International Journal of Basic and Applied Medical Sciences ISSN: 2277-2103 (Online)
An Open Access, Online International Journal Available at http://www.cibtech.org/jms.htm
2014 Vol. 4(1) January-April, pp.127-133/Menon et al.
Research Article

COMPARATIVE EVALUATION OF ^{1}HNMR SPECTROSCOPY AND GAS LIQUID CHROMATOGRAPHY IN RAPID DETECTION OF NONSPORING ANAEROBES FROM CLINICAL SPECIMENS

***Sarala Menon[1], Renu Bharadwaj[1], AS Chowdhary[1], DV Kaundinya[1] and DA Palande [2]**
[1]*Department of Microbiology, Grant Medical College & Sir J.J. Hospital, Mumbai-400008*
[2]*Department of Neurosurgery, Grant Medical College & Sir J.J. Hospital, Mumbai-400008*
**Author for correspondence*

ABSTRACT
The nonsporing anaerobes are commonly implicated as significant pathogens in human infections especially brain abscesses. Conventional laboratory diagnosis of anaerobes is time-consuming. Rapid detection of anaerobes from the clinical specimens is the need of the hour. Direct Gas Liquid Chromatography (GLC) has been reported to be useful in rapid detection of anaerobes from the clinical specimens. The present study was carried out to evaluate a new technique, Proton Nuclear Magnetic Resonance (^{1}HNMR) for the direct detection of anaerobes from clinical specimens. A total of 34 pus specimens collected from brain abscesses were subjected to Conventional Microbiological Culture, Proton Nuclear Magnetic Resonance (^{1}HNMR) and Gas Liquid Chromatography (GLC). The results of these two rapid techniques were correlated with the culture. Out of 34 pus specimens, eight pus specimens showed presence of anaerobes by culture. NMR (100%) and GLC (75%) correlated well with the culture in the detection of anaerobes. Seven pus specimens which were negative for anaerobes by culture showed presence of anaerobes by this two rapid techniques.NMR seems to have the potential in direct detection of anaerobes. NMR correlated well with culture and the traditional GLC which is usually used for the rapid detection of anaerobes. An important advantage of NMR is that the pus specimens do not require pre-treatment as that of GLC. Both rapid techniques could detect presence of anaerobes where culture failed.

Keywords**:** *Nonsporing Anaerobes, Rapid Diagnosis, GLC, NMR, Brain Abscesses*

INTRODUCTION
Non-sporing anaerobes have been found to be associated with various human infections (Willis, 1977). Conventional methods to identify anaerobic bacteria have often relied on unique clinical findings, isolation of organism, and the laboratory identification by morphology and various biochemical tests. Diagnosing common pathogens usually includes Gram staining, simple tests (e.g., determination of oxidase and catalase activity) and phenotypic tests requiring commercial identification kits or automated systems taking several hours to days. Thus isolation and identification of anaerobes is a tedious and cumbersome exercise. Moreover the culture may remain sterile as the anaerobes die during transit. End product analysis by gas-liquid chromatography (GLC) was established to overcome the viability issue of anaerobes during transit (Sutter *et al.*, 1985). GLC is usually performed either from culture media following isolation of the organisms or from the pus specimens directly. The production of volatile and non-volatile fatty acids is the primary metabolic activity of anaerobes. Holdman and associates (1977) used GLC for the taxonomic classification of obligate anaerobes. Bacteria contain a number of macromolecules that could potentially contribute to various peaks giving rise to MR spectra (Costerton *et al.*, 1974). 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 (Delpassand *et al.*, 1995). The microenvironment of the organism depends on 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 (Sutter *et al.*, 1985). These are reflected as different peaks of NMR spectra. Hence the technique could be a major tool for identification of anaerobes. Keeping this in mind, in the present study, NMR and GLC techniques were evaluated for the rapid detection of anaerobes from pus specimens. An attempt was also made to study the utility of these two rapid techniques in culture negative abscesses.

MATERIALS & METHODS

A total of 34 pus specimens collected during neurosurgery were subjected to Conventional Microbiological culture, NMR and GLC. This is a part from the thesis titled Bacteriological spectrum of intracranial abscesses with special reference to anaerobes and evaluation of some rapid diagnostic techniques, submitted to University of Pune for the doctoral degree after obtaining necessary ethical clearance from the institutional ethical committee.

Conventional Microbiological culture

Pus was inoculated at the bedside immediately wherever possible, if this was not possible, pus was collected in plain sterile tube for aerobic culture and pus for anaerobic culture was collected in Robertson's cooked meat medium (RCM). Pus was inoculated on Blood agar and Mac'Conkey's agar and incubated aerobically at 37°C for 24-48 hours. Similarly, pus sample was also inoculated on chocolate agar at 37°C in 5-10% CO_2 for 24-48 hours. It was plated on Blood agar, Neomycin blood agar and Bacteroides bile Esculin agar for anaerobes. All the anaerobic culture plates were incubated for 48-72 hours in a gas-pack system with Fildes & McIntosh Indicator. All the isolates obtained were identified by conventional biochemical reactions (Willis, 1977; Baron *et al.*, 1994).

Nuclear Magnetic Resonance (NMR): One hundred µl of pus sample was loaded in 5 mm NMR tube and D_2O was added to make the approximate volume of 0.4 ml, and then subjected to NMR analysis using Mercury plus Varian 300 MHz (7.05 T) nuclear MR spectrometer. The spectral analysis was done according to the published literature (Delpassand *et al.*, 1995; Maheshwari *et al.*, 2002; Lai *et al.*, 2005). Referencing was done with the water peak (4.8 ppm).

Gas Liquid Chromatography: One milliliter of pus was acidified with 0.1 ml of 50 % aqueous H_2SO_4. This sample was used for detection of volatile and non-volatile fatty acids by the method described by Willis & Phillips (1988).The equipments used was Chromatograph – Packard 804 model using Column – 10 % free fatty acid phase (FFPA) – Pye Unican Ltd packed in a 6 feet, 3 mm iD glass column. For volatile fatty acids, 1 ml of Diethyl ether was added to acidified sample and then vortex mixed for 15 seconds. The mixture was briefly centrifuged to break the ether-medium emulsion. The upper ether layer was removed .Two µl of the ether extract was then injected into the column. The chromatogram was run approximately for 12-15 minutes. The peaks obtained were interpreted by comparing to control chromatogram. For non-volatile acids, 2 ml of methanol was added to pus. Then 0.5 ml of 50 % H_2SO_4 was also added. Then this mixture was placed in a water bath at 55°C for 30 minutes. One ml of water was then added. 0.5 ml chloroform was also added and vortex mixed for 15 seconds. Chloroform layer was removed. Two µl was then injected into the column. The chromatogram was run approximately for 12-15 minutes. The peaks obtained were interpreted by comparing to control chromatogram. Multiple volatile fatty acids in a pus specimen were taken as the evidence for presence of anaerobes in the pus specimen (Willis and Phillips, 1988). The results of NMR were then compared with culture and GLC.

RESULTS AND DISCUSSION

Conventional identification of anaerobes is time-consuming and often tedious, hence poses a great challenge to Microbiologists. If stringent measures are not taken during the transport of specimens from hospital to laboratory, anaerobes loose their viability making the isolation still difficult. Hence rapid identification of these anaerobes is highly desirable. Several rapid identification methods for anaerobes

are described in the literature either directly from specimen or from cultures (Kurtiza *et al.*, 1986; Harplod *et al.*, 1987; Stoakes *et al.*, 1991; Negy *et al.*, 2006; Menon *et al.*, 2007; Akesbzeci *et al.*, 2010; Lin *et al.*, 2010; Ricky, 2010). However; all these methods have their own limitations.

The production of volatile and non-volatile fatty acids is the primary metabolic activity of anaerobic bacteria. Analysis by GLC of volatile and non-volatile fatty acids was initially used by Holdman and associates for the taxonomic classification of obligate anaerobes and is commonly used in many clinical laboratories to supplement biochemical tests for anaerobic identification. A wide range of anaerobic bacteria, showed the qualitative and quantitative differences in the end products of metabolism, that are associated with different genera and species and since these characteristics are stable, they are of considerable taxonomic value. The use of direct GLC as a procedure for the rapid diagnosis of suspected anaerobic infection has been reported by several researchers (Gorbach *et al.*, 1976; Phillips *et al.*, 1976; Ladas *et al.*, 1979; Watt *et al.*, 1982; Gupta *et al.*, 1983; Hunter *et al.*, 1985; Gupta *et al.*, 1986). In contrast to current diagnostic methods in microbiological laboratories, 1^H NMR is an emerging tool, which has shown its potential in identification of microorganisms. MR spectra are due to characteristic signals given by the cell wall constituents or metabolites of the microorganism. Since this pattern varies with the organisms, the spectra obtained could contribute to the identification of the organism.

In the present study, this technique was evaluated for its usefulness in the rapid identification of anaerobes from the clinical specimens. A total of 34 brain abscess pus specimens were processed for anaerobes by conventional culture, GLC &1^H NMR. Table 1 shows the results of all the three methods employed for detection of anaerobes. Out of 34 pus specimens, eight showed the growth of anaerobes while 17 pus specimens yielded facultative anaerobes. Remaining nine specimens showed no growth on culture. The spectra of the pus specimens which yielded anaerobes on culture and MR Spectra of pure cultures of anaerobes including the control strain NCTC *B fragilis* were used to decide the criteria for anaerobes. Thus an algorithm was developed for identification of nonsporing anaerobes (Menon *et al.*, 2007). Thus the pus specimen showing the peaks at 0.9, 1.3, 1.9, 3.0 ppm along with or without succinate peak at 2.4ppm was taken as suggestive of presence of anaerobes. Similarly, the presence of multiple peaks of volatile fatty acids on GLC was taken for the presence of anaerobes (Willis A and Phillips KD, 1988). Table 1 clearly shows that the specimens which yielded anaerobes on culture showed the presence of anaerobe using 1^H NMR (100%). GLC was suggestive of anaerobes in 6(75%) specimens. In another four specimens which showed facultative anaerobes on culture both these rapid techniques suggested of presence of anaerobes. To evaluate the utility of GLC and 1^H NMR in culture negative abscesses, we processed pus from nine such abscesses by both the techniques. Out of nine culture negative abscesses, three showed presence of anaerobes using these rapid techniques. Culture negative abscesses are the ones, which create a therapeutic dilemma. Table 1 clearly shows that both these techniques could detect presence of anaerobes in additional seven cases where culture failed. The most probable reason culture negativity for anaerobes can be attributed to death of anaerobes during transport of specimens.

Gupta *et al.*, (1986) performed direct GLC analysis of 60 brain abscess cases. All the 13 specimens, which revealed anaerobes on culture, showed presence of multiple peaks on GLC. Thus, their finding revealed a good (100%) correlation of multiple volatile fatty acids with isolation of anaerobic bacteria. Ladas *et al.*, (1979) studied 18 pus specimens from various sources. They also found that the detection of multiple volatile fatty acids by direct. GLC of pus specimens was strong evidence for anaerobic infection; Gorbach *et al.*, (1976) also found good correlation between the recovery of anaerobic Gram negative bacilli & the presence of multiple volatile fatty acids in pus specimens. They analyzed 98 specimens of pus or serous fluid. Nineteen of twenty specimens showed the significant amount of these acids (>0.11 mol/ml). Hunter *et al.*, (1985) used GLC to find out the evidence of anaerobic infection in 83 patients with pulmonary disease. Clinically significant anaerobic pleuropulmonary infection were not found in their patients with bronchitis, bronchiectasis & cystic fibrosis & occurred in only some of their patients with empyema & lung abscess. GLC of pleural fluid (empyema) or sputum (lung abscess) was helpful in identifying anaerobic infection when multiple peaks were observed on GLC.

Delpassand *et al.,* (1995) used NMR for rapid identifi cation of facultative anaerobes such as *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Staphylococcus aureus* and *Enterococcus faecalis* while Bourne *et al.,* (2001) described MR spectra of *Enterococcus, Streptococcus and Staphylococcus*. Himmelreich *et al.,* (2003) also found this technique useful or rapid identification of *Candida species*. There are also some scanty reports describing the spectra of anaerobes using this technique (Garg *et al.,* 2003; Lai *et al.,* 2005; Menon *et al.,* 2007). However, there are hardly any studies which study the comparison of GLC and NMR for rapid detection of anaerobes. GLC and 1^{H} NMR are rapid as compared to conventional culture where results are available within an hour. GLC requires pre-treatment of specimens to extract volatile and non-volatile fatty acids, while there is no such treatment required for NMR. The only limitation for these rapid techniques is the basic cost of the additional machinery required.

Table 1: Showing the results of culture, ^{1}HNMR & GLC

Culture Results	NMR suggestive of anaerobe*	GLC suggestive of anaerobe**
Anaerobes (8)	8 (100%)	6(75%)
Facultative anaerobes (17)	4 (23.5%)	4 (23.5%)
No Growth (9)	3 (33.33%)	3 (33.33%)

Note: ***** Peaks at 0.9, 1.3, 1.9, 3.0 ppm along with or without succinate peak at 2.4ppm was taken suggestive of presence of anaerobes ****** The presence of multiple peaks of volatile fatty acids on GLC was taken for the presence of anaerobes.

Table 2 showing the detailed MR spectra and GLC analysis of the specimens which were suggestive of presence of anaerobes

Culture Results	**NMR results**	**GLC results**
B fragilis	0.9**m**, 1.3**d**, 1.4, 1.6, 1.92, 2.4, 3.0 ,3.0-3.5**(m)** 3.5	A, PA, B, IV, V, MM, S, P
B fragilis	0.9**m**, 1.3**d**, 1.4, 1.6, 1.92, 2.4, 3.0 ,3.0-3.5**(m)** 3.5	A, PA, IB, B, IV, IC, L, O, MM, M, S, F
B fragilis	0.9**m**, 1.3**d**, 1.4, 1.6, 1.92, 2.4, 3.0 ,3.0-3.5**(m)** 3.5	A, PA, B, IV
P melaninogenica	0.9**m**, 1.04, 1.3**d**, 1.4, 1.92, 2.4, 2.8, 3.0	A, PA, IB, B, IV, L, O, MM, S, F
P denticola	0.9(m), 1.3(d), 1.4, 1.6, 1.92, 2.4, 3.0 , 3.5	**NO PEAKS**
F necrophorum	0.9**m**, 1.3**d**, 1.4, 1.6, 1.92, 3.0 , 3.5	**PYR**
P niger	0.9**m,** 1.3**d**, 1.4, 1.6, 1.92, 2.1,2.4, 3.0 ,3.5	A,B, MM, S
Peptostreptococcus	0.9**m**, 1.3**d**, 1.4, 1.6, 1.92, 2.4, 3.0 ,3.1	A, S
Facultative anaerobe	0.9**m**, 1.3**d**, 1.4, 1.6, 1.92, 2.4, 3.0 ,3.0-3.5**(m)** 3.5	A,B, B, IV, V, IC,M
Facultative anaerobe	.9**m**, 1.3**d**, 1.4, 1.6, 1.92, 2.4, 3.0 ,3.0-3.5**(m)** 3.5	B, IV, S
Facultative anaerobe	0.9**m**, 1.3**d**, 1.4, 1.6, 1.92, 2.4, 3.0 ,3.0-3.5**(m)** 3.5	A, PA, B, IV, V, IC
Facultative anaerobe	0.9**m**, 1.3**d**, 1.4, 1.6, 1.92, 2.4, 3.0 ,3.0-3.5**(m)** 3.5**,**	B, IV
No growth	0.9**m**, 1.3**d**, 1.4, 1.6, 1.92, 2.4, 3.0 ,3.0-3.5**(m)** 3.5	A,P, A, O, F
No growth	.9**m**, 1.3**d**, 1.4, 1.6, 1.92, 2.4, 3.0 ,3.0-3.5**(m)** 3.5	A, B, L
No growth	0.9**m**, 1.3**d**, 1.4, 1.6, 1.92, 2.4, 3.0 ,3.0-3.5**(m)** 3.5	A, PA, B, IV, L

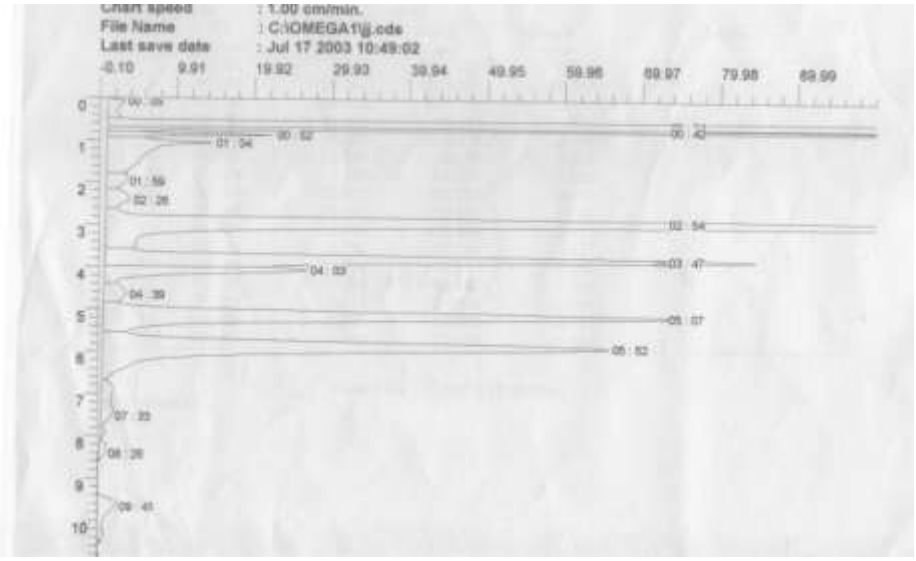

Figure 1: GLC of pus specimen which yielded *B fragilis* on culture
Note the presence of multiple peaks of volatile fatty acids

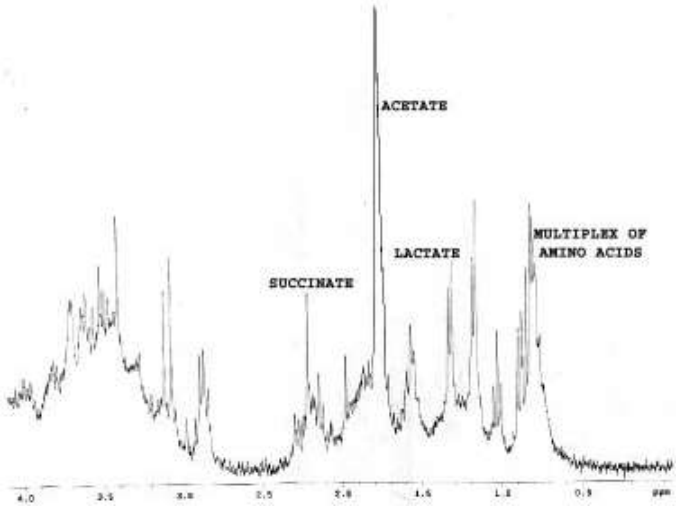

Figure 2: ^{1}HNMR Spectra of pus specimen which yielded *B fragilis* on culture
Note the presence multiplex of amino acids at 0.9 ppm, duplex of lactate at 1.3ppm, acetate at 1.9,and succinate at 2.4ppm

ACKNOWLEDGEMENTS
We appreciate the help of Dr. M. K. Lalitha, CMC,Vellore for providing the standard strain. Our sincere thanks to Mr. Moombasawallah, Ms.Nutan and Ms. Sonali, RSIC, IIT, Powai, Mumbai for obtaining the NMR spectra.

REFERENCES
Aksebzeci BH, Asyali MH, Kahraman Y, Ozgur Er, Kaya Esms, Ozblige H and Kara S(2010). Classification of root canal microorganisms using electronic-nose and discriminant analysis. *Biomedical Engineering Online* **9** 77. Available: http:/www.biomedical engineering.online.com/content/9/1/77.
Baron EJ, Peterson LR and Finegold SM (1994). *Bailey and Scott's Diagnostic Microbiology,* 9th Edition (The CV Mosby Company: St Louis) 474-550.

Bourne R, Himmelreich U, Sharma A, Mountford C and Sorrell T (2001). Identification of *Enterococcus, Streptococcus and Staphylococcus* by multivariate analysis of proton magnetic resonance spectroscopic data from plate cultures. *Journal of Clin*ical *Microbiology* **39** 2916-23.
Costerton JW, Ingram JM and Cheng KJ (1974). Structure and function of the cell envelope of gram-negative bacteria. *Bacteriolical Reviews* **38** 87-110.
Delpassand ES, Chari MV, Stager CE, Morrisett JD, Ford JJ and Romazi M (1995). Rapid identification of common human pathogens by high resolution proton magnetic resonance spectroscopy. *Journal of Clinical Microbiology* **33** 1258-62.
Garg M, Misra MK, Chawla S, Prasad KN, Roy R and Gupta RK (2003). Broad identification of bacterial type by 1HMR spectroscopy. *Europian Journal of Clinical Investigation* **33** 518-24.
Gorbach SL, Mayhew JW, Barttett JG, Thadepalli H, Onderdonk AB (1976). Rapid diagnosis of anaerobic infections by direct GLC of clinical specimens. *The Journal of Clinical Investigation* **57** 478-84
Gupta U and Murugesan K(1983). Direct GLC of clinical specimens for rapid diagnosis of anaerobic infections. *Indian Journal of Medical Research* **77** 19-23.
Gupta U, Murugesan K, Bhatia R, Mukundan K, Hazarika M, and Jotwani R(1986). GLC in rapid diagnosis of anaerobic brain abscess. *Indian Journal of Medical Research* **84** 502-507.
Harpold DJ and Wasilauskas BL(1987). Rapid identifi cation of obligately anaerobic gram-positive cocci using high-performance liquid chromatography. *Journal of Clinical Microbiology* **25** 996-1001.
Himmelreich U, Somorjai RL, Dolenko B, Lee OC, Daniel HM and Murray R *et al.*, (2003). Rapid identification of *Candida species* by using nuclear magnetic resonance spectroscopy and a statistical classifi cation strategy. *Applied and Environmental Microbiology* **69** 4566-74.
Holdman LV, Kato EP and Moore WEC (1977). Anaerobe Laboratory Manual. *Blacksburg; Virginia Polytechnic Institute, Anaerobe Laboratory*, 4th Edition.
Hunter JV, Chadwick M, Hutchinson G and Hodson ME (1985). Use of GLC in the clinical diagnosis of anaerobic pleuropulmonary infection. *British Journal of Diseases Chest* **79** 1-8.
Kurtiza AP, Getty CE, Shaughnessy P, Hesse R and Salyers AA (1986). DNA probes for identification of clinically significant Bacteroides spp. *Journal of Clinical Microbiol*ogy **23** 343-9.
Ladas S, Arapakis G, Ladas HM, Palikaris G and Arseni A (1979). Rapid diagnosis of anaerobic infections by GLC. *Journal of Clinical Pathology* **32** 1163 – 1167.
Lai PH, Li KT, Hsu SS, Hsiao CC, Yip CW and Ding S *et al.*, (2005). Pyogenic brain abscess: findings from in vivo 1.5-T and 11.7-T in vitro proton MR spectroscopy. *American Journal of Neuroradiology* **26** 279-88.
Lin YT, Vaneechoutte M, Huang AH, Teng LJ, Chen HM, Su SL and Chang TC (2010). Identification of clinically important anaerobic bacteria by an oligonucleotide Array. *Journal of Clinical Microbiology* **48** 1283-90.
Maheshwari S and Mukherji S (2002). Proton MR spectroscopy: Clinical applications. *Journal of Imaging Technology Management* 1-13. Available: http://www.imagingeconomics.com/issues/articles/2002- 08_04.asp. [Last accessed on 2007 Jun 10].
Menon S, Bharadwaj R, Chowdhary A, Kaundinya D and Palande D(2007). Rapid identification of non-sporing anaerobes using nuclear magnetic resonance spectroscopy and an identification strategy *Indian Journal of Medical Microbiology* **25**(4) 330-5.
Negy E, Urbán1 E, Sóki J, Terhes G and Nagy K (2006). The place of molecular genetic methods in the diagnosis of human pathogenic anaerobic bacteria. *Acta Microbiologica ET Immunologica Hungarica* **53** 183-194.
Ng HoNg Ho Yin Ricky (2010). Identification of anaerobic, non-sporulating, Gram-positive bacilli from blood cultures by 16S rRNA gene sequencing. This thesis is submitted to the Department of Microbiology of The University of Hong Kong In partial fulfillment of the requirements for The Degree of Master of Medical Sciences.

Phillips KD, Tearle PV and Willis AT (1976). Rapid diagnosis of anaerobic infections by GLC of clinical material. *Journal of Clinical Pathology* **29** 428 – 432.

Stoakes L, Kelly T, Schieven B, Harley D, Ramos M and Lannigan R *et al.*, (1991). Gas liquid chromatographic analysis of cellular fatty acids for identifi cation of gram-negative anaerobic bacilli. *Journal of Clinical Microbioogy* **29** 2636-8.

Sutter VL, Citron DM, Edelstein MA and Finegold SM (1985). *Wadsworth Anaerobic Bacteriology Manual*, 4th edition (Star Publishing Company: Belmont, CA) 71-78.

Watt B, Geddes P, Greenan O, Napier S and Mitchell A (1982). Can direct GLC of clinical samples detect specific organisms? *Journal of Clinical Pathology* **35** 706 –708.

Watt B, Geddes P, Greenan, Napier and Mitchell (1982). GLC in the diagnosis of anaerobic infections: a three year experience. *Journal of Clinical Pathology* **35** 709-714.

Willis AT (1977). *Anaerobic bacteriology: Clinical and Laboratory Practice*, 3rd edition (Buttersworths: London-Boston) 173-204, 232

Wills A & Phillips KD (**1988).** Gas liquid chromatography. In: *Anaerobic Infections Clinical & Laboratory Practice* (Public Health Laboratory Service) 128-139.