic studies was reported in 1977, by Brown et al., who determined the presence of lactate, pyruvate, creatinine, and alanine in a blood cell suspension by proton NMR (^1H NMR) [10]. Since then, the analysis of biological fluids, tissues, and cell extracts has been carried out successfully by NMR, especially in the context of research on diseases and evaluation of toxicological processes [11, 12].

2.1. Basics of NMR spectroscopy

The NMR phenomenon can only be carefully described and fully understood using quantum mechanics. Therefore, a complete understanding of the technique would require an exhaustive knowledge of the properties of the angular momentum in the quantum mechanics field, along with statistical thermodynamics knowledge to describe the floating processes needed in the liquid state.

However, since the theory and fundamentals of NMR have been fully developed over the last few years [13, 14] and its detailed description would get away from the objective of this chapter,

we describe below some aspects of the technique, which could help to understand the equipment and methodologies used in metabolic research.

The basis of the technique is to use the magnetic properties of atomic nuclei that are defined by their angular momentum and their associated magnetic moment. Both moments are vector quantities, and they are related by a constant called gyromagnetic constant (γ), which is specific to each type of atomic nucleus. According to quantum mechanics, both moments are quantized and their value depends on a quantum number called spin (I). Not all nuclei are valid to get NMR signals, only those whose spins are greater than zero are valid, but ^1H or proton is the most used nucleus in NMR studies. Moreover, higher the value of constant γ , more sensitive will be the active nucleus in NMR. So, ^1H is the nucleus used in NMR studies because of its great abundance (100%) and high value of γ . However, other magnetically active nuclei with lower sensitivity may be used, such as ^2H (or D), ^{13}C , ^{19}F , ^{15}N , ^{31}P , and ^{23}Na . Focusing on the proton, in the presence of an external magnetic field (B_0), two different energy levels appear. The magnetic moments of these nuclei try to align with B_0 , resulting in two possible orientations at that time. Each of these orientations corresponds to a different energy level. That is, in the presence of B_0 , the cores can be arranged in two new states with different energies. This phenomenon, from a vectorial viewpoint is known as magnetization.

To obtain a NMR signal, the sample is irradiated with a radiofrequency wave, perpendicular to B_0 , which compels it to reach the state of resonance, where the nuclei gyrate with a resonance frequency (f_0), specific to each atomic nucleus and called Larmor frequency. For this reason, NMR spectrometers are designated by the ^1H resonance frequency instead of the magnetic field (for example, on a 14.1 T field, ^1H resonates at 600 MHz). After the pulse, the excited nuclei return to the initial equilibrium state emitting a radiofrequency signal, which decays with the time, a phenomenon known as relaxation. The resonance of the excited nuclear magnets is detected as an oscillating current in a coil placed around the sample. This signal is the FID (free induction decay), which arrives at the receiver and provides a spectrum formed by lines defining frequencies and widths by a mathematical operation known as Fourier transform. The widths are formed from the contributions of all nuclei of the sample, so that this quality allows quantitative measurements. A line in the NMR spectrum obtained at a certain frequency (or chemical shift) corresponds to an atomic nucleus with a given chemical environment, which allows structural information about the molecule it belongs.

2.2. Equipment

The NMR spectrometer involves several parts such as a superconducting magnet, a radio transmitter, a probe, a radio receiver, an analog-to-digital converter (ADC), and finally, a computer. The magnet is the main element and consists of a solenoid of superconducting Nb/Ti alloy wire immersed in liquid helium (4 K) that is charged to generate the essential field strength. The helium is protected with a vacuum jacket and further cooled by an outer dewar of liquid nitrogen (77 K).

The probe head is a coil of wire positioned around the sample (NMR tube) that alternately transmits and receives radio frequency signals. The probe head is usually hosted into the magnet from the bottom and is connected to at least three radiofrequency channels provid-

ing the ^2H lock, ^1H frequency, and one X-nucleus frequency. In general, devices to control temperature (heater, thermoelement, and air) are needed. New developments include the digital transmission of the probe-head parameters to the console.

The computer addresses the transmitter to send a high-power and very short duration pulse of radio frequency to the probe-head. Instantly, after the pulse of radio frequency, a weak signal (FID) from the sample received by the probe-head is amplified and sampled at intervals by the ADC to produce a digital FID signal, which is just a list of numbers. The computer automatically determines the timing and power of pulses output by the transmitter and receives and processes the digitalization. After the computer performs the mathematical processes of Fourier transform in order to convert time domain into frequency domain, the resulting spectrum can be displayed on the computer monitor, transferred to other computers or plotted on a paper.

2.3. Sample preparation

The sample volume should be about 0.6 mL, which gives a 4.0 cm depth in a standard 5 mm NMR tube. Volume of the samples is less important than the concentration of targets. Very small volumes could not be studied by NMR equipment with low magnetic field but once the volume is established, it is more important to ensure that the metabolites to be studied are in

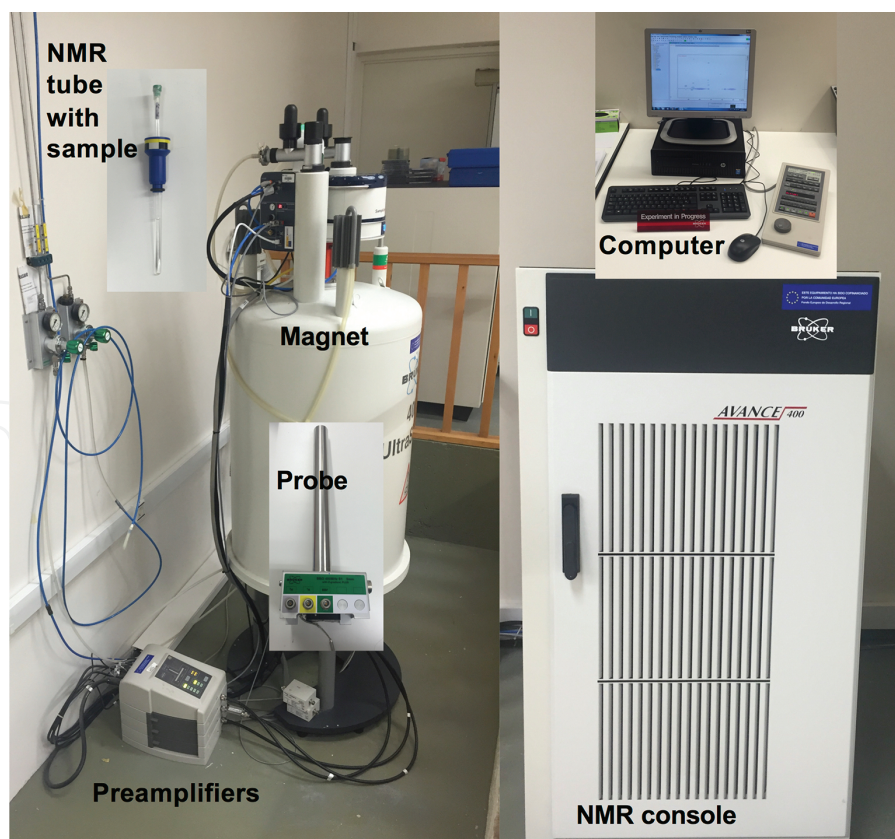


Figure 1. Components of an NMR equipment.

an appropriate concentration. For biological samples, the ideal solvent is D_2O or a mixture of $\text{H}_2\text{O}/\text{D}_2\text{O}$. In this latter case, the ^1H NMR spectra will be recorded using the pulse sequence for presaturation or the equivalent in order to avoid the signals from water. This is the typical and most simple method used to record NMR spectra of biological samples.

Usually, the protocol of preparation for biological and aqueous samples is simple, quick and involves two steps. Samples are prepared from inocula of the microorganisms and the bacterial concentration is adjusted using 0.5–2 McFarland standards [15, 16]. Cultures are incubated at the optimum temperature and time to preserve the same growth conditions than the reference method, if possible. After incubation, the suspensions are removed by centrifugation and the supernatants are decanted and used for NMR experiments. Because the measurements are carried out on supernatants, there is no need to quench cell growth rapidly. Furthermore, pH is measured and fixed by the slow addition of aqueous 1 M HCl and 1 M NaOH solutions or with a buffer solution in order to fix the chemical shifts. Next, a biological sample is added to a 5 mm NMR tube together with D_2O with the addition of the sodium salt (trimethyl)propionic-2,2,3,3- d_4 acid (TSP) for the chemical shift calibration. **Figure 1** shows an overview of the components of NMR equipment.

2.4. Sample treatment

The quality of the obtained NMR spectra depends on several variables that influence the process from sample collection to final data collection. The sample collection involves the sample, containers used, additives (preservatives, stabilizers), and time (collection, transport, storage) [17].

Depending of the source of the biological sample, two different methodologies can be used for the experiment acquisition:

(A) Sample concentration by lyophilization and subsequent reconstitution with a deuterated solvent: using this methodology, NMR experiments can be performed without solvent suppression, allowing an increase in sensitivity and stopping enzyme activity. The risks in using this method include the possibility of introducing contaminants into the sample and more importantly, the loss of volatile compounds.

(B) The addition of a small amount of D_2O to the aqueous sample: the corresponding NMR experiment is performed with a pulse program that can remove the water signal, which would otherwise mask the signals from the rest of the sample. This method involves minimal sample handling and the ability to detect volatile compounds, making it a more suitable method for metabolite analysis of biological samples.

In addition to these processes, due to the infectious potential of microorganisms contained in the sample, all steps of sample processing must be performed safely, through protocols and in laboratories appropriate to the biosafety level for the organism—until the organism is inactivated.

2.5. Data processing

Interpretation of the NMR data is essential to complete metabolomics studies to draw conclusions and trends. The first thing is to perform a pre-processing of NMR data in which NMR spectra are cleaned up in standard ways. After treatment of the spectra, it is possible to get information of metabolites, either through direct quantification by parameters [18, 19] or by applying the methods of data analysis and modelling. In this case, chemometric techniques and multivariate analysis are used to identify and quantify the different metabolites present in the sample. **Figure 2** shows an example of NMR spectrum with the main metabolites obtained. The existence of NMR databases of metabolites can greatly facilitate the latter processes.

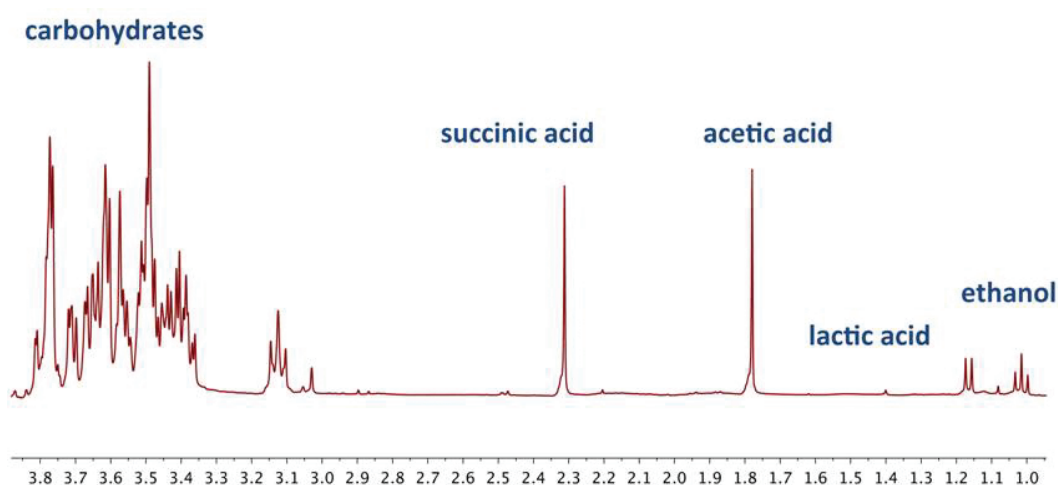


Figure 2. ^1H NMR spectrum showing the main metabolites.

3. Applications of ^1H NMR spectroscopy in clinical microbiology

The application of ^1H NMR to living cells is used to determine metabolites in complex mixtures and has been widely used for identification and quantification of the bacterial species [15, 20]. This technique has also been applied for antimicrobial drug susceptibility studies on different species of yeast, and in the last few years, it has also been developed for bacterial studies. Furthermore, other determinations directly in body fluids have emerged to help in the diagnosis of different diseases and conditions.

3.1. Bacterial identification and metabolic studies

^1H NMR spectroscopy has been used for bacterial identification and quantification and for metabolic pathways studies. Several studies have been conducted for the diagnosis of the bacteria that cause urinary tract infections (UTI). These focus on the use of ^1H NMR spectroscopy for the identification and quantification of common uropathogens such as *Pseudomonas*

aeruginosa, *Klebsiella pneumoniae*, *Escherichia coli*, and *Proteus mirabilis* in urine samples. These studies are based on specific properties of the metabolism of the studied bacteria, and the results showed that ^1H NMR is a simple and fast tool compared with the traditional methods [16, 21, 22].

The qualitative and quantitative determination of *P. aeruginosa* using NMR spectroscopy is based on the specific property of the bacteria to metabolize nicotinic acid (NA) to 6-hydroxynicotinic acid (6-OHNA). Only this bacterium can produce this reaction. The addition of NA to urine samples after incubation and the subsequent analysis by ^1H NMR spectroscopy showed that NA signals disappeared from the medium after some time, while the appearance of new signals of the metabolite 6-OHNA indicated the presence of *P. aeruginosa*. The increase in the intensity of the metabolite signals, together with the decrease in the NA signals, involved a proportional increase in the number of bacteria. This shows the potential offered by this technique for quantitative and qualitative identification, simultaneously, on the bacteria [21].

A similar process occurs in the determination of *K. pneumoniae*. In this case, the specific metabolic reaction is the transformation of glycerol into 1,3-propanediol, so the substance that is added into the medium is glycerol. Despite *Citrobacter freundii* also being capable of carrying out this reaction, both bacteria can be easily differentiated using microscopic examination by observing their motility. *K. pneumoniae* is not mobile while *C. freundii* is. In addition, *C. freundii* is not a common nosocomial urinary tract infection agent. The combination of both methods showed very good sensitivity and specificity (90 and 100%, respectively), suggesting the potential usefulness of NMR for bacterial diagnosis [22].

The same experiment carried out on *E. coli* and *P. mirabilis* revealed that the specific metabolites of the bacteria are lactate and 2-hydroxy-4-(methylthio)butyric acid (MOBA) after incubation with lactose and methionine, respectively [16].

The results obtained using this alternative technique provided the warrant for the development of this method in bacterial identification and quantification and the technical development with other microorganisms. With experience, spectral analysis and data interpretation could be quick and reliable [16].

3.2. Antimicrobial susceptibility assays

The use of ^1H NMR spectroscopy for antimicrobial susceptibility tests has been not highly studied despite its powerful utility in this area of study [5]. Application of ^1H NMR spectroscopy to antimicrobial susceptibility studies was first carried out on different species of yeast. The standardized methods currently available for fungal susceptibility studies are unreliable and relatively slow, so, ^1H NMR spectroscopy can be a simple indicator, an objective and fast method (metabolic changes detected by this method are more easily observed than growth inhibition in broth). ^1H NMR spectroscopy is potentially valuable in determining the metabolic composition of yeast suspensions incubated with a drug. In addition, it is a high performance automated method with low operating costs, so that both operator time and reagent cost are greatly reduced. Therefore, it has great potential to emerge as an alternative method for the antifungal drug susceptibility determination of different yeast species [15, 20].

One of the few studies in which the ^1H NMR technique has been applied to observe the effect on microorganisms upon exposure to several drugs, has been carried out with medically relevant fungi. The fungal species analysed were *Cryptococcus neoformans*, different species of *Candida* and *Aspergillus* spp. These studies are based on the identification of the fungal metabolites produced, on their comparative profile implementation and on the monitoring of the nutrient utilization of the incubation medium in the presence of certain drug concentrations (casposfungin, amphotericin B, and voriconazole). The spectra obtained after subjecting the sample to the ^1H NMR were interpreted based on the metabolites produced (fumarate, malate, ethanol, etc.) and/or the metabolites consumed (tyrosine, phenylalanine, valine, etc.). This interpretation established a measurable parameter, the metabolic end point (MEP), from the spectral peak area. The MEP is defined as the lowest drug concentration at which nutrient utilization from the medium or the production of fungal metabolites is inhibited $\geq 50\%$ and compared with minimum inhibitory concentrations (MIC) used in the reference method. The results of MEP generally showed a good correlation with MICs, which were determined by a modification of the reference method in broth microdilution M27-A of the CLSI. Discrepancies, which may arise between MEPs and MICs, could be due to differences in the culture medium and incubation time. In addition, ^1H NMR spectroscopy is a potentially valuable method for determining the metabolism of microorganisms incubated with the drug because it is a reproducible and relatively quick method (it takes 16 h versus 48 h required by the reference method) suggesting its consolidation as a platform for rapid determination of antifungal susceptibility [15, 20].

In reference to bacteria, there are very few studies based on their antimicrobial susceptibility according to metabolic profiling by ^1H NMR. One of these studies focused on a bacterial disease called 'Withering Syndrome' of abalone (a type of mollusc belonging to *Haliotis* spp. important in aquaculture). It is caused by a pathogen of the Rickettsiaceae family, '*Candidatus Xenohaliotis californiensis*' that infects digestive epithelial cells [23, 24]. On this basis, the effects of the antibiotic oxytetracycline in the metabolic profiles were observed by ^1H NMR. This drug, used to treat bacterial infections in aquatic species, reduces the severity and mortality of the Withering Syndrome. The aim of this study was to observe whether the recovery of the metabolism during treatment with oxytetracycline coincided with the disappearance of the disease caused by the bacteria. To this end, they examined the metabolic constituents present in the foot muscle of the mollusc after oxytetracycline treatment during several established days at two different seawater temperatures (13.4 and 17.3°C) [24]. Metabolic changes were observed at both temperatures: levels of taurine, glycine-betaine, and homarine increased and the amino acid and carbohydrate levels decreased. The detection of metabolic differences between animals treated and untreated with antibiotics was observed only at the highest temperature. Therefore, oxytetracycline eradicated the infection and at the highest temperature it reduced the metabolic changes due to the syndrome. The conclusions drawn from these experiments drive the development of ^1H NMR based on metabolic studies and its complementarity with other techniques, such as the histology for the identification of pathological processes in the aquatic species and for the optimization of drug therapy. This tool displays the performance by analysing quickly and cheaply the functional status of an organism [23, 24].

We have analysed the metabolism and antimicrobial susceptibility of *Escherichia coli* ATCC 25922 in the presence of gentamicin using ^1H NMR and compared with a reference method [25]. The MIC, determined by the reference method used in this study, would correspond to the termination of the bacterial metabolism observed using NMR. To carry out these experiments, serial dilutions of gentamicin were tested. Furthermore, two controls were also analysed (one was the medium with an inoculum of bacterium (control I), and the other was only the medium (control II)). The comparison of the two control ^1H NMR spectra showed different signals. Succinic acid, acetic acid, and ethanol were only detected in the control I spectra and threonine was only detected in the control II medium. According to the results obtained by visual turbidity, the lowest concentration of drug that completely inhibited visible growth (MIC) was $0.5\ \mu\text{g/ml}$ (MIC: $0.25\text{--}1\ \mu\text{g/ml}$) [26]. When we registered antibiotic spectra at different concentrations, we detected the presence of succinic acid, acetic acid, and ethanol only in samples with concentrations of gentamicin lower than the MIC. Moreover, when the concentration of gentamicin was greater than the MIC, we detected the presence of threonine. These data suggested that the results obtained by ^1H NMR spectroscopy were in agreement with those obtained by visual turbidity. These results confirm that *E. coli* is able to metabolize components of the medium to produce succinic acid, acetic acid, and ethanol. Furthermore, threonine only appeared in the spectra of those samples with gentamicin concentrations of $\geq 0.5\ \mu\text{g/ml}$. Differences in peak intensities for the metabolites observed in spectra allowed the determination of the MIC for gentamicin using ^1H NMR spectroscopy. Consumption of the amino acid threonine, present in the culture medium, was interrupted when MIC was performed. Therefore, we assume that succinic acid, acetic acid, and ethanol are metabolites produced by the bacteria and threonine is the amino acid consumed by *E. coli*.

Furthermore, to evaluate the potential of this tool, we also performed the same biological experiments but using an NMR tube as the incubation reactor. The bacterial activity occurred effectively within the NMR tube, and the metabolic process started around 3 h 20 min and ended at 6 h. Moreover, when samples containing gentamicin were analysed in the same way the ethanol signal appeared later using the lowest concentration of gentamicin (4 h 40 min) compared with the experiments performed in the absence of the antibiotic (3 h 20 min) and much later (8 h 40 min) when the gentamicin concentration used was close to MIC. Similarly, threonine consumption by bacteria was delayed when the concentration of antibiotic in the medium was higher [25].

3.3. Applications in biofluids

In the last few years, ^1H NMR has been used to directly analyse biofluids and to diagnose different diseases directly from body fluids. In this sense, it has been applied to analyse human microbiota from faeces and urine samples, to study the metabolic implications that take place in sepsis, or even to diagnose hepatitis C virus infection, distinguish HIV-1 positive patients from negative individuals or to diagnose pneumonia from urine [27–33].

As mentioned above, ^1H NMR has been employed to study gut microbiome focusing on the metabolite profiling obtained by the analysis of different human samples. In this sense, Jacobs et al. [27] studied faeces from healthy subjects after consuming placebo, grape juice, or a mix

of grape juice and wine during four weeks by ^1H NMR. The comparison of the NMR profiling of the samples indicated that only the mixture of wine and grape juice was able to modulate gut microbiota assessed by the reduction in isobutyrate observed only in the samples from subjects who had consumed the mixture. These results confirm that ^1H NMR could determine the impact of the nutrition in human microbiome [27]. Furthermore, the use of ^1H NMR to understand gut microbiota has been also extended to the study its impact on obesity by analysing metabolite profiling obtained from urine samples [28, 34].

^1H NMR has been also used to study different conditions as sepsis. Stringer et al. used ^1H NMR to compare the metabolic profile of whole blood from serum. This study revealed that the use of ^1H NMR in whole blood allowed obtaining more metabolic details than serum, and, in some cases, the metabolites detected were in higher concentrations in whole blood than in serum. Furthermore, whole blood allowed the determination of metabolites associated with red blood cell metabolism and observed that alterations in their metabolism could be in relationship with sepsis due to the haemolysis they cause [29]. The same authors have carried out several experiments in the same way. In these experiments, they were able to observe that blood samples of sepsis-induced acute lung injury patients were measurable and distinguishable from healthy blood due to differences in metabolites using ^1H NMR [35]. Other studies have been carried out to evaluate sepsis by ^1H NMR, but in rats [30]. Metabolic profiles obtained from the ^1H NMR analysis reveal changes in the metabolites involved in energy metabolism, especially in the serum of septic rats. From these results, authors concluded that according to the metabolomic approach, ^1H NMR has the potential for the early prognostic evaluation of sepsis [30].

As discussed above, ^1H NMR has been applied to the diagnosis of hepatitis C virus infection [31]. This study performed in urine samples was able to identify infected patients and negative individuals with good sensitivity and specificity using a metabolomics model based on the spectra obtained from the urine samples analysis. In this study, although the differences observed in the spectra allowed comparison of both groups, the chemical structures showed in the spectra are still being analysed [31]. In a similar way, ^1H NMR has been also used to distinguish between HIV-1 positive/AIDS patients on antiretroviral treatment and HIV-1 negative individuals [32]. These experiments were carried out in serum samples and differences in the metabolite profiling showed the distinction between the two groups. The authors also suggested that ARV-associated side effects could be monitored using ^1H NMR [32].

Pneumococcal pneumonia is another condition that has been diagnosed using ^1H NMR [33]. The use of the technique applied to urine samples of patients diagnosed with pneumonia has enabled to distinguish pneumonia caused by *Streptococcus pneumoniae* from that caused by other microbes such as viruses or other bacteria. This distinction is observed due to the differences in the metabolomic profiles. So the use of ^1H NMR-metabolite profiling could result in a rapid, specific and sensitive tool for the diagnosis of pneumococcal pneumonia. In this study, it is also observed that the metabolic profile shown in the samples of patients with pneumonia caused by *S. pneumoniae* changed to a more normal metabolite profiling when specific treatment is administered, suggesting that the urinary profiles were specific to the infection [33].

3.4. Other types of analyses

The combination of NMR spectroscopy, with the use of isotopically substituted molecules as tracers is a well-established protocol in microbiology. These NMR analyses appear to be the most appropriate for such studies because of their analytical power (provided that the labelling of products can be easily monitored non-invasively), their non-destructive features, and the large number of compounds that can be analysed simultaneously [6, 36–40]. However, despite the great potential of this combination in clinical practice, these analyses are out of the aim of this chapter.

4. Conclusion

In conclusion, ^1H NMR is an emerging technique in the microbiological field that promises to be a useful tool for the diagnosis of a broad range of conditions, including rapid identification of microorganisms, antimicrobial susceptibility and infectious-related syndromes. It can be also employed for knowing the metabolic pathways used by microorganisms, allowing the performance strategies for fighting against the infection.

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