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Content

PREFACE

The present rapidly changing healthcare environment demands every individual and organization to make an impact. This "Proceedings" takes pride in presenting such exemplary efforts by individuals and organizations all over the world.

Nobel Laureate Prof. Dr. Harald zur Hausen, was present in this conference. He was awarded the Nobel Prize in medicine for his work on cervical cancer activated by virus infections in the year 2008. His research made it possible to develop a vaccine against the third most frequent kind of cancer affecting women. According to him increased risks for colorectal cancers have been associated with prolong consumption of cooked and processed red meat. Therefore this identification could have important proposition for prevention, risk assessment and therapy of one of the most frequent human cancers. In this manner the articles in this book deals with diverse health care issues like the assessment of Automated Devices applied in measurement of Blood Pressure, Review on Tuberculosis Cases on Central Nervous System, Oral Toxicity and Bioactive Compounds Identification. The book contains paper depicting perspective on Learning through Smartphone. There is an article on DNA extraction methods from cerebrospinal fluid for the detection of *Streptococcus pneumoniae*, Hexa-plex PCR Optimization method, Development and Optimization of Multiplex PCR and also Reiki Therapeutic Nursing Program. The book also depicts the effect of Apitherapy application in Cervical Cancer Patient. The eminent authors are from Universiti Sains Malaysia, MAHSA University, Pantai Hospital Cheras, University of Malaya, INTI International University, Malaysia, University of Basrah, Iraq, and Prince of Songkla University, Thailand. As a result the book assimilated innovative papers and novel works by leading researchers having direct clinical relevance to the latest scientific advances.

Thus the book focuses on the science of lifestyle changes so as to provide a forum for exchange among the many disciplines involved in health promotion and formed an interface between researchers and practitioners. It provides state of art information to make the programs more effective and to make genuine and consistent contributions to the scientific community. Scientists and scholars can keep themselves updated regarding the latest developments in their respective fields, and get the required exposure for their own work through this book. All the conference delegates can thus access the papers from all over the world both during and post conference. It may help to shape perspectives for a broader interest for non-specialists and may enhance personal insight in the respective fields.

We are thankful to the authors for submitting their manuscript and helping us in the compilation of the book. We are furthermore, thankful to Nobel Laureate Prof. Dr. Harald zur Hausen for his contributions and Dato' Tan Yoke Hwa, Director of Allied Health Sciences and Ministry of Health, Malaysia for the support. We are also thankful to Datuk Dr Hjh. Bibi Florina Abdullah, Chief Advisor and Pro Chancellor, Dr Amiya Bhaumik, CEO and Vice Chancellor, Datuk Dr Abdul Gani Bin Mohamed Din, Deputy Vice Chancellor (Academic), Mr. Aditya Bhaumik, Deputy Vice Chancellor (Administration), Dato (AMB) Mohd Yusoff A. Bakar Deputy Vice Chancellor (Research & Internationalization)Lincoln University College for actively supporting us and giving their suggestions on each every endeavor. We wish to extend our thanks to Ministry of Home Affairs, Malaysia to kindly give necessary permission to publish the book.

MESSAGE FROM PRO CHANCELLOR

DATUK DR HAJJAH BIBI FLORINA ABDULLAH

First of all I would like to congratulate ourselves because of our never ending search for excellence in terms of skilled knowledge and also in the area of research and development. We like to encourage the people to pursue research, promote critical and analytical mind to solve problems in the field of research and innovation. So this book stresses upon the issues so as to learn from the practice. We can share whatever we have practiced or do some research on any areas for the betterment of mankind. I hope that we will move forward collectively to ensure that every country is doing more to fulfill its obligation to provide high quality Physicians, Health care workers and working with clinical colleagues in the right place at the right time with the right skills. We can continue to provide the best Health care practice and also offer the opportunity to do work on global crisis in Healthcare resources and to support the under developing countries.

We have a lot of Healthcare Programs in Lincoln University College and we try to get the right people in this institution so that they can deliver the curriculum according to the needs and requirements of the country and also globally. I still remember that when I was the secretary to the Midwifery Board of Malaysia and I was attending the conference in Sydney, Australia, it was mentioned in the WHO that Malaysia has the best Mental and Child Healthcare in the world. We have the benchmark curriculum in the global arena and broad range of Health and Medical Research Platform. Our faculty of Pharmacy does research on ongoing issues and on ongoing diseases.

This book will help in the improvement of the research area. Basically we are focusing on the diseases, affecting large proportion of population particularly Cancer, the Cardio-vascular diseases, Diabetes, Infectious diseases, Mental illness, Obesity, Arthritis, and Asthma. These are all healthy lifestyle diseases and we have skill in Medical and Health Sciences areas to research on such diseases. Subsequently, we try developing Medical Aids to assist in the treatment and prevention along with cure.

As for example in the curriculum of Pharmacology, we will not only to put the area of clinical elements but also terms of drugs delivery and industrial issues. So that it can meet the needs of the every level of Healthcare. Malaysia has the potential to develop new treatments for sport injuries and new treatments for potential pandemic or endemic breaks. For sports injuries and related fields we have program like Physiotherapy to look into all these issues.

We have to be very creative and innovative, and have the responsibility to make leadership approach in research. So, we, Lincoln University College, like to complement the Government effort in that area. This conference is not only highlighting the modern way of Healthcare Practice and Healthcare Research Development, but also the important issues of present age. Here we share our deep insights on how it can be done in present and in which direction for the positive change in the Healthcare System around the world. It offers the opportunity to present the best scientific works of our Nobel laureates and share their experience in Fundamental Medicine, Dentistry, Pharmacy, Nursing, Physiotherapy and Public Health with the students of Malaysia and other countries. We are very confident that this book will help the Health Sciences professionals, researchers and students for more development in their respective fields.

MESSAGE FROM VICE CHANCELLOR

DR AMIYA BHAUMIK

"Malaysia-Truly Asia" is the place where we can explore multi-ethnic culture. It is our duty as a researcher to find out new avenues that we can include to our country. Many people think that the scientists or the researcher are different from businessman. But I denied the statement and I would like to say that the researchers, the innovators, the scientists are the most important businessman or business woman because to do anything new, anything away from regular duties and responsibilities we need entrepreneur spirit by which we can take the challenges and believe that we can do it. The researchers, scientists, innovators have that kind of motivation and spirit with that they can go against all odds and even also against closed family members who often would say 'Oh! What you are doing? This is nonsense. You would not get anything.' But they believe themselves and think that 'Yes, it is there. I will find it.' So the nobelauriate, the researcher, the scientist, all are fantastic entrepreneur who without such zeal would not be able to write papers, to speak in front of people, to invent something new.

We welcome you to expose, to explore your entrepreneur zeal, motivation and inspiration to search for more new innovation, knowledge, idea and so forth. Lincoln University College is a private higher educational institution and started with humble beginning in 2002 with only 3 students. Today we have more than 8000 students worldwide. We started our journey with Computer Science and today we have Medicine, Dentistry, Pharmacy, Nursing, Physiotherapy, Engineering, Business Hospitality departments. We can say you will have everything under one roof and be able to achieve further with the help of our dedicated Deans, Professors, Lecturers and staffs. As a result, in this endeavor we will try to organize this conference almost every year. This book is just not for reading articles, but to acquire knowledge which will help you in the long run.

Our country inspires the local institutions to go for various research and innovation and we arrange collaborative research work with other institution to expand and make deeper our knowledge.

The book is the outcome of our International Conference on Recent Advances in Health Sciences on, held on August, 2014. It helps to spread the message among the healthcare professionals, scientists, researchers and students to pursue work on this basis and to get an idea about modern trends and fields of studies. I hope that the readers will achieve the objectives of this book.

ASSESMENT OF ROSSMAX AUTOMATED UPPER ARM AND WRIST DEVICES FOR MEASUREMENT OF ARTERIAL BLOOD PRESSURE

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Abstract:

Background: The best indicator of arterial blood pressure is a reading of central arterial pressure, but this is not practical, so traditionally blood pressure is measured by using mercury manometer with stethoscope from the brachial artery as the value of measurement is not different significantly from that of the central aortic pressure. But by using this method only single measurement can be performed by a physician during the patients visit. Then the recent advancement have lead to the development of other non-invasive self-monitoring of BP by patients at home.

Aim of the study: To evaluate the automatic Rossmax upper arm and wrist devices in measurement of arterial BP in comparison with the arterial pressure method.

Subjects and methods: It is a cross sectional study conducted on 47 patients , the blood pressure was measured simultaneously intra arterially and by using the Rossmax upper arm and wrist devices. Then to evaluate these two automated devices, two process were used:

a. Systematic error: To evaluate the accuracy of these 2 devices, a comparison was made between the values of BP measured by invasive method against the values measured by these 2 devices using unpaired *t*-test.

b. Random error: To evaluate the reproducibility of these 2 devices, a duplicate estimation of arterial BP were conducted in other 41 individuals using these 2 devices and then a paired difference between each 2 estimates have been made.

Results:

In systemic error the values of systolic, diastolic and mean arterial B.P. measured by these devices, were higher than those measured by the central method and statistical anal-

ysis using unpaired *t*-test showed significant difference in these parameters by using wrist device ($p<0.05$,<0.001 and <0.001 respectively).While by using the brachial device, there is significant difference regarding diastolic ($P<0.001$) and mean arterial blood pressure ($P<0.005$) and no significant difference regarding systolic blood pressure ($P>0.05$).

In random error, a duplicate estimation of the systolic, diastolic and mean arterial blood pressure by these 2 devices were made and a paired t-test was done. The result showed no significant differences between the 2 readings in all these parameters by using the wrist device ($P>0.05$). By using brachial device there is no significant difference regarding systolic blood pressure ($P>0.05$) while there is such a difference between the readings in diastolic and mean arterial blood pressure ($P<0.05$).

In conclusion these devices are not reliable for diagnosis and follow up of hypertensive patients because they are inaccurate. Even if they are accurate for certain values, their pressure sensors should be calibrated periodically to maintain accuracy.

Keyword : Automated upper arm, wrist devices, arterial BP

Introduction

It is very important to have accurate measurement of arterial blood pressure for screening, management of hypertension, assessing a person's suitability for a sport or certain occupation to avoid both observer and methodological errors which can occur by blood pressure measurements. Furthermore, it is a good indicator for cardiovascular diseases. [20, 27]

The best indicator of arterial blood pressure is a reading of central aortic pressure (direct or invasive method) [28, 29, 3]. But this method is not practical due to its invasiveness and inability to be applied to large groups of a symptomatic individuals for hypertension screening.

Traditionally blood pressure is measured by using mercury manometer with stethoscope from the brachial artery, as the value of measurement is not different significantly from that of central aortic pressure,[1] provided that the doctor minimizes patients related factors such as anxiety which is the cause of white coat hypertension.[30, 10, 26]

But by using manual sphygmomanometer, only single measurement can be performed by a physician during the patient's visit.

Then recent advancement have led to the development of other non- invasive detection methods for self-monitoring of blood pressure by patients at home, which are very important in own health care and lead to significant reduction in blood pressure.[8]

In addition, mercury seems distend to disappear from the workplace because of its hazardous effect on the environment [14].

The new methods for measurement include ambulatory blood pressure monitoring (ABPM) [23].

Semi-automated B.P monitors (inflated manually) and automated upper arm devices which replaced the auscultation of Korotkoff sounds by the digital processing of pressure oscillation transmitted from the arteries to the cuff [12,32].

Another automated device is the wrist device which record the pulse wave originated from stretching of the wall of aorta when the heart beats [15].

Aim

To evaluate the automatic Rossmax upper arm and wrist devices in measurement of arterial B.P. No studies have been done to evaluate the accuracy of these devices which are widely used in our country.

Subjects and methods

It is across sectional study in which we tested the Rossmax upper arm and wrist devices (produced by Rossmax international Ltd.Rossmax Swiss Gmbh / Switzerland, model AJ701) in measurement of arterial B.P in 47 patients (29 males and 18 females with mean age of 54.27±9.29years and 56±6.32 years respectively) undergoing cardiac catheterization (in whom arterial blood pressure and blood sugar were controlled before catheterization for patients who were hypertensive or diabetics) in Al-sader teaching hospital, every Sunday through the period from November 2012-June 2013.

Blood pressure was measured simultaneously intra arterially (in which a wide stiff and short catheter were used with the avoidance of catheter movement and bubble formation) [11] and by using the Rossmax upper arm and wrist devices (in which a suitable cuff size was used with correct site for wrapping the arm) [27,4,9,33], other measurements were also taken into account to avoid wrong readings as body and arm positions [27,25,14,21,22], avoid talking or excessive movement of the patient [16,17,18,34] with insurance of a one minute gap between repeated inflation of the cuff to avoid venous congestion [34]. Then to evaluate these two automated devices, two processes were used [1].

a. Systemic error: To evaluate the accuracy of these 2 devices, a comparison was made between the values of blood pressure measured by invasive method with those measured by these two devices. Systematic error may be very importance in clinical situations involving monitoring of acutely ill patients or in taking decision about the treatment for patients with hypertension.

b. Random error : A duplicate estimates of blood pressures by using the two automated devices were made for other 41 patients (12 males and 29 females) with mean age group of 50.58±12.27 and 46.86±12.05 respectively (22 of them were diabetics and hypertensive but they were under treatment and controlled). Random error may matter more in epidemiological studies, then the paired differences of each two estimates were determined in the order to evaluate the reproducibility of these devices.

Results

The results of this study concerning the evaluation of a two automated non-invasive blood pressure devices in comparison with the central method of measurement of arterial blood pressure was as following:

a. Systemic error

Comparison have been made regarding values of systolic, diastolic and mean arterial blood pressure measured by the Rossmax upper arm and wrist devices with those measured by the invasive method of the aortia in 47 subjects undergoing cardiac catheterization.

The values were expressed as mean±S.D, in which those that were measured by the wrist device were higher than those measured by the invasive method & statistical analysis using unpaired *t*-test showed significant differences among their mean values ($P<0.05$,$P<0.001$and $P<0.001$ regarding systolic, diastolic and mean arterial blood pressure respectively) as shown in table (1), while the values which were measured by the brachial device were also higher than those measured by central method but statistical analysis using unpaired *t*-test showed no significant difference regarding systolic blood pressure($P>0.05$) and significant differences regarding diastolic and mean arterial blood pressures($P<0.01$and <0.005 respectively) as shown in table (2).

b. Random error

The reproducibility of the two automated devices was measured by a duplicate estimation of arterial blood pressure by these two devices for other 41 individuals and then a paired difference between each two estimates have been made. Itshowed no significant differences between the 2 measured values of systolic, diastolic and mean arterial blood pressures regarding the wrist device($P>0.05$), as shown in table (3), while in case of the brachial device, the results showed no significant difference between the 2 measured values of systolic BP ($P>0.05$), but significant differences between the 2 measured values of diastolic and mean arterial BP ($P<0.05$) as shown in table (4).

Table (1) *Comparison of values of arterial blood pressure (mean±S.D.) measured by central method and automated wrist device.*

Parameters	BP by invasive method Mean±S.D.	BP by wrist device Mean±S.D.	Mean of unpaired differences±S.D.	*P* value
Systolic BP(mmHg)	143.68±27.28	154.85±16.51	11.17±4.66	0.018
Diastolic BP(mmHg)	78.66±12.91	96.79±13.94	18.13±2.77	0.000
Mean arterial BP(mmHg)	100.33±15.26	116.14±14.23	15.81±3.04	0.000

Table (2) *Comparison of values of blood pressure (mean±S.D.) measured by central method and automated brachial device.*

Parameters	BP by invasive method Mean±S.D	BP by brachial device Mean±S.D	Mean of unpaired differences±S.D.	*P* value
Systolic BP(mmHg)	143.68±27.28	149.68±24.88	6.00±5.38	0.268
Diastolic BP(mmHg)	78.66±12.91	85.45±10.56	6.79±2.43	0.006
Mean arterial BP(mmHg)	100.33±15.26	106.86±13.32	6.53±2.95	0.030

Table (3) *Comparison of values of BP measured by automated wrist device (random error)*

Parameters	1st reading of BP	2nd reading of BP	Mean of unpaired differences±S.D.	*P* value
Systolic BP(mmHg)	123.73±18.99	125.27±18.15	-1.54±11.98	0.42
Diastolic BP(mmHg)	77.07±15.55	77.24±11.26	-0.17±13.79	0.94
Mean arterial BP(mmHg)	92.63±15.83	93.25±13.46	-0.63±12.86	0.76

Table (4) *Comparison of values of BP measured by brachial device (Random error)*

Parameters	1st reading of BP	2nd reading of BP	Mean of unpaired differences±S.D.	*P* value
Systolic BP(mmHg)	126.31±24.65	124.93±25.40	1.39±9.4	0.349
Diastolic BP(mmHg)	78.37±14.73	76.10±13.88	2.27±6.56	0.03
Mean arterial BP(mmHg)	94.35±16.93	92.36±16.36	1.99±5.09	0.016

Discussion

In this study and regarding systemic error, the results of systolic, diastolic and mean arterial blood pressures measured by the automated Rossmax wrist and brachial devices were significantly higher than those measured by the central method, except the systolic BP measured by the automated brachial device which is not significantly higher than that measured by the central method. This indicates either intrinsic inaccuracy of these instru-

ments, or because they produce inaccurate readings in patients with heart and circulation problems like arterial sclerosis, arrhythmias, preeclampsia, hypotension, hypertension, pulse alternaus and pulses paradoxus [5,7,35,27], or the readings of these devices depend on the standard on which a lgorithms was based [31,13,19].

Systemic error may be of great importance in clinical situations involving monitoring of acutely ill patient or treatment decision for hypertensive patients [6].

While regarding random error, the results showed no significant differences between the values of each duplicate measurement of systolic, diastolic and mean arterial BP in case of the wrist instrument, which indicates reproducibility of this instrument. But with the brachial device, there also no significant difference between the values of duplicate measurement of systolic BP but significant differences between the values of duplicate measurement of diastolic BP and between the duplicate measurement of mean arterial BP, which indicates that this instrument is neither reproducible nor accurate and this may matter more in epidemiological studies [6].

In conclusion these devices are not reliable for diagnosis and follow up of hypertensive patients because they are inaccurate and even if they are accurate for certain values, their pressure sensors should be calibrated periodically to maintain accuracy.

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A REVIEW ON CENTRAL NERVOUS SYSTEM TUBERCULOSIS CASES IN TERTIARY CARE HOSPITAL, KELANTAN

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Abstract:

Central nervous system tuberculosis (CNS TB) is the most serious form of tuberculosis, which accounts approximately 1% of all tuberculosis cases. Due to its high mortality rate despite of given anti-tuberculosis treatment, CNS TB remains a great public health concern worldwide. The objective of the review was to study the socio-demographic background, clinical and laboratory data of CNS TB patients in Hospital Universiti Sains Malaysia (HUSM). A cross-sectional study of registered CNS TB patients (n = 46) from January 2009 to February 2014 were reviewed from Chest Clinic, HUSM. From the analysis, it was found that 95.7% of CNS TB patients in HUSM were Malay. Majority of the patients were male which is 32 (69.5%). The mean age was 47.76 years (3 to 85 years). Four cases (8.7%) had microbiological confirmation for CNS TB by culture, acid fast bacilli smear and/or polymerase chain reaction (PCR) while others were diagnosed solely based on clinical evidences. 8.7% of patients had concurrent pulmonary TB, 8.7% had TB with diabetes mellitus and none of patients had TB with Human Immunodeficiency Virus (HIV). 89.1% of the patients had Meningitis while 10.9% had Tuberculoma. This review also revealed that most of patients were dead 56.5% while 26.1% completed the treatment and another 6.5% were still in treatment. For intensive treatment, most of patients were on 2SHRZ (58.7%) followed by 2EHRZ (26.1%) and 2HRZ (6.5%). We conclude that, CNS TB is associated with high mortality rate and commonly intensively treated with 2SHRZ. Meningitis was found to be the most common CNS TB manifestation in this review.

Introduction

Tuberculosis is a grave infectious disease caused by *Mycobacterium tuberculosis* (MTB) complex infection. It is predominantly attacks the lungs and has the ability to infect other organs and tissue known as Pulmonary Tuberculosis (PTB) and Extra-Pulmonary Tuberculosis (EPTB) respectively. Central nervous system tuberculosis (CNS TB) is the most serious form of EPTB, which accounts for 5 to 15% of the EPTB cases and approximately 1% of all Tuberculosis cases.

The most common clinical manifestation of CNS TB is Tuberculosis Meningitis (TBM) followed by other clinical manifestation such as tuberculoma, cerebral military tuberculosis and Tuberculosis Encephalitis. It is reported that CNS Tuberculosis cause significant disability and fatality in adults and children despite of given anti-Tuberculosis treatment [4]. According to [11], the fatality rate of CNS Tuberculosis represented in different studies ranged from 20 to 50%.

Due to its high mortality rate despite of given anti-Tuberculosis treatment, CNS TB remains a great public health concern worldwide. The objective of the review was to study the socio-demographic background, clinical and laboratory data of CNS TB patients in Hospital Universiti Sains Malaysia (HUSM).

Methodology

Study Design

A cross-sectional study of registered CNS TB patients from January 2009 to February 2014 was reviewed from Chest Clinic, HUSM. Data were collected from their medical records.

Study population

All registered CNS TB patients (n = 46) at Chest Clinic, HUSM from January 2009 to February 2014 who are confirmed CNS TB or were being investigated for CNS TB were included in this study.

Data collection

Most of the data was collected from medical records. The data collected include demographic information (age, gender, race, BCG vaccination status, smoking and alcohol) and clinical data (symptoms, CNS TB with diabetes, CNS TB with PTB, clinical manifestations, type of cases, microbiological confirmation, outcome of treatment).

Definition

As for anti-TB drug regimen, treatment consist of a minimum of 6 month daily-regimen using Streptomycin or Ethambutol in combination with Isoniazid, Rifampicin and Pyrazinamide for 2 months (initial phase) followed by Isoniazid and Rifampicin for 4 months (continuation phase). Duration of treatment for TBM is prolonged period from 9 to 12 months depends on the clinical decisions.

Standard abbreviations are adopted: S=Streptomycin, H=Isoniazid, R=Rifampicin, Z=Pyrazinamide, and E=Ethambutol; and the numbers used, e.g., 2SHRZ refers to "2 months of daily SHRZ"; 4H3R3 refers "three times a week for 4 months".

Data Analysis

Data analysis was carried out using descriptive statistical procedures by Statistical Package for the Social Sciences (version 22) and Microsoft Excel.

Result

Table 1: *Demographic characteristic*

Characteristic	Patients, n (%)
Age	
1-14	4 (8.7)
15-54	23 (50.0)
>54	19 (41.3)
Median age: 49	
Mean age : 47.76 (3-85 years)	
Gender	
Male	32 (69.6)
Female	14 (30.4)
Race	
Malay	44 (95.7)
Others	2 (4.3)
BCG	
Vaccinated	30 (65.2)
Non vaccinated	2 (4.3)
Data not available	14 (30.4)
Smoking	
Smoker	10 (21.7)
Non smoker	21 (45.7)
Data not available	15 (32.6)
Alcoholic	
Alcoholic	2 (4.3)
Not alcoholic	29 (63.0)
Data not available	15 (32.6)

Table 2: *Clinical and treatment data*

Variables	Patients, n (%)
CNS TB with diabetes	
With diabetes	4 (8.7)
Without diabetes	28 (60.9)
Data not available	14 (30.4)
CNS TB with PTB	
With PTB	4 (8.7)
Without PTB	42 (91.3)
Clinical manifestation of CNS TB	
Meningitis	41 89.1
Tuberculoma	5 10.9
Type of cases	
New case	43 (93.5)
Recurrence case	3 (6.5)
Microbiological confirmation (culture/AFB/PCR)	
Yes	4 (8.7)
No	42 (91.3)
Treatment **Intensive phase**	
2SHRZ	27 (58.7)
2EHRZ	12 (26.1)
2HRZ	3 (6.5)
Data not available	4 (8.7)
Continuous phase	
4 HR	11 (23.9)
$4H_3R_3$	3 (6.5)
Data Not available	32 (69.6)

Outcome treatment	
Recovered	2 (4.3)
Completed treatment	12 (26.1)
Dead	26 (56.5)
In treatment	2 (4.3)
Stopped treatment	4 (8.7)

Discussion

Based on Table 1, the prevalence of CNS TB was found to be highest among Malay (95.7%) as it might be due to that Malay was the majority of the population in Kelantan state. In terms of age- distribution of the cases, two-third of patients were between 15 to 54 years age group. It is comparable with the previous studies that also reported majority of the cases were in 15-54 years age group [3,1]. This findings are consistent with WHO estimation that TB affects the most economically productive age group [10]. However, all age groups are at risk. Majority of the patients were found to be male (69.5%) compared to female (30.4%). According to [7], it is postulated that the difference might be due to higher mobility of the male group due to work requirements.

In Malaysia, National BCG Vaccination Program was initiated as a part of neonatal immunization (at birth) in order to prevent severe forms of TB since 1961. It is reported that the efficacy of BCG vaccination among newborns towards TBM ranges from 52% to 84% depends on geographical settings [6]. However, there is considerable ambiguity with regard to the efficacy and extent of protection offered by the vaccination towards developing the disease in adult. Based on this review, approximately two-third of the patients that were given BCG vaccination developed the disease. It is suggest that protective response of BCG vaccination in adults varied depends on several factors such as exposure to environmental mycobacteria, usage of the vaccine and host-related factors [9].

As shown in Table 2, small percentages (8.7%) of CNS TB patients had concurrent pulmonary TB. None of patients had reported to have TB with Human Immuno Deficiency virus (HIV) while 8.7% had CNS TB with diabetes. Majority of CNS TB patients in this review developed TBM (89.1%) while another 10.9% had Tuberculoma. It is consistent with previous reports as CNS TB manifest itself primarily as TBM [5]. Most of the cases in this study were new cases, 93.5% while only 6.5% were recurrence cases. Among all 46 cases, only four cases (8.7%) had microbiological confirmation for CNS TB by Cerebro Spinal Fluid (CSF) culture, acid fast bacilli smear and/or Polymerase Chain Reaction (PCR) while others were diagnosed as CNS TB solely based on clinical suspicion and evidences. The scenario is due to the diagnosis of CNS TB is rather difficult as sample collection is not

easily done compared to PTB cases. Furthermore, those samples have a low bacterial load and only a few proportions of cases have positive microscopy and culture for *M. tuberculosis* [2].

For intensive phase of treatment, majority of patients were on 2SHRZ (58.7%) followed by 2EHRZ (26.1%) and 2HRZ (6.5%). This is in compliance with [8], recommendation as Streptomycin should be used instead of Ethambutol in treating adult TB Meningitis. Meanwhile for continuous phase of treatment, initially 23.9% of patients were on 4HR while another 6.5% were on 4H3R3. Prolonged treatment then was noted for these cases as TBM were treated for 9 to 12 months. This review also revealed that most of patients were dead (56.5%) before completion of treatment while 8.7% stopped the treatment and 4.3% were still under treatment.

As most of our data were collected retrospectively, our main concern is incompletion of the data. Data might be missing either by misplacing the records or they were not recorded. Besides, our study is a hospital- based study. Hence, the findings could not be generalized to the community. In spite of that, it gives us valuable information on socio-demographic, clinical and laboratory data of CNS TB patients in HUSM. An extensive data collection nationwide should be done in order to determine the actual pattern of CNS TB in Malaysia.

Conclusion

The review found that the majority of CNS TB patients were Malay, male and vaccinated with BCG. Meningitis was found to be the most common CNS TB manifestation. Treatment wise, most patients were commonly intensively treated with SHRZ and have high mortality rate.

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BIOFILM INHIBITORY EFFECT, ORAL ACUTE TOXICITY AND BIOACTIVE COMPOUNDS IDENTIFICATION OF CRUDE CULTURE EXTRACT FROM A NOVEL BACTERIAL SPECIES OF *Paenibacillus*

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Abstract

The effectiveness of many antimicrobial agents is currently decreasing therefore it is important to search for alternative therapeutics. Our study was carried out to assess the *in vitro* antibiofilm activity using microtiter plate assay, to characterize the bioactive compounds using Ultra Performance Liquid Chromatography-Diode Array Detection and Liquid Chromatography-Mass Spectrometry and to test the oral acute toxicity on Sprague Dawley rats of extract derived from a novel bacterial species of *Paenibacillus* strain 139SI. Our results indicated that the crude extract and its three identified compounds exhibit strong antibiofilm activity against a broad range of clinically important pathogens. Three potential compounds were identified including an amino acid antibiotic $C_8H_{20}N_3O_4P$ (MW 253.237), phospholipase A2 inhibitor $C_{21}H_{36}O_5$ (MW 368.512) and an antibacterial agent $C_{14}H_{11}N_3O_2$ (MW 253.260). The acute toxicity test indicates that the mortality rate among all rats was low and that the biochemical parameters, hematological profile and histopathology examination of liver and kidneys showed no significant differences between experimental groups ($p > 0.05$). Overall, our findings suggested that the extract and its purified compounds derived from novel *Paenibacillus sp.* is non-toxic exhibiting strong antibiofilm activity against Gram-positive and Gram-negative pathogens that can be useful towards new therapeutic management of biofilm-associated infections.

Keywords: Biofilm Inhibitory Effect, Acute Toxicity, Bioactive Compounds, Culture Extract Paenibacillus.

Introduction

The effectiveness of many antimicrobial agents is currently decreasing due to the prevalence of multidrug-resistant pathogens [43]. The emerging of these pathogens remains a serious challenge to medicine and healthcare [16]. One of the mechanisms for such resistance is the formation of biofilms which are layers of microbial cells attached to a surface and embedded in a matrix of exopolysaccharide [13]. Therefore, it is important to search for alternative therapeutics to control biofilm-associated infections. Although several plant-based compounds are receiving attention for their therapeutic properties [26], only few are reported to exhibit antibiofilm activity [15]. Natural ecosystems are rich sources of microbes that produce a wide range of compounds that exhibit diverse and versatile biological effects [6, 32]. Many marine and soil microorganisms were recently documented for their effective antibiofilm property against pathogens [33, 40, 3]. The genus *Paenibacillus* represent one of the important soil bacteria that comprises strains of medical, industrial and agricultural importance [39]. Interest in *Paenibacillus* species as a source of new antimicrobials has been increasing and the probability of finding novel antibiofilm compounds from these bacterial strains is promising [16]. It is worth mentioning that the administration of antimicrobial agents and biocide compounds in the local sites of some infection has been a useful approach to combat microbial biofilms [12]. However, prolonged persistence of these compounds in the environment could induce toxicity towards non-target organisms and resistance among microorganisms within biofilms [38]. Moreover, some of these compounds may exhibit toxic effects even at therapeutic doses which makes it necessary to test their toxicity in experimental animals [1]. This aspect has led to the development of more environment friendly compounds to combat with the issue. Acute toxicity is the toxicity produced by a compound when it is administered in one or more doses during a period of 24 hours [9]. These studies are usually necessary for any compound intended for human use and the information obtained from them is useful in identifying the organs of toxicity and choosing the safe doses [9]. The objective of acute studies can usually be achieved in rodents using small groups of experimental animals [9]. Therefore, our study was carried out to assess the *in vitro* antibiofilm activity, charecterization of the bioactive compounds and test the acute toxicity on Sprague Dawley rats of an extract derived from novel bacterial species of *Paenibacillus* strain 139SI.

Materials and Methods

Bacterial isolates

The clinical bacterial isolates were collected from patients undergoing tonsillectomy for chronic and recurrent tonsillitis at University Malaya Medical Centre (UMMC) upon approval by the medical ethics committee (PPUM/UPP/300/02/02Ref. No. 744.11). Reference bacterial strains used were *Staphylococcus aureus* (ATCC 25923), *Pseudomonas aeruginosa* (ATCC 27853) and *Escherichia coli* (ATCC 25922) [36]. A bacterial strain 139SI originally isolated from a local agricultural soil was

identified as *Paenibacillus* via 16S rRNA gene sequencing and deposited at the American Type Culture Collection (ATCC) with a cataloguing number (ATCC–BAA-2268) [37].

Experimental animals

A total of 36 adult male and female Sprague Dawley (SD) rats were obtained from the Animal Care Unit Center (ACUC) at the Faculty of Medicine, University of Malaya. The Animals weighed 150-200 gm and were kept in wire-bottomed cages at 25°C temperature, 50% humidity and a 12 hours light-dark cycle for at least 3 days before the experiment to allow their acclimatization to the conditions of experiments. Animals were maintained at standard housing conditions and free access to standard diet and water ad libitum during the experiment. The experimental protocol was approved by the animal ethics committee (PM/27/07/2010/MAA (R)) and all animals received humane care according to the guide for the care and use of laboratory animals [20] and the guide for the control of experiments on animals (CPCSEA) [11].

Preparation of Paenibacillus sp. crude culture extract

A single colony from the culture of *Paenibacillus* species strain 139SI was transferred into sterile brain heart infusion (BHI) broth (BD Difco™) followed by incubation at 37°C. We have prepared the growth curve of *Paenibacillus* 139SI supernatant (extract) in three different incubation periods after 24, 48 and 72 hours. However, only the 72 hours extract showed the highest activity compared to the 24 and 48 hours. This was due to the longer incubation period that allows the maximum secretion of bioactive metabolites by the *Paenibacillus* 139SI colonies into the culture media. Therefore, only the 72 hours incubation extract was used in our study. The *Paenibacillus* extract was then transferred aseptically into 50ml conical bottom centrifuge tube (Jet Bio Fil) followed by centrifugation at 8000 rpm in 4°C for 20 mins to separate the cell from the supernatant. The obtained supernatant was then subjected to sterile filtration to remove all unwanted particles using syringe filter with a pore size of 0.22 µm (Minisart® Sartorius) [31]. The obtained cell free supernatant was then freeze-dried and dissolved in ultra-pure water (MilliQ, Millipore) and stored at -20°C as a stock to be used for all experiments. For each 1mg freeze dried supernatant powder, the amount of ultra-pure water used to re-suspend the powder was 1ml.

In vitro antibiofilm activity

To assess the antibiofilm activity of *Paenibacillus* sp. strain 139SI extract and its purified compounds against clinically important pathogens, microtiter plate assay (MTP). The assay was carried out using 96 well-flat bottom polystyrene titer plates as described previously [10], [25]. Each well was filled with 100 µl sterile BHI broth and 50 µl overnight culture for each clinical pathogenic isolate followed by adding 150 µl *Paenibacillus* sp. crude extract and 150 µl of purified compounds separately with concentrations of 1000, 1500, 2000, 2500, 3000, 3500, 4000 and 4500 µg/ml before incubation at 37°C for 24 hours. After incubation, plates were gently washed three times with phosphate-buffered saline and

the planktonic cells were discarded while the weakly adherent cells were removed through two round of thorough washing with de-ionized water and allowed to air dry before being stained. The adherent biofilm were stained by 200 µl of 0.4% crystal violet solution (w/v) for 10 mins. The optical density (OD) of the biofilm was measured at 570 nm (OD570) with a microtiter absorbance reader (iMark™, Bio-Rad) [44]. To compensate for possible differences in growth rates under the different incubation conditions and/or for strains with different characteristics, the adherence index was adjusted as an estimate of the density of the biofilm which would be generated by a culture with an OD600 of 0.5 [4]. Calculation of the adherence index was done according to the following formula: Adherence index = mean density of biofilm (OD570) x 0.5/mean growth (OD600).

Biofilm inhibitory concentration

In order to determine the lowest concentration of strain 139SI extract that can cause visible inhibition in the biofilm formation, the biofilm inhibitory concentration (BIC) test was carried out using 6 well-flat bottom polystyrene titer plates as described previously with few modifications [22]. A piece of glass cover slip (1×1 cm) was placed inside each well to allow the growth of bacterial isolates on the surface and to visualize the inhibitory effect of 139SI extract on the biofilm formation. Each well was filled with 300 µl sterile BHI broth followed by inoculation with 150 µl of overnight culture for each clinical pathogenic isolate then addition of 150 µl *Paenibacillus* sp. extract and 150 µl of its purified compounds separately with concentrations ranging from 1000 to 4500 µg/ml before incubation at 37°C for 24 hours. After incubation, biofilm inhibition was determined spectrophotometrically using a microtiter absorbance reader (iMark™, Bio-Rad) and visualized microscopically using an upright light microscope (Eclipse LV150L, Nikon).

Characterization and identification of bioactive compounds

The *Paenibacillus* sp. cell-free supernatant was subjected to High Performance Liquid Chromatography (HPLC). Briefly, the extract solution was filtered using an SRP-4 membrane 0.45µm and injected into the HPLC column (Agilent Zorbax XDB-C18, 4.6 x 250 mm, 5.0 µm) at a 100µl injection volume with a flow rate of 1.2 ml/min. The standard solvent system was a combination of acetonitrile and water at a pH of 3.55. Furthermore, the spectrum range was 200-500 nm with UV absorption of 200, 230, 254 and 320 nm. Data acquisition time was between 0 to 32 mins yielding a total of 32 fractions (compounds). Further analysis to identify the chemical structure of each of the purified fractions was conducted using Ultra Performance Liquid Chromatography-Diode Array Detection (UPLC–DAD) and Liquid Chromatography-Mass Spectrometry (LC-MS). An Acquity UPLC system (Waters Corporation) equipped with a photo diode array detection detector was used for the analysis and quantification. The UPLC–ESI-MS peak identification was recorded using the UPLC system coupled with a LCQ DECA plus mass spectrometer equipped with an electrospray interface (Thermo-Finnigan Corporation). The quantification of UPLC–DAD was performed on a reversed-phase

column Acquity UPLC BEH C-18 (2.1 × 50 mm) with 1.7 μm spherical porous particles. The UPLC–ESI-MS analysis was operated in positive ESI modes and compounds were identified on the basis of their UV spectra and MS fragmentation patterns by searching the dictionary of natural products on DVD, Version 20:2 *(CRC Press, Taylor & Francis Group)*.

Oral acute toxicity

The virulence of our novel bacterial species of *Paenibacillus* strain 139SI was tested in experimental mice using the LD50 test as described previously [1]. For acute toxicity test, the selected experimental rats were randomly divided into six groups of six rats each as the following :

Group 1 - Normal saline (5ml/Kg, oral) daily for 14 days (male control group).

Group 2 - Normal saline (5ml/Kg, oral) daily for 14 days (female control group).

Group 3 - 139SI extract (5ml/Kg, oral) with a concentration of (2gm/Kg) daily for 14 days (male low dose group).

Group 4 - 139SI extract (5ml/Kg, oral) with a concentration of (2gm/Kg) daily for 14 days (female low dose group).

Group 5 - 139SI extract (5ml/Kg, oral) with a concentration of (4gm/Kg) daily for 14 days (male high dose group).

Group 6 - 139SI extract (5ml/Kg, oral) with a concentration of (4gm/Kg) daily for 14 days (male high dose group).

The body weight of all animals was measured daily. Mortalities, clinical signs and time of onset were recorded. In addition, gross general observations were observed on the basis of behavioral signs such as food intake, salivation, muscular weakness, reflexes, piloerection, respiration (dyspnea), convulsion and changes in locomotion [1]. All rats were sacrificed 24 hours after last oral administration and overnight fasting prior to anesthesia with an intra-muscular combination of Ketamine and Xylazine (1 ml of 100 mg/ml Xylazine and 9 ml of 100 mg/ml Ketamine) given at a dose of 0.1 ml/100 gm of body weight followed by necropsy. Blood samples were collected and the liver and kidneys were harvested, washed in normal saline, blotted with filter paper, and weighed. Gross examination was conducted in a blind fashion to examine the macroscopic abnormalities on the organs compared to the control. Moreover, liver and kidneys were subsequently subjected to a histopathological evaluation to examine the microscopic abnormalities on the organs compared to the control.

Biochemical parameters and hematological profile

Upon sacrifice, blood was drawn from the jugular vein under anesthesia and samples were immediately collected then referred to the clinical diagnostic laboratories (CDL) at University Malay Medical Centre (UMMC) for assessment of the biochemical parameters and hematological profile. For the biochemical parameters, blood was collected into yellow caped VACUETTE® clot activator. Liver function tests were assessed including Total Protein, Albumin, Globulin, Total Bilirubin, Conjugate Bilirubin, Alkaline Phosphatase, Alanine Aminotransferase, Aspartate Aminotransferase, G-Glutamyl transferase. In addition, renal function tests were assessed including Sodium, Potassium, Chloride, Carbon Dioxide, Anion Gap, Urea and Creatinine. For the hematological profile, blood was collected into violet caped VACUETTE® EDTA tubes and the complete blood count (CDC) test was assessed including Hemoglobin (HGB), Hematocrit (HCT), Red Blood Cells (RBC), Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH), Mean Corpuscular Hemoglobin Concentration (MCHC), Red blood cell Distribution Width (RDW) and White Blood Cell (WBC) and platelet. In addition, differential blood count test was assessed including Neutrophil, Lymphocyte, Monocyte, Eosinophil and Basophil.

Histopathology Examination

Upon sacrifice, the thoracic cavity was opened by an excision through the peritoneum that was extended through the sternum and the rib cage was fully opened followed by the collection of liver and kidneys. The collected organs were fixed with 10% neutral buffered formalin (NBF) for 24 hours then sliced into smaller pieces to be fixed again with NBF for another 24 hours. Histopathology examination was performed as described previously [19]. Briefly, fixed tissues were embedded in paraffin wax using an embedding center (Leica EG1160, Leica Biosystems), sectioned using a microtome (Leica RM2135, Leica Biosystems) and fixed onto glass slides using a water bath (Leica HI1210, Leica Biosystems). The paraffin sections were then stained with Hematoxylin & Eosin (H&E) stain, mounted with diphenyl xylene (DPX) and visualized using an upright light microscope (Eclipse LV150L, Nikon).

Statistical analysis

Statistical analysis was carried out using the Statistical Product and Service Solutions software (IBM SPSS statistics 21). Categorical data were compared by the $\chi 2$ test, while unpaired differences in continuous data were compared by both the Mann-Whitney U test and the analysis of variance (ANOVA) test. All values were reported as standard error mean (S.E.M±) and a probability value of $p < 0.05$ was considered to be statistically significant.

Results

In vitro antibiofilm activity

The results of MTP assay for the crude extract of *Paenibacillus* sp. strain 139SI showed significant inhibition of the biofilms when assessed spectrophotometrically. The lowest and most effective concentration that caused significant reduction in the biofilm's adherence index was found to be 4500µg/ml. Among all the 32 purified fractions (compounds) of 139SI crude extract, only 3 compounds showed the highest antibiofilm activity selected against Gram-negative (Table 1) and Gram-positive (Table 2) clinical isolates. Moreover, compound number 5 (FR5) was the most active with significant decrease in the adherence index when compared to other compounds and controls. The results of MTP assays were compatible with the BIC test in which there was an 80% inhibition in the biofilm when visualized under light microscope showing scattered bacterial cells with no extracellular matrix.

Table 1 *Antibiofilm activity of potential compounds of 139SI against Gram-negative clinical isolates.*

Experimental Treatment	*Haemophilus influenzae*	*Haemophilus parainfluenzae*	*Klebsiella pneumoniae*	*Pseudomonas aeruginosa*	*Citrobacter* sp.	Biofilm-forming strain *P. aeruginosa* (ATCC 27853)	Non-biofilm-forming strain *E. coli* (ATCC 25922)
	OD ± SD	OD ± SD	OD ± SD	OD ± SD	OD ± SD	OD ± SD	OD ± SD
With compound FR4	0.235 ± 0.005	0.245 ± 0.004	0.266 ± 0.004	0.194 ± 0.003	0.175 ± 0.004	0.225 ± 0.004	0.164 ± 0.004
Without compound	0.311 ± 0.002	0.459 ± 0.015	0.369 ± 0.056	0.439 ± 0.052	0.235 ± 0.015	0.539 ± 0.052	0.244 ± 0.113
With compound FR5*	0.192 ± 0.007	0.228 ± 0.009	0.245 ± 0.004	0.177 ± 0.005	0.165 ± 0.002	0.204 ± 0.003	0.147 ± 0.003
Without compound	0.584 ± 0.002	0.355 ± 0.038	0.244 ± 0.006	0.254 ± 0.003	0.391 ± 0.003	0.309 ± 0.114	0.202 ± 0.099
With compound FR13	0.255 ± 0.003	0.206 ± 0.004	0.257 ± 0.005	0.215 ± 0.004	0.182 ± 0.002	0.245 ± 0.003	0.155 ± 0.004
Without compound	0.304 ± 0.003	0.284 ± 0.006	0.355 ± 0.003	0.254 ± 0.003	0.277 ± 0.001	0.539 ± 0.052	0.211 ± 0.002
With 2(5H)-Furanone (+ve Control)	0.120 ± 0.004	0.092 ± 0.004	0.106 ± 0.004	0.116± 0.004	0.119 ± 0.002	0.124 ± 0.003	0.105 ± 0.004
Without compound	0.262 ± 0.003	0.487 ± 0.003	0.486 ± 0.004	0.559 ± 0.015	0.564 ± 0.002	0.377 ± 0.122	0.216 ± 0.005
With BHI broth (-ve Control)	0.057 ± 0.038	0.050 ± 0.006	0.021 ± 0.002	0.069 ± 0.020	0.095 ± 0.012	0.084 ± 0.006	0.084 ± 0.006
Without compound	0.276 ± 0.004	0.369 ± 0.056	0.257 ± 0.024	0.363 ± 0.079	0.316 ± 0.056	0.304 ± 0.003	0.163 ± 0.001

OD > 0.24 = Positive biofilm former isolate
OD > 0.12 - < 0.24 = Weak biofilm former isolate
OD < 0.12 = Negative biofilm former isolate

* represent the most active compound

Table 2 *Antibiofilm activity of potential compounds of 139SI against Gram-positive clinical isolates.*

Experimental Treatment	*Staphylococcus aureus*	*Streptococcus agalactiae*	Group G Streptococci	*Streptococcus pyogenes*	*Streptococcus pneumoniae*	Biofilm-forming strain *S. aureus* (ATCC 25923)	Non-biofilm-forming strain *E. coli* (ATCC 25922)
	OD ± SD	OD ± SD	OD ± SD	OD ± SD	OD ± SD	OD ± SD	OD ± SD
With compound FR4	0.254 ± 0.004	0.205 ± 0.005	0.199 ± 0.010	0.191 ± 0.003	0.230 ± 0.004	0.246 ± 0.004	0.186 ± 0.004
Without compound	0.484 ± 0.008	0.215 ± 0.002	0.253 ± 0.002	0.304 ± 0.003	0.371 ± 0.002	0.395 ± 0.003	0.216 ± 0.073
With compound FR5*	0.224 ± 0.004	0.166 ± 0.005	0.158 ± 0.002	0.153 ± 0.005	0.224 ± 0.004	0.166 ± 0.005	0.158 ± 0.002
Without compound	0.368 ± 0.028	0.304 ± 0.003	0.377 ± 0.122	0.363 ± 0.079	0.445 ± 0.042	0.030 ± 0.038	0.224 ± 0.005
With compound FR13	0.208 ± 0.004	0.205 ± 0.004	0.195 ± 0.004	0.216 ± 0.004	0.235 ± 0.004	0.254 ± 0.004	0.165 ± 0.004
Without compound	0.404 ± 0.003	0.276 ± 0.056	0.262 ± 0.003	0.257 ± 0.024	0.277 ± 0.002	0.378 ± 0.003	0.254 ± 0.003
With 2(5H)-Furanone (+ve Control)	0.119 ± 0.002	0.123 ± 0.003	0.116 ± 0.004	0.117 ± 0.001	0.121 ± 0.001	0.107 ± 0.004	0.116 ± 0.002
Without compound	0.257 ± 0.003	0.216 ± 0.005	0.206 ± 0.055	0.202 ± 0.099	0.214 ± 0.030	0.216 ± 0.005	0.211 ± 0.002
With BHI broth (-ve Control)	0.057 ± 0.038	0.050 ± 0.006	0.021 ± 0.002	0.069 ± 0.020	0.095 ± 0.012	0.084 ± 0.006	0.084 ± 0.006
Without compound	0.254 ± 0.003	0.243 ± 0.045	0.211 ± 0.002	0.243 ± 0.045	0.270 ± 0.042	0.243 ± 0.002	0.208 ± 0.074

OD > 0.24 = Positive biofilm former isolate
OD > 0.12 - < 0.24 = Weak biofilm former isolate
OD < 0.12 = Negative biofilm former isolate
* represent the most active compound

Characterization of potential bioactive compounds

The results of characterizing of the compounds from *Paenibacillus* sp. extract using HPLC showed a total of 32 purified fractions in which only 3 fractions exhibited antibiofilm activity *in vitro* when assessed spectrophotometrically [2]. From these 3 fractions, a total of 3 potential compounds were identified in which the first compound was Leucine 2-(hydroxymethoxyphosphinyl)-2-methylhydrazide with a molecular weight of 253.237 and a molecular formula of $C_8H_{20}N_3O_4P$ described as an amino acid antibiotic with an

activity against Gram-positive and Gram-negative bacteria. The second compound was 4-Hydroxy-5-(Hydroxymethyl)-3-(14-methylpentadecanoyl) tetronicacid-2(5H)-Furanone with a molecular weight of 368.512 and a molecular formula of $C_{21}H_{36}O_5$ described as a phospholipase A2 inhibitor. The third compound was 6-(Hydroxymethyl)-1-phenazinecarboxyamide with a molecular weight of 253.260 and a molecular formula of $C_{14}H_{11}N_3O_2$ described as an antibacterial agent.

Gross general observation of experimental rats

Gross general observations showed that experimental rats grew at relatively constant rates. Following the 14 days oral ingestion of *Paenibacillus* sp. extract, there was no significant difference ($p > 0.05$) in the overall growth among the groups except for high dose group where a decrease in growth was observed in the last two days of experiment. These results suggested that the 14 days acute oral ingestion of extract did not affect the weight of rats. Moreover, there was an irregular dose-dependent mortality in both sexes for which only one rat from each sex died after 72 hours ingestion of the high dose (1 out of 6 males and 1 out of 6 females). Moreover, the observed symptoms of toxicity included minor hypo-activity, loss of appetite, hyperventilation, convulsion, dizziness and salivation; however, they were statistically insignificant when compared to the controls.

Biochemical parameters, complete and differential blood counts

Despite minor discrepancies between sexes, the results of biochemical parameters (Table 3 and Table 4) showed no significant differences ($p>0.05$) in the liver and kidney function tests except for elevated levels in Globulin, Alkaline Phosphatase, Alanine Aminotransferase, Aspartate Aminotransferase, G-Glutamyltransferase, Potassium, Urea and Creatinine. Moreover, there were increased levels of Anion Gap among female rats only. Overall, our results indicated that the 139SI extract has no detectable differences on both liver and kidney functions. Moreover, the results of hematological profile (Table 5 and Table 6) showed no significant differences ($p>0.05$) in both the complete and differential blood count except for elevated levels in Red Blood Cells (RBC), White Blood Cell (WBC), Platelet, Neutrophil, Lymphocyte and Monocyte particularly among the high dose groups (4 gm/Kg).

Table 3 *Liver and Renal Function tests among male rats*

Liver Function Test	Male Experiment Groups			Control (Reference Range)	International Unit (IU)
	Vehicle (0.9% NaCl)	Low Dose (2 gm/Kg)	High Dose (4 gm/Kg)		
Total Protein	**57.50 ± 2.45***	**67.33 ± 1.72**	**65.83 ± 2.79**	**64 - 82**	**g/L**
Albumin	**10.00 ± 2.04***	**12.46 ± 1.35***	**16.31 ± 5.98***	**35 - 50**	**g/L**
Globulin	**42.33 ± 4.77***	**54.33 ± 1.83***	**50.83 ± 4.14***	**23 - 35**	**g/L**
Total Bilirubin	**3.66 ± 0.76**	**0.31 ± 0.47**	**3.75 ± 1.12**	**3 - 17**	**µmol/L**
Conjugate Bilirubin	**1.50 ± 0.34**	**1.33 ± 0.21**	**1.50 ± 0.34**	**0 - 3**	**µmol/L**
Alkaline Phosphatase	**256.16 ± 29.30***	**229.66 ± 22.48***	**195.50 ± 22.10***	**50 - 136**	**IU/L**
Alanine Aminotransferase	**74.50 ± 10.59***	**83.16 ± 5.67***	**107.16 ± 16.66***	**30 - 65**	**IU/L**
Aspartate Aminotransferase	**210.50 ± 22.81***	**188.16 ± 20.13***	**201.16 ± 17.64***	**15 - 37**	**IU/L**
G-Glutamyltransferase	**11.00 ± 3.35***	**8.33 ± 2.06***	**10.33 ± 4.27***	**15 - 85**	**IU/L**
Renal Function Test					
Sodium	**139.00 ± 1.12**	**138.66 ± 0.91**	**137.83 ± 1.40**	**136 - 145**	**mmol/L**
Potassium	**6.93 ± 0.79***	**6.03 ± 0.25***	**6.45 ± 0.72***	**3.6 - 5.2**	**mmol/L**
Chloride	**101.83 ± 1.01**	**102.00 ± 1.03**	**102.16 ± 1.24**	**100 - 108**	**mmol/L**
Carbon Dioxide	**28.05 ± 1.50**	**29.21 ± 0.72**	**26.41 ± 1.38**	**21.0 - 30.0**	**mmol/L**
Anion Gap	**16.16 ± 1.37**	**14.76 ± 0.74**	**16.76 ± 2.11**	**10 - 20**	**mmol/L**
Urea	**7.78 ± 0.81***	**8.71 ± 0.71***	**9.35 ± 1.77***	**2.5 - 6.4**	**mmol/L**
Creatinine	**18.16 ± 2.38***	**21.00 ± 3.29***	**20.00 ± 4.67***	**61.9 - 115**	**µmol/L**

Values are expressed as the standard error mean ± S.E.M. and the significant value was at $p<0.05$
* indicates values that are above or below the control reference range
g/L= gram per Liter, µmol/L = micromole per Liter, IU/L = International Unit per Liter

Table 4 *Liver and Renal Function tests among female rats*

Liver Function Test	Female Experiment Groups			Control (Reference Range)	International Unit (IU)
	Vehicle (0.9% NaCl)	Low Dose (2 gm/Kg)	High Dose (4 gm/Kg)		
Total Protein	61.00 ± 3.81*	71.66 ± 0.95	63.16 ± 2.46*	64 - 82	g/L
Albumin	15.83 ± 3.00*	13.66 ± 1.02*	16.66 ± 5.74*	35 - 50	g/L
Globulin	45.83 ± 3.77*	57.50 ± 1.64*	48.16 ± 4.85*	23 - 35	g/L
Total Bilirubin	6.83 ± 2.15	2.83 ± 0.83*	4.66 ± 1.76	3 - 17	µmol/L
Conjugate Bilirubin	1.83 ± 0.40	1.33 ± 0.33	1.50 ± 0.34	0 - 3	µmol/L
Alkaline Phosphatase	156.33 ± 38.10*	136.50 ± 21.53*	140.00 ± 23.42*	50 - 136	IU/L
Alanine Aminotransferase	67.50 ± 11.44*	67.50 ± 3.19*	82.33 ± 16.32*	30 - 65	IU/L
Aspartate Aminotransferase	150.33 ± 19.10*	196.66 ± 19.69*	196.83 ± 24.25*	15 - 37	IU/L
G-Glutamyltransferase	5.66 ± 0.71*	4.50 ± 0.56*	7.66 ± 1.68*	15 - 85	IU/L
Renal Function Test					
Sodium	139.50 ± 0.99	138.00 ± 1.06	138.50 ± 0.84	136 - 145	mmol/L
Potassium	6.80 ± 0.59*	6.10 ± 0.65*	5.56 ± 0.25*	3.6 - 5.2	mmol/L
Chloride	96.66 ± 4.70*	100.66 ± 1.05	98.50 ± 2.61*	100 - 108	mmol/L
Carbon Dioxide	24.68 ± 1.04	27.03 ± 1.35	26.66 ± 1.37	21.0 - 30.0	mmol/L
Anion Gap	24.68 ± 1.04*	27.03 ± 1.35*	26.66 ± 1.37*	10 - 20	mmol/L

Values are expressed as the standard error mean ± S.E.M. and the significant value was at $p<0.05$
* indicates values that are above or below the control reference range
g/L= gram per Liter, µmol/L = micromole per Liter, IU/L = International Unit per Liter

Table 5 *Complete blood count and differential blood count tests among male rats*

Complete Blood Count (CBC) Test	Male Experimental Groups			Control (Reference Range)	International Unit (IU)
	Vehicle (0.9% NaCl)	Low Dose (2 gm/Kg)	High Dose (4 gm/Kg)		
Hemoglobin (HGB)	144.333 ± 3.323	147.166 ± 3.709	139.500 ± 4.295	130 - 170	g/L
Hematocrit (HCT)	0.421 ± 0.006	0.465 ± 0.013	0.466 ± 0.016	0.40 – 0.50	L/L
Red Blood Cells (RBC)	5.583 ± 0.241*	5.733 ± 0.187*	6.173 ± 0.246*	4.50 – 5.50	10^{12}/L
Mean Corpuscular Volume (MCV)	57.500 ± 1.543*	65.666 ± 2.011*	65.833 ± 3.590*	77 – 97	fL
Mean Corpuscular Hemoglobin (MCH)	23.050 ± 0.755*	23.333 ± 1.227*	25.083 ± 1.549*	27.0 – 32.0	pg
Mean Corpuscular Hemoglobin Concentration (MCHC)	326.000 ± 1.807	328.500 ± 1.522	337.666 ± 2.245	315 – 345	g/L
Red blood cell Distribution Width (RDW)	12.483 ± 0.335	12.600 ± 0.265	13.683 ± 0.514	11.6 – 14.0	%
White Blood Cell (WBC)	5.516 ± 0.286	6.216 ± 0.613	11.333 ± 0.792*	4.0 – 10.0	10^9/L
Platelet	247.833 ± 12.605	297.333 ± 20.397	411.333 ± 19.022*	150 - 400	10^9/L
Differential Blood Count Test					
Neutrophil	5.666 ± 0.714	8.500 ± 0.763*	9.166 ± 1.077*	2.00 – 7.00	10^9/L
Lymphocyte	2.833 ± 0.307	3.666 ± 0.421*	7.166 ± 0.477*	1.00 – 3.00	10^9/L
Monocyte	1.183 ± 0.079*	1.650 ± 0.168*	2.366 ± 0.164*	0.20 – 1.00	10^9/L
Eosinophil	0.188 ± 0.032	0.270 ± 0.024	0.258 ± 0.054	0.02 – 0.50	10^9/L
Basophil	0.011 ± 0.007*	0.026 ± 0.010	0.045 ± 0.010	0.02 – 0.10	10^9/L

Values are expressed as the standard error mean ± S.E.M. and the significant value was at $p<0.05$
* indicates values that are above or below the control reference range
g/L=gram per Liter, L/L Liter per Liter, fL=femtoliters, pg = pictogram, %=percentage

Table 6 *Complete blood count and differential blood count tests among female rats*

Complete Blood Count (CBC) Test	Female Experimental Groups			Control (Reference Range)	International Unit (IU)
	Vehicle (0.9% NaCl)	Low Dose (2 gm/Kg)	High Dose (4 gm/Kg)		
Hemoglobin (HGB)	140.666 ± 2.577	135.000 ± 2.840	150.833 ± 4.158	130 - 170	g/L
Hematocrit (HCT)	0.433 ± 0.009	0.458 ± 0.015	0.455 ± 0.011	0.40 – 0.50	L/L
Red Blood Cells (RBC)	5.566 ± 0.252*	6.350 ± 0.232*	7.300 ± 0.343*	4.50 – 5.50	10^{12}/L
Mean Corpuscular Volume (MCV)	63.500 ± 2.667	60.333 ± 2.564	60.500 ± 2.753	77 – 97	fL
Mean Corpuscular Hemoglobin (MCH)	21.216 ± 0.820*	23.683 ± 0.603*	20.500 ± 0.940*	27.0 – 32.0	pg
Mean Corpuscular Hemoglobin Concentration (MCHC)	328.833 ± 2.329	335.833 ± 2.358	343.666 ± 5.129	315 – 345	g/L
Red blood cell Distribution Width (RDW)	12.533 ± 0.289	12.500 ± 0.163	12.866 ± 0.401	11.6 – 14.0	%
White Blood Cell (WBC)	5.983 ± 0.411	8.216 ± 0.503	10.916 ± 0.925*	4.0 – 10.0	10^9/L
Platelet	322.833 ± 28.703	375.833 ± 17.284	433.666 ± 27.690*	150 - 400	10^9/L
Differential Blood Count Test					
Neutrophil	6.000 ± 0.966	10.166 ± 0.703*	11.166 ± 0.600*	2.00 – 7.00	10^9/L
Lymphocyte	3.333 ± 0.421*	2.833 ± 0.477	6.500 ± 1.056*	1.00 – 3.00	10^9/L
Monocyte	1.350 ± 0.133*	1.800 ± 0.146*	2.016 ± 0.280*	0.20 – 1.00	10^9/L
Eosinophil	0.333 ± 0.030	0.246 ± 0.049	0.316 ± 0.030	0.02 – 0.50	10^9/L
Basophil	0.015 ± 0.007*	0.030 ± 0.010	0.040 ± 0.013	0.02 – 0.10	10^9/L

Values are expressed as the standard error mean ± S.E.M. and the significant value was at $p<0.05$
* indicates values that are above or below the control reference range
g/L= gram per Liter, L/L Liter per Liter, fL=femtoliters, pg = pictogram, % = percentage

Histopathology examination of liver and kidneys

Upon histological examination of liver and kidneys, the organs showed normal architecture, no changes in colour and no morphological disturbances. Liver tissue sections showed regular cellular architecture with distinct hepatic cells, sinusoidal spaces and a central vein. Ordinary patterns with normal parenchyma and reduced fibrous septa and lymphocyte infiltration were seen (Figure 1). Overall, the examination showed no detectable differences in the integrity of tissue among all groups and that the 139SI extract had no effects on the cellular structures and thus do not cause degeneration of cells in these particular organs.

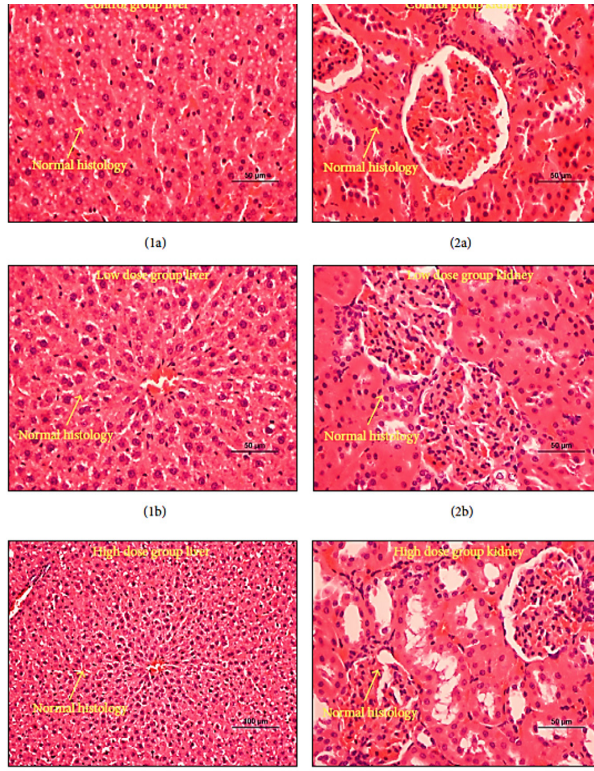

Fig. 1 - *Microscopic images of representative tissue sections showing the histopathology evaluation of the organs of toxicity among SD rat groups. 1A and 2A, vehicle (control) group with normal histology of liver and kidneys respectively. 1B and 2B, low dose (2gm/Kg) groups with normal histology of liver and kidneys respectively. 1C and 2C, High dose (4gm/Kg) groups with normal histology of liver and kidneys respectively. Sections were stained with H&E stain.*

Discussion

Bacteria that inhabits the soil is a potential source for the isolation of novel antibiofilm compounds [28]. It has been estimated that among all the microbes isolated from soil, *Bacillus and Paenibacillus* species are the most frequently found members with antimicrobial and antibiofilm activities [45, 24]. Therefore the report of a taxonomically novel species of *Paenibacillus* strain 139SI having antibiofilm activity is not surprising. Our study demonstrates the occurrence of a broad range antibiofilm activity in the crude extract and in three identified compounds of an extract from a novel *Paenibacillus sp*. strain 139SI. These identified compounds included an amino acid antibiotic, phospholipase A2

inhibitor and antibacterial agent. To our knowledge, no literature has reported the finding of such compounds with such activity from soil bacteria. We found that the addition of the crude extract and its bioactive compounds to a range of Gram-positive and Gram-negative bacteria results in inhibitory effect on their biofilm development. This broad spectrum of antibiofilm activity might help *Paenibacillus sp.* in the soil environment to establish itself on the surface of plant roots and critically influence the development of unique bacterial community.

It has been previously reported that some bacterial compounds such as extracellular-polysaccharides (EPS) can be involved in the antibiofilm activity. For example EPSs from the marine bacterium *Vibrio sp.* QY101 display selective or broad spectrum antibiofilm activity [21]. However, the potentiality of the compounds described in this study against a wide range of pathogenic and non-pathogenic organisms suggests that these compounds might be a powerful alternative among the previously identified compounds. Based on the findings, the first compound reported here as an amino acid antibiotic with the name Leucine 2-(hydroxymethoxyphosphinyl)-2-methylhydrazide has a phosphate group in it and thus can be proposed that its electronegative property might modulate the surface of the tested organism in such a way that there is an inhibition of the cell-surface attachment. This was similar to a previous study where it was reported that the identified polysaccharide compounds might interfere with the cell-surface influencing cell-cell interactions of a wide range of bacterial isolates [38]. The second compound reported here was phospholipase A2 inhibitor with the name 4-Hydroxy-5- (Hydroxymethyl)-3-(14-methylpentadecanoyl) tetronicacid-2(5H)-Furanone has similar chemical structure to the previously identified quorum-sensing antagonist (5Z)-4-bromo-5-(bromomethylene)-3-butyl-2(5H)-furanone from the marine alga *Delisea pulchra* which was reported to inhibit the biofilm formation in *E. coli* without inhibiting its growth [35]. The third compound reported here as an antibacterial agent with the name 6-(hydroxymethyl)-1-phenazinecarboxyamide might modify the physicochemical characteristics and the architecture of the outer membrane of biofilm-forming organisms which is the phenomenon observed for some antibiotics as reported previously [14]. In the BIC test that included the use of cover slip, a gradual decrease of biofilm development was visualized with the increase of the concentration of crude extract from *Paenibacillus sp*. strain 139SI.

In recent years, many studies have focused on the acute toxicity of antimicrobial metabolites isolated from different soil microorganisms for the purpose of identifying new sources of bioactive compounds [23,5]. Acute toxicity studies in experimental animals are useful to provide the primary data supporting single dose safety and kinetic in humans [9]. In our study, the oral acute toxicity and compound characterization of an antibiofilm extract from a novel bacterial species of *Paenibacillus* strain 139SI was assessed in Sprague Dawley (SD) rats. In the present study, a total of 36 rats were selected in which 12 rats of both sexes were treated with a low dose (2gm/Kg) and 12 rats were treated via a gastro-gavage with a high dose (4gm/Kg) of an antibiofilm extract from the novel bacte-

rial species of *Paenibacillus* strain 139SI at a concentration of 4500 µg/ml for a duration of 14 days. In rodents, a decrease in food and water consumption is an important sign of health deterioration which generally results in the loss of bodyweight [41]. Changes in bodyweight have also been used as an indicator of the effect of drugs and chemicals [18]. Overall, the gross general observation indicated that the effect of orally administered extract was not affected by sex and that the mortality rate was low among experimental rats. These finding were similar to a previous study in which the effects of a new compound from novel soil bacterial species of *Streptomyces* was investigated on Long Evan's rats showing no adverse effects at a dose of 300µg/rat/day [43]. It is known that both liver and kidney play significant roles in various metabolic processes. However, if too many demands are made on the capacities of these organs, the function of their cells will eventually be adversely affected [29] The liver plays an important role in xenobiotic function whereas the kidneys are the main organs involved in drugs elimination [41]. Moreover, the enzymes Alanine Aminotransferase (ALT) and Aspartate Aminotransferase (AST) are usually used as biomarkers to predict possible toxicity in the liver [34]. Therefore, any damage to the parenchymal liver cells will result in elevations in both these enzymes [42]. In our study, the elevated levels of ALT and AST especially among high dose groups (4gm/Kg) seemed to suggest that the *Paenibacillus* extract did affect the liver cells' mitochondria. However, this appeared to be an acute and short lasting response that did not cause significant mortalities among experimental groups. This was similar to a previous study in which the acute oral administration of an aqueous plant extract of *Artemisia afra* to mice induced the same insignificant symptoms in both sexes. Furthermore, it was noticed that the levels of anion gap increased among female rats only. Although the significance of this result is unclear, determining the concentration of anion gap may be susceptible to specific errors such as the delay in processing blood samples after collection or when particular pathological condition occur like diarrhea that will eventually cause dehydration which will eventually lead to some alterations in the renal function. Hematopoietic system is one of the most sensitive targets for toxic compounds [17], it was thus important to investigate the effect of our *Paenibacillus* sp. extract on the hematological profile. Our results showed that here were no significant differences (p>0.05) in the Haematocrits, Mean Cell Haemoglobin Concentration, Platelet, RBC and WBC counts among all experimental rats. The hemoglolobin and the RBC levels were not affected suggesting that haemolytic anemia and polycythemia, that are characterized by decreases and increases in RBC count, Haematocrits and Hemoglobin, respectively, were not likely to be induced by the extract. The Platelet levels despite being slightly elevated were also not significant indicating that the extract also did not affect the production of platelets nor induced thrombocytopenia, the latter normally being the first evidence of drug-induced toxic effects on haematopoiesis [40]. Moreover, the levels of WBC which serve as scavengers that destroy microorganisms at infection sites [30] were also not changed suggesting that the extract was also not toxic to the immune system and did not affect leucopoiesis. Collectively, all the results suggest that the acute ingestion of the extract of novel species of *Paenibacillus* strain 139SI did

not alter the haematological parameters of our SD rats. Upon visual histological examination of both liver and kidneys, the acute oral administration of the extract had no adverse effects on these organs and that it was well tolerated over the 14 days study period. Therefore, it is being considered as the material that should be safe for use in oral formulations on pre-clinical and clinical studies. The bioactive compounds produced by bacteria in natural environments could be a mixture of several class of chemical compounds that can be either amino acids, peptides, nucleosides, alkaloids, terpenoids, sterols, saponins or polycyclic [7]. There is little information in the literature on the toxic or lethal levels of crude extract from bacteria belonging to the genus *Paenibacillus*. Our extract did not show any toxicity against experimental rats; the more likely explanation is that the toxic compounds in the crude extract were very low to induce death. However, some of the rats did die from ingesting the high dose extract. This can be due to high concentrations of one or more of the three chemical compounds characterized by UPLC–ESI-MS. In addition to the above findings, our study raises the following concerns. Firstly, although the acute oral doses of *Paenibacillus* sp. extract did not produce any significant adverse effects in rats, further studies using higher doses of the characterized compounds may be needed. Secondly, to confirm the non-toxic nature of the extract and its derivative compounds, the effect of various factors such as type of soil, bacterial growth stage, type of growth media and storage conditions may also need to be investigated. Thirdly, the effects of extract and its compounds in pregnant animals, on the reproductive capacity of animals and on causing tumors need to be assessed. Overall, our findings provide valuable preliminary data on the toxicity profile and potential bioactive compounds of an antibiofilm extract from a novel soil bacterial species of *Paenibacillus* that can be useful for the planning of future pre-clinical and clinical studies towards new therapeutic management of biofilm-associated infections.

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PHYSIOTHERAPY STUDENTS PERSPECTIVE ON LEARNING THROUGH SMARTPHONE

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Abstract:

Objectives: Smartphone technology has transformed our lives in many ways and it is also reflected in learning. So the main objective of this pilot study is to find out the physiotherapy students' perspective on use of smartphone and medical related applications (apps) for learning in a private university in Malaysia.

Methods: The method used in this study was an online survey. The link for the online survey form was posted on the Facebook page of the University Physio Club and the participation in the survey was voluntary. The students have to answer close ended questions related to the ownership of the smartphone; frequency, duration and purpose of medical related apps use in the smartphone for their learning, in university and clinical environment. Open ended questions were also used to explore the students' perception about the usefulness of the applications, and their recommendations.

Results: 51 students responded to the questionnaire out of which 68.6% were females and 31.4% were males. The very popular Smartphone type among physiotherapy students was Google Android (60.8%) variety followed by iPhone (23.5%). The majority (74.5%) of the students owned 1 to 5 medical related apps in their Smartphone. Their preferred use was, for educational learning (86.7%) and revising (75.6%), with less usage in clinical ward (31.1%) and clinic (17.8%) environment. Their usage frequency was once or twice a day with duration lasting between 1 and 10 minutes, in university as well as in a clinical environment. They often used the applications to find out the disease diagnosis/management and they found it most useful due to easy accessibility of information.

Conclusion: This pilot study shows that most of the physiotherapy students use medical related apps in their Smartphone for learning activity, and they mostly recommended dictionary and physiotherapy apps.

Keywords: Physiotherapy students, Smartphones, Medical App, Survey.

Introduction

A mobile device which has advanced capabilities beyond ordinary mobile phone is called a smartphone. Smartphone technology has transformed our lives in many ways and its influence can be seen in various fields like medicine teaching and learning [12,3]. Smartphone applications (apps) have changed the way people do their jobs. Since the

introduction of medical related applications, smartphone has been used widely in the medical field for assessment [10, 12] and management of patients [1,4,12]. It is also used to educate the patients about health related conditions and self management of chronic diseases [12]. The use of a smartphone for learning is also increasing and various studies explored by the use of a smartphone among university students [2, 3, 6] The increasing number of smartphone apps brings the solution of many complex problems in the student's hands and it's no wonder that smartphones is popular among the student community. Its use is also echoed in the medical students' community for learning.

Previous studies explored the usage of smartphone among medical students and their use for learning [15]. Medical students are using smartphone and medical related apps for learning not only in university campus but also in the clinical setting [13]. Likewise, Medical related apps usage among nursing students and physiotherapy students has to be assessed.

With the use of body measurement smartphone apps students can measure the patient's joint angles accurately without any sophisticated equipment [10]. Recent research has proved that posture and gait assessment can also be done with a smartphone app [8,11]. Smartphone apps can also be used for fall prevention [9] activity monitoring [5] and providing treatment like balance training [7]. As rehabilitation related apps are increasing, we presume that physiotherapy students also using smartphone for their learning in academic as well as in a clinical setting. Even though previous studies analyzed the use of smartphone among medical students, the physiotherapy students' perspective was not analyzed specifically, especially in Malaysia. Hence this pilot study is planned to find out the use of smartphone and medical related apps among physiotherapy students which will help us to transform our course delivery in teaching.

Methods

The method used in this study was a descriptive online survey. The questionnaire used in this study was adopted and modified from the questionnaire used by [13] with permission. The original questionnaire was piloted with 20 physiotherapy students and modified accordingly. Then the 12 items questionnaire were created in the Google docs (www.docs.google.com) and the link for the online survey form was posted on the Facebook page of the INTI International University Physio Club for 3 weeks from 23rd June 2014 to 14th July 2014. The participation in this survey was voluntary. All the physiotherapy students of INTI International University are the members of the INTI Physio club page on Facebook. Hence anyone from year 1 to year 4 can access and answer the questionnaire. When they click the online survey form link on Facebook, a separate page in Google docs will open with informed consent and the questionnaire. After reading the informed consent if the student wishes to continue, then they have to answer close ended questions related to their gender, current year of study, ownership of the smartphone and medical related applications, frequency, duration and purpose of medical related apps use in the smartphone

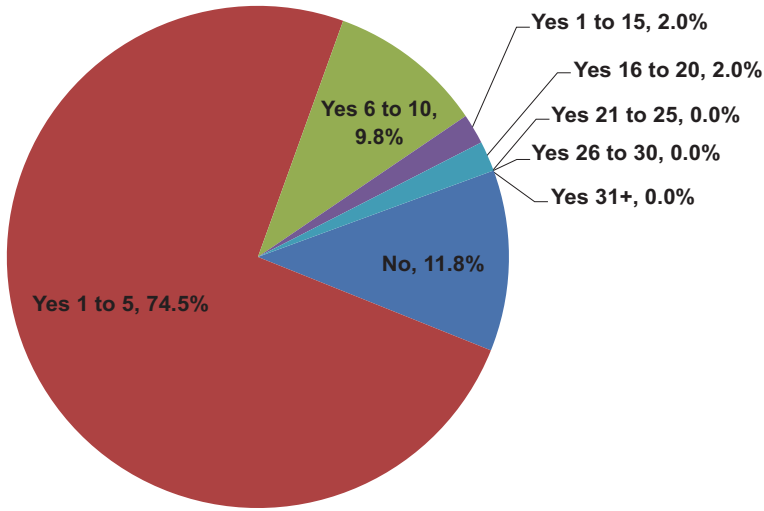
for their learning, in university and clinical environment. The usage of university linked application among students' is also explored with a closed ended question. Open ended questions were also used to discover the students' perception about the usefulness of the applications, and their recommendations. Please find the questionnaire in the appendix.

After 3 weeks the link was closed and the numerical data were analyzed using Microsoft Excel (MS Office 2010) and Statistical Package for Social Sciences (SPSS version 22). The descriptive statistics were analyzed for frequency and percentage by entering data from Google docs into Microsoft excel. The inferential statistics for non- parametric Chi square test was run using SPSS as appropriate. The response to the open ended questions were organized into key themes and discussed in the results.

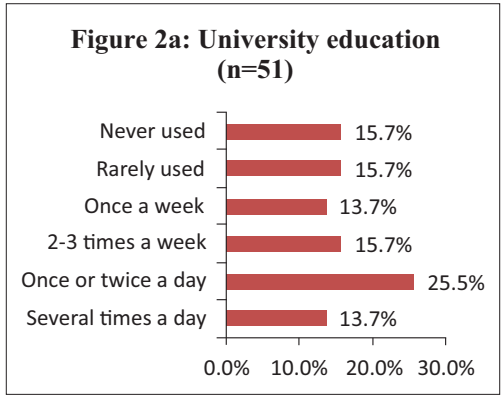
Results

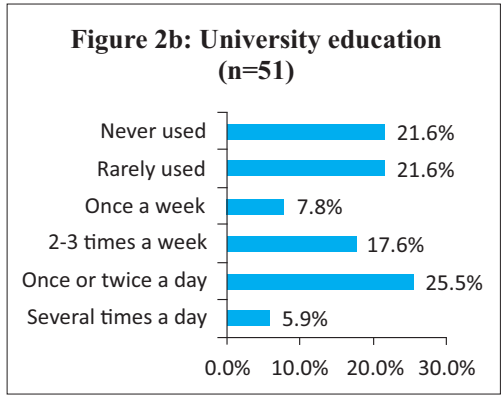
Of the undergraduate physiotherapy students surveyed 51 students responded, out of 156 registered physiotherapy students; with a response rate of 32.7%. Out of 51 responses 68.6% (n=35/51) of the students were females and remaining 31.4% (n=16/51) were males. The recorded responses were from 4th year (35.3%), 3rd year (45.1%), 2nd year (19.6%) and no first year students answered the questionnaire. Google Android (60.8%, n=31/51) was the most popular smartphone followed by iPhone (23.5%, n=12/51) and other smartphone (15.7%, n=8/51) among physiotherapy students.

Figure 1: Percentage of physiotherapy students owning medical related smartphone apps. (n=51)



The majority of the students (74.5%, n=38/51) owned 1 to 5 medical related apps in their smartphone. Whereas 11.8% (n=6/51) students did not own any medical related apps in their smartphone. There was no association between gender and ownership of medical related apps (Chi-Square=0.558, df=1, *P*>0.05). Similarly no association was found between the type of phone and ownership of medical related apps (Chi-Square=4.392, df=2, *P*>0.05). The students used smartphone mostly for educational learning (86.7%, n=39/45) and educational revision (75.6%, n=34/45), and their usage in the clinical area was limited representing 31.1% (n=14/45) in a ward environment and 17.8% (n=8/45) in a clinic environment. The figure 2a and 2b shows the frequency of usage of medical related apps in university education and clinical education.





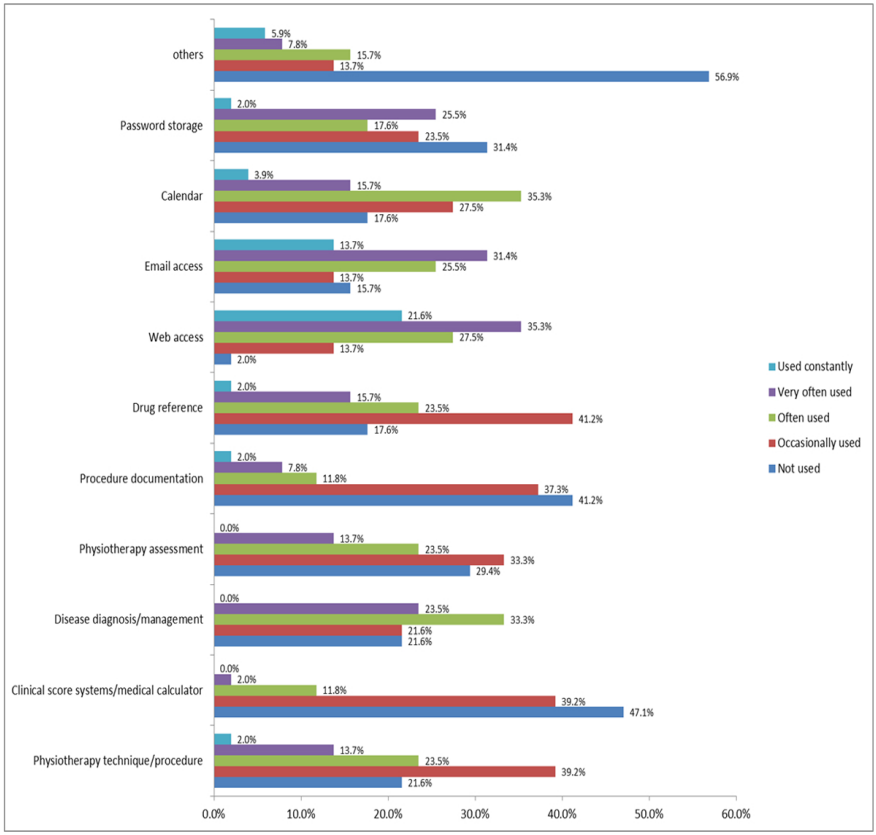
The purpose and frequency of medical related apps as reported by the students is given in the figure 3. The students stateded other uses such as notes, presentation slides, photo, BMI calculator, stopwatch, games and social media (Facebook, Twitter, YouTube, WhatsApp). Daily use (in minutes) of medical related apps among physiotherapy students is

reported in the figure 4a and 4b. Out of 51 responses 56.9% (n=29/51) of students like to use smartphone apps specific to their university and 43.1% (n=22/51) did not like to use it.

More than one third of the students' (n=19/51) responded to the open ended questions about their comments on the use of medical related application in a clinical environment. 9 participants stated that apps are easily accessible and convenient to use. The students also revealed that it is helping to get further information on the lecture, assisting to learn technology with education and helping to revise the topic. Four students were not sure about their answers to this question.

The physiotherapy students recommended following apps: human anatomy atlas, PubMed, BMI calculator, disease dictionary, physiotherapy exercises, goniometer, medical diagnosis, medical dictionary, Google and more physiotherapy related apps.

Figure 3: The purpose and frequency of medical related apps use among physiotherapy students. (n=51)



Daily use (in minutes) of medical related apps among physiotherapy students is reported below in figure 4a and 4b. (n=51)

Figure 4a: Daily use in university setting

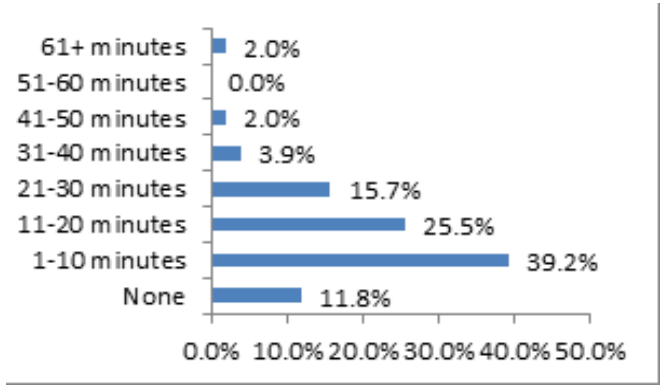
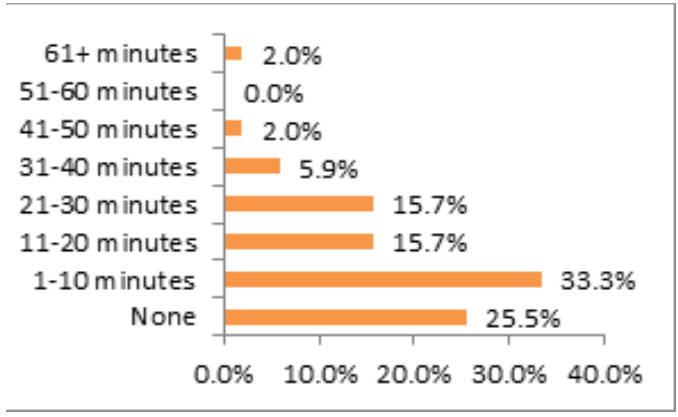


Figure 4b: Daily use in clinical setting



The most useful features of medical related apps described by physiotherapy students was drug information and disease diagnosis, medical dictionary with diagram or picture, precise diagrams and explanations, convenient and quick access, summarized information, quick reference, easy to use, and reliable source of information.

Discussion

This pilot study discovers the medical related apps usage among physiotherapy students for the first time in Malaysia. Understanding the perception of students about smartphone in learning will help the academicians to modify their teaching and course delivery in an exclusive way. It will also help smartphone companies and apps developers to design and modify their software according to consumer preferences.

Even though only one third of the students responded to the questionnaire, all of them owned a smartphone. So the smartphone usage is high among physiotherapy students. This trend is supported by Sedek, Mahmud, Jalil, & Daud, (2013) in their study, which reported high amount of the smartphone, tablet and laptop ownership among undergraduate students from Malaysian Technical Universities. Similarly research studies by Kim *et al*., (2013) from Korea and Alfawareh & Jusoh, (2014) [2] from Saudi Arabia stated the peak use of a smartphone among university students.

Google Android phone (60.8%) was popular among physiotherapy students in Malaysia. On the contrary Apple iPhone was the most popular smartphone among Canadian medical students and residents [15] and United Kingdom medical students [13]. The slightly cheaper price of android phone when compared to the iPhone may be influencing consumers to buy android phones in a developing country like Malaysia.

74.5% of the Malaysian students owned 1 to 5 medical related apps in their smartphone which is higher when compared to United Kingdom medical students and junior doctors as reported by Payne *et al*., (2012). However ownership of more than 5 medical related apps is less among Malaysian physiotherapy students when compared to UK medical students. This study does not show any significant association between types of smartphone and ownership of medical related apps which is contrary to the UK study. As medical related apps on Apple App store and Google Play store are numerous with identical functionality, the app can use by both types of smartphone iPhone and Android users which are almost similar. Only 25.5% of Malaysian physiotherapy students used medical apps, once or twice a day for their clinical as well as university education, which is less than the use of Canadian medical student [15]. Even though the physiotherapy students in the pre-clinical years (second year) had limited clinical posting, the overall use in university and clinical education for once or twice in a day were similar.

The percentage of Malaysian students using smartphone several times a day for medical purpose is less; 15.7% and 21.6% of students never used in their university education and clinical education respectively. This displays that physiotherapy students are using their smartphone for medical related educational purpose, but not very frequently.

A review by Ozdalga *et al*., (2012) stated many uses of smartphone in medicine. In that he reported about the use of a smartphone for patient care and monitoring in various fields

like neurology and rehabilitation. The results from this current pilot study reveal that physiotherapy students are often used smartphone for disease diagnosis/management, but they used occasionally for physiotherapy assessment and procedures. But they used very often for web access and email access. Regarding the duration of use, 1-10 minutes per day was reported by most of the Malaysian students. Although this duration is less, it is understandable that the current generation of students wants to enjoy their time by using social media and email which can be seen through peak usage of smartphone for web access and email access in this study. Hence the duration they use their smartphone for medical related apps is limited.

For open ended questions, most physiotherapy students reported that smartphone is very convenient to use and easy to access and they preferred dictionary and physiotherapy related apps. Only half of the Malaysian students (56.9%) are using university linked apps in their smartphone. This shows that the university linked app is not attractive for the students to use. So the university has to consider this and make the app more attractive and useful for the students.

No first year students answered the questionnaire. It may be due to lack of awareness of medical related apps use as they just enter the course. Because of this we do not know the first year physiotherapy students' perception about using smartphone for learning.

Limitations

This pilot study was conducted in small groups of students in one university, which may not reflect the larger physiotherapy student population in Malaysia. So studies with larger samples and in different locations have to be conducted in order to confirm the results reported in this study.

These study samples are younger generation students who are tech savvy. So the usage by students does not reflect the usage in the physiotherapist community. Hence practicing physiotherapist also has to be included in future studies.

No first year students answered the questionnaire. Hence the result may not reflect the complete picture because first year students do not have clinical posting which may affect the result of clinical setting usage.

Conclusion

From this study it is clear that physiotherapy students are using smartphone for their learning in university education as well as in a clinical setting. But their preference concerning the type of smartphone varies from western country students. Physiotherapy students prefer to use medical related apps in their university education for learning, but their usage in clinical setting is less. The increasing trend of smartphone and medical related apps use among students urges physiotherapy academicians to incorporate smartphone technology in their teaching. As physiotherapy students desiring more physiotherapy related apps, the app developers including university have to consider the students' opinion for their future business and development.

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Appendix

** Required*

1. Please state your gender (*Mark only one box)* :* ☐ Male ☐ Female

2. Please state your current year of study (*Mark only one box)* :*

☐ 1st Year ☐ 2nd Year ☐ 3rd Year ☐ 4th Year

3. Do you own an application smartphone (*Mark only one box)*?*

☐ No ☐ Google Android Yes-

☐ Yes-iPhone Yes- ☐ Other smartphone

4. Concerning your smartphone, do you own medical related applications? *
Mark only one box.

☐ No ☐ Yes 11 to 15 ☐ Yes 26 to 30

☐ Yes 1 to 5 ☐ Yes 16 to 20 ☐ Yes 31+

☐ Yes 6 to 10 ☐ Yes 21 to 25

5. Please indicate how you use medical related app:
(You may choose more than one answer)
Check all that apply.

☐ Education-revising ☐ Clinical-ward environment

☐ Education-learning ☐ Clinical-clinic environment

6. Please estimate the frequency you utilize medical applications on your smartphone during clinical attachment compared to university educational time.*
(one of the below options chosen for the categories: 'university education' and 'clinical attachment')
Mark only one box per row.

	Several times a day	Once or twice a day	2-3 times a week	Once a week	Rarely used	Never used
University education	☐	☐	☐	☐	☐	☐
Clinical education	☐	☐	☐	☐	☐	☐

7. In relation to the following types of applications, please indicate how often you use them during educational and/or clinical hours: *
(choice of 'not used', 'Occasionally used', 'often used', 'very often used' and 'used constantly' for each of below. Please select your choice.)
Mark only one box per row.

	Not used	Occasionally used	Often used	Very often used	Used constantly
Physiotherapy technique/Procedure	☐	☐	☐	☐	☐
Clinical score systems/medical calculator	☐	☐	☐	☐	☐
Disease diagnosis/management	☐	☐	☐	☐	☐
Physiotherapy assessment	☐	☐	☐	☐	☐
Procedure documentation	☐	☐	☐	☐	☐
Drug reference	☐	☐	☐	☐	☐
Web access	☐	☐	☐	☐	☐
Email access	☐	☐	☐	☐	☐
Calendar	☐	☐	☐	☐	☐
Password storage	☐	☐	☐	☐	☐
Other (please detail in comment box)	☐	☐	☐	☐	☐

Please detail in comment box if you have selected Other

8. Please estimate the time you spend per day (in minutes) using smartphone applications related to clinical and educational activities *
(one of the below options chosen for the categories 'education' and 'clinical', please circle your option) **Mark only one box per row.**

	None	1-10 minutes	11- 20 minutes	21-30 minutes	31- 40 minutes	41-50 minutes	51- 60 minutes	61+ minutes
Education	☐	☐	☐	☐	☐	☐	☐	☐
Clinical	☐	☐	☐	☐	☐	☐	☐	☐

9. Would you utilize a smartphone app specific to your university? *
Mark only one box.

☐ Yes

☐ No

Please detail any further comments you have regarding your use of medical related smartphone application in the clinical environment:

10. What characteristics would you find useful in a university linked app?

11. Which specific apps would you recommend?

12. What features do you find most useful in a medical related app?

13. If you would like a summary of my findings, please fill in your name and email address on this form and I will be happy to forward my findings to you when the study is completed.

COMPARISON OF FOUR DNA EXTRACTION METHODS FROM CEREBROSPINAL FLUID FOR THE DETECTION OF *Streptococcus pneumoniae* BY POLYMERASE CHAIN REACTION IN MENINGITIS.

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Abstract

Meningitis is defined as inflammation of the meninges that surrounds the brain and spinal cords. The development of Polymerase Chain Reaction (PCR) techniques based on the detection of target genes offers a rapid, DNA-based test for the diagnosis of meningitis from CSF sample. Since clinical specimens have PCR inhibitors, the presence of inhibitory factors are able to influence the performance of PCR. The availability of pure DNA lacking PCR inhibitors as well as a rapid and easy-to-perform DNA extraction protocols are vital tenable performing with the reliable PCR-based diagnostic test. The purpose of the present study was to compare with the four DNA extraction protocols including boiling method, lysis + centrifugation, chelex method, and DNA extraction kit from the spike CSF and cell suspension for the detection of *Streptococcus pneumoniae* by PCR. The target DNA was successfully amplified frochelex method, DNA extraction method and boiling method, however there is no band observed in lysis+centrifugation method. Boiling the method was the most suitable method due to it cost effectiveness, and also have added value because it is simple, and easy to prepare.

Keywords: Meningitis, Streptococcus pneumoniae, Polymerase Chain Reaction (PCR), DNA extraction.

Introduction

Meningitis is an infectious disease associated with high rates of morbidity. Early identification of causative pathogens is vital and useful in preventing life-threatening clinical outcomes and for monitoring purposes. Bacterial meningitis is known to cause disease of early childhood in which more than 50 per cent incidence were reported in children below five years old [8].

The majority of cases of bacterial meningitis have been related to *N. meningitidis, Streptococcus pneumonia,* or *Haemophilus influenza* Type B [4]. Meanwhile less frequently encountered bacterial causes meningitis includes *Mycobacterium tuberculosis, Listeria monocytogenes, Staphylococcus aureus,* Group B haemolytic *Streptococci, Escherichia coli*, and other Coliforms, *Salmonella* species, and Spirochetes. *Streptococcus pneumoniae* is a Gram positive bacteria, which belongs to the species of streptococci. It produces a range of colonization and virulence factors, including the polysaccharides capsule, surface proteins, enzyme and the toxin pneumolysin (ply). *S.pneumoniae* is reported to be the second most common frequent bacterial causing meningitis after Neisseria meningitidis and prevalent cause of sinusitis and otitis media [5].

Polymerase chain reaction method have been widely used worldwide since decades for the diagnosis and detection of infections with *Streptococcus pneumonia* [14]. Ingeneral, PCR appears to be more sensitive than cultures as it is less time consuming. It offers rapid turn-around time as compared to culture and minimizes the need of labors [7]. However the application of PCR to clinical specimens has many potential pitfalls because of susceptibility of PCR to inhibitors, contamination and experimental conditions. The efficiency of DNA extraction is also a critical factor in detection of bacteria using PCR [12,14]. Therefore, sample preparation and DNA extraction methods can affect the result and reliability of a PCR assay and play a vital factor in diagnostic sensitivity [1].

Therefore, we evaluated PCR by comparing four extraction methods for the detection of *S. pneumoniae* in cerebrospinal fluid (CSF) specimen. The objective of this study was to compare four DNA extraction methods including boiling, lysis with centrifugation, chelex and DNA extraction kit (Qiagen) with respect to its rapidity, cost-effectivess and easy to carry out, and analytical sensitivity for the detection of *Streptococcus pneumoniae* in cerebrospinal fluid (CSF) using polymerase chain reaction assay (PCR).

Materials And Methods

Streptococcus pneumoniae

Streptococcus pneumoniae (ATCC strains 41619) was grown on blood agar overnight at CO_2 and grown on Brain heart infusion (BHI) broth at CO_2. The cells were harvested by spin down at 1500rpm for two to three minutes. The pellet was resuspended and rinse twice in 5ml phosphate buffer saline (PBS) and adjusted based on OD: 0.25-3 (~106 cfu/ml of *S. pneumoniae*).

Spiked CSF

A pool of negative CSF-culture specimen for *S. pneumoniae* were used in this study. All negative CSF-culture, CSF specimen were stored at -20°C. It was thawed immediately before spiking to known concentration of *S. pneumoniae*. A small loopful of *S. pneumoniae* was inoculated into 20% BHI. 4ml of pool negative CSF-culture were

added into 4ml of 20% BHI, vortexed and centrifuged at room temperature. After centrifuge, the pellet were washed twice with normal saline and store at -20°C until use.

DNA extraction method

Boiling method: A cell pellet, obtained by centrifugation, was lysed by incubation for five minutes at 100°C in 1:1 cell pellet/ spike CSF : dH_2O.

Lysis+centrifugation: A cell pellet, obtained by centrifugation was resuspended in 15µl of lysis buffer as described by Alfonso Y *et al.* (2008). It was then incubated in 55°C with shaking for 90 minutes. The pellet was incubated with proteinase K for inactivation at 94°C for 10 minutes, centrifuged at 10000 x g for five minutes. The supernatant obtained prior to centrifugation was transferred into a new tube.

Chelex method: A 250µl of cell suspension or spike CSF were extracted by adding 200 µl of 5% chelex and was incubated at 100°C for eight minutes as described by Bahador A. *et al*. (2004).

DNA extraction kit: DNA extraction was performed using QIAamp DNA mini kit (Qiagen, Hilden, Germany) according to manufacturer's instruction.

The DNA pellet obtained by each method were subjected to amplification by PCR to evaluate which extraction method gave best-quality for PCR. All four extraction methods using *S. pneumoniae* strains and spike CSF were run in PCR and compared.

Polymerase chain reaction (PCR)

A 349 bp fragment of *pneumolysin* (ply) gene of *S. pneumoniae* was used as the target for PCR. The following oligonucleotide primers were used: forward ply (3′TTG ACC CAT CAG GGA GAA AG-5′) and reverse ply (5′- CTT GAT GCC ACT TAG CCA AC-3′). The reactions were performed using Gradient Cycler MJ Research (PT 200) in a volume of 20µl reaction mixture contained sterile PCR water, 10X Taq polymerase buffer, magnesium chloride, 5U/µl TaqDNA polymerase, 10 mM deoxynucleoside triphosphates (dNTPs), 25 pmol/ µl of ply *Streptococcus pneumoniae* primers (forward and reverse). PCR amplifications were performed using the following parameters: initial denaturation at 95°C for 5 minutes, followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 58°C for 30 seconds, extension at 72°C for 30 seconds, and the final cycle of extension was carried out at 72°C for 5 minutes and was maintained at 18°C. From the PCR product, an aliquot of 8µl was subjected to a 1.5% agarose gel electrophoroseis containing 0.5 x TBE buffer (pH 8.0) and fluorosafe DNA was used to stain the gel. Electrophoresis was carried out at 90 voltage for 60 minutes, with 3µl of 100bp DNA marker. Gel documentation system was used to observe for the DNA bands under ultraviolet light.

Sensitivity

Cell stock suspension or spiked cerebrospinal fluid was diluted from 10-1 to 10-7 and use as templates in PCR lowest detection limit. One ml of dilution was added to 1 ml of CSF and then mixed by vortexing. Phosphate Buffer Saline was then added until final volume 5mL and mixed. The analytical sensitivity of the PCR was determined using tenfold dilutions ranging from 10-1 to 10-7.

Result

A comparison between four extraction methods was carried out to determine the best method yielding more reliable result. The result of four extraction methods used in this study shows in .

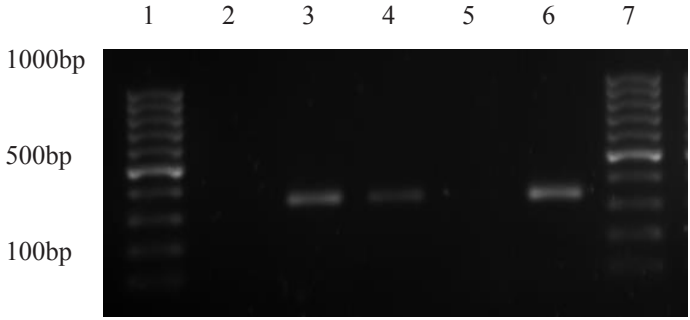

Figure 1. *PCR indicated that all the extraction methods gave expected band at 349bp exceptionally for lysis+centrifugation*

Figure 1 shows PCR amplicons of different extraction methods from celebrospinal fluid for the detection of *Streptococcus pneumoniae*. Lane 1 and lane 7 were 100bp DNA ladder, lane 2 was negative control, lane 3 was chelex method, lane 4 was DNA extraction using kit, lane 5 was lysis+centrifugation, and lane 6 was boiling method.

Discussion

The PCR assay have been widely used in detecting *S. pneumoniae* and other organisms that caused diseases. The assay is a sensitive method and able to detect most of microbial pathogens in clinical specimen including *S. pneumoniae* [14,2]. How be it, the application of PCR to clinical specimens could be influenced by many potential pitfalls and the sensitivity and specificity of the assay largely depends on target genes, primer sequence, PCR techniques, DNA extraction procedures and PCR product detection method [13,14]. The extraction yield of target DNA plays an important role in the PCR amplification in detecting of bacteria in clinical specimens [14].

A comparison between four extraction procedures was carried out to determine which method yielded best result in PCR in detection of *pneumolysin* gene fragment of *Strep-*

tococcus pneumoniae. The best extraction assay can be adopted in future as the standard procedure for the diagnosis of *S. pneumoniae* in CSF. As shown in Figure 1, the DNA obtained from each extraction method used in this study showed a high enough quality to be assayed in PCR, except for lysis+centrifugation method that showed no band observed.

Boiling method is the fastest and easy to perform, it only needs 15 minutes to complete, requiring least labor-consuming method and very inexpensive method. This technique does not require any organic solvent, and the whole procedure only requires the use of one test tube, decreasing the time and the possibility of cross-contamination. In this study, the amplification product was observed and showed best result as the band observed in chelex method.

Lysis+centrifugation is simple method as well, howbeit it is costly as requires the use of proteinase K and time-consuming because it needs to be done in two hours. In addition, as compared to the finding reported by Alfonso Y *et al* (2008)[1], there is no band observed using this method for detection of *S. pneumoniae.*

Chelex method is a fast, cheap and effective method for DNA extraction. Chelex method is a favored extraction method as it is quick. It does not require multiple tube transfers as using extraction kit and it does not use toxic organic solvents such as phenol–chloroform method. But Phillips *et al*. (2012) and Aygan (2006) explained that this method is not able to remove inhibitors (such as haem) which can be detrimental to downstream processes. In this study, the amplification product was observed and the band showed a good intensity as same as boiling method.

As compared to other methods, DNA extraction using QIAamp mini kit have been frequently used in the research field, it is safer. However, it is a demanding procedure as required to transfer into few tubes for several times during the procedure and use more processing time upon completion as compared to others.

Ten CSF-culture negative specimens were pooled and spiked with a known concentration of *S. pneumoniae* and were subjected to amplification. Prior to pooling process, all the specimens were exposed to UV lights to avoid false-positive result.

In order to detect PCR amplification even at lower concentration of bacteria/DNA, the assay have tested the lower detection limit into eight different concentration starting from 20ng, 10ng, 1ng, 100pg, 10pg, 100fg, 10fg and 1fg using 100-fold dilution. The analytical result showed the limit of detection was at 10^{-3} for cell suspension and spiked CSF samples.

Conclusion

From the four extraction methods evaluated, chelex and boiling method provided the greatest yield of DNA. Additionally, boiling procedure was technically simple and was done in less than 1 hour, with approximately in 20 mins of hands-on time. Although extraction with *lysis+centrifugation* was easy to carry out and inexpensive, it produced the poorest DNA yield. DNA extraction using kit also provided good DNA yield however it takes few hours to be completed and very costly. Therefore it would best to suggest boiling method which is the best to be used for DNA extraction due to its rapidity to be perform and cost-effectiveness.

Acknowledgements

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HEXA-PLEX PCR OPTIMIZATION: TOWARDS THE DEVELOPMENT OF A READY-TO-USE DETECTION ASSAY FOR BACTERIAL RESPIRATORY PATHOGENS

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Abstract

A multiplex polymerase chain reaction (mPCR) assay was developed in this study for the simultaneous detection of five common bacteria associated with RTIs; *Haemophilus influenzae, Klebsiella pneumoniae, Pseudomonas aeruginosa, Staphylococcus aureus and Streptococcus pneumoniae.* The aim of the present study was to optimize the mPCR conditions for the best amplifiable target DNA. Five sets of primers were designed to specifically bind to the target bacteria. The bacterial genomic DNA was extracted using commercialized kit and their concentrations were standardized to the final 2 ng/µl. Each of the PCR reagents was optimized one by one while keeping the others constant. An internal control (IC) was incorporated in this hexa-plex PCR assay to rule out false negative result from PCR inhibitors. The mPCR amplicons ranged around 100-600 bp were amplified using the optimized concentrations of primers: *H. influenzae* P6 (0.5 µM); *K. pneumoniae* Mdh (0.2 µM); *P. aeruginosa* OprL (0.2 µM); *S. aureus* FemA (0.2 µM), *S. pneumoniae* Ply (0.2 µM) and IC GlmM (0.2 µM). The other mPCR reagents were optimized as follow: dNTPs (0.2 mM), $MgCl_2$ (2.0 mM) and Taq DNA polymerase (0.75 units). The initial optimization will be used for the development of a rapid, specific and sensitive mPCR-based diagnostic test for RTIs.

Keywords: Respiratory tract infections, Hexa-plex PCR, Haemophilus influenzae, Klebsiella pneumoniae, Pseudomonas aeruginosa, Staphylococcus aureus and Streptococcus pneumoniae.

Introduction

The burden of respiratory tract infections (RTIs) due to the high rate of worldwide morbidity and mortality indicates the importance of established rapid and accurate diagnostic tests, not only for providing early efficient treatments, but also to monitor the diseases tendency [5]. RTIs can be classified into two major classes based on the site of infections; i) the upper RTIs, i.e., pharingitis, laryngitis, sinusitis, colds,

influenza, whooping cough and throat infection, and ii) the lower RTIs, including bronchitis, bronchiolitis and pneumonia, whereby the causal pathogens are mostly bacteria. Although less common in populations, the lower RTIs are more severe and are more likely to cause morbidity and mortality [1], especially during infancy and late-adulthood.

Most of the fatality and severe illness episodes of RTIs are due to pneumonia and other acute lower RTIs. Around 4.2 million deaths of lower RTIs occurred worldwide among all age groups; with 1.8 million of these are children between age 1 to 59 months [6]. The commonest bacteria associated with RTIs are *Haemophilus influenzae, Klebsiella pneumoniae, Staphylococcus aureus, Streptococcus pneumoniae* and *Pseudomonas aeruginosa* [2]. *S. pneumoniae* and *H. influenzae* are frequently isolated in adult community-acquired pneumonia [4]. In contrast, *P. aeruginosa, S. aureus and K. pneumoniae* are the common pathogens of hospital-acquired pneumonia [4].

Identification of the bacteria pathogens using the gold standard bacterial culture followed by confirmation via biochemical tests usually requires two to five days. The delay may urge for the use of broad-spectrum antibiotics, which indirectly results in potentially unnecessary costs, adverse medication effects, and the emergence of multidrug-resistant bacteria. Hence, rapid, sensitive, and specific assays for the detection of RTIs' pathogens is needed for earlier and more accurate diagnosis, which then could lead to better treatment

Research Objective

This study focused on the development of multiplex PCR assay for the detection of five common bacteria respiratory pathogens; *H. influenzae, K. pneumoniae, S. aureus, S. pneumoniae* and *P. aeruginosa*, with an internal control incorporated to monitor PCR inhibition that may lead to false-negative result. In the present study, we aimed for the optimization of PCR reagents used in the hexa-plex PCR assay.

Materials And Methods

1) Primers

Six pairs of primers were designed for *H. influenzae, K. pneumoniae, P. aeruginosa, S. aureus* and *S. pneumoniae*, in which their sizes were distinguishable to one another, approximately between 50 to 100 base pairs. Table 2.1 shows the primer sequences used in this study.

2) Bacteria strains and plasmid

To develop the hexa-plex PCR, the American Type Culture Collection (ATCC™) bacteria strains were used as the reference strains and served as the template DNA in the mPCR. An IC was constructed using Helicobacter pylori's specific glmM gene cloned into pTOPO plasmid. The list of bacteria strains and plasmid was showed in Table 2.2.

3) Standardization of single-plex PCR

The designed primers were tested individually by single-plex PCR and were found to be specific with no alternate binding, neither internally nor in between the target amplicons. The standard single-plex PCR was performed in 20 µl reactions containing final concentrations of 1X PCR buffer, 2.5 mM $MgCl_2$, 0.16 mMdNTPs, 1µM of each sense and anti-sense primers and 0.75 units Taq DNA polymerase enzyme. Using gradient PCR thermal cycler, the optimum annealing temperature was selected at 60°C. PCR program consisted of initial denaturation at 95°C (5 min.), 30 repeating cycles consisting of denaturation at 95°C (30 sec.), annealing at 60°C (30 sec.) and elongation at 72°C (30 sec.), and final elongation at 72°C (10 min.).

Table 1 : List of Primers

PRIMERS	SEQUENCE (5'-3')	TARGET AMPLICONS	SIZE (bp)
GlmMIC	F: AAC TTA TCC CCA ATC GCG CA	Internal control (IC)	110
	R: GCC CTT TCT TCT CAA GCG GT		
Mdh	F : CCG ACC TGT TTA ATG TGA AT	*K. pneumoniae* (KLPN)	204
	R: AAG GTA TTG GAG CGG ATA AT		
FemA2	F: CGC AAA CTG TTG GCC ACT AT	*S. aureus* (STAU)	293
	R: CTC GCC ATC ATG ATT CAA GT		
Ply2	F: TTG ACC CAT CAG GGA GAA AG	*S. pneumoniae* (STPN)	349
	R: CTT GAT GCC ACT TAG CCA AC		
OprL3	F: GAT GGA AAT GCT GAA ATT CG	*P. aeruginosa* (PSAE)	414
	R: GGA CGC TCT TTA CCA TAG GA		
P6	F: TAG CTG CAT TAG CGG CTT GT	*H. influenzae* (HEIN)	584
	R: AAC GAC CCA CTG TGT TGA TGA		

Note:
F - Forward/sense sequence
R - Reverse/anti-sense sequence
bp - basepair

Table 2 : Source of Bacterial DNA Used in Hexa-Plex PCR

TARGET AMPLICONS	SOURCE/TYPE*	CONCENTRATION
Internal control	Extracted pTOPO plasmid cloned with *glm*M gene from *H. pylori*	10 pg
K. pneumoniae	Extracted genomic DNA from ATCC™ 1706	20 ng
S. aureus	Extracted genomic DNA from ATCC™ 25923	20 ng
S. pneumoniae	Extracted genomic DNA from ATCC™ 49619	20 ng
P. aeruginosa	Extracted genomic DNA from ATCC™ 27853	20 ng
H. influenzae	Extracted genomic DNA from ATCC™ 49247	20 ng

Note:

ATCC™- American Type Culture Collection

pg - picogram

ng - nanogram

*** - Plasmid DNA was extracted by using**

Genomic DNA were extracted by using

4) Development of hexa-plex PCR

By using the standard PCR parameters used in the single-plex PCR, the optimization of hexa-plex PCR reagents was done one-by-one, while keeping the other conditions unchanged. To develop a pre-optimized mastermix for the hexa-plex PCR, optimization of the PCR reagents was done as follow:

I. Concentration of the primers

Sense- and antisense-primer mixes were separately prepared by combining GlmMIC, Mdh, FemA2, Ply2 and OprL3 primers into the respective tubes. Concentration of each primer in the mixture was 4 µM. One microlitre of the primer mix was used in the hexa-plex PCR, where the final concentration of each primer was 0.2 µM. Due to the low intensity, the concentration of P6 HEIN primer was tested individually at 0.5 µM, 1.0 µM and 2.0 µM.

II. Concentration of dNTPs

Referring to the standard concentration of dNTPs used in the single-plex PCR, the range was tested between 0.05 to 0.25 mM and run with the optimized concentration of primers.

III. Concentration of $MgCl_2$

Different concentrations of $MgCl_2$ were tested where the final concentrations ranged between 0.5 mM to 3.5 mM. The optimized primer and dNTPs concentrations were used in this stage, together with the fixed concentration of other reagents used in the standard single-plex.

IV. Concentration of Taq DNA polymerase

Taq DNA polymerase was also optimized using a range of concentrations: 0.5, 0.75, 1.0, 1.25, 1.5 and 1.75 (units). The previous optimized reagents were used in this stage.

Results

By using the standardized PCR parameters, hexa-plex PCR assay was developed under these optimizations:

1. Different final concentrations of primer P6 (Figure 1A, Lane 1-3:0.5, 1.0 & 2.0 µM, respectively) were tested with 0.2 µm final concentration of other primers. The result showed that 0.5 µM of primer P6 (Lane 2) was sufficient to amplify *H. influeanzae* target DNA.

2. Using the fixed concentrations of primers, 0.1 mM dNTPs (Figure 1B, Lane 2) was chosen to be used in the hexa-plex PCR assay. Concentrations of dNTPs being tested were 0.05, 0.1, 0.15, 0.2 and 0.25 mM (Figure 1B, Lane 1-5).

3. 2.0 mM $MgCl_2$ (Figure 1C, Lane 3), which showed comparable band intensities, was selected among other tested concentrations (Lane1-7: 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 and 3.5 mM, respectively).

4. When different units of Taq DNA polymerase enzyme were compared (Figure 1D, Lane 1-6: 0.5, 0.75, 1.0, 1.25, 1.5 and 1.75 (units), respectively), 0.75 units (Lane 2) was ideal for all the target band intensities.

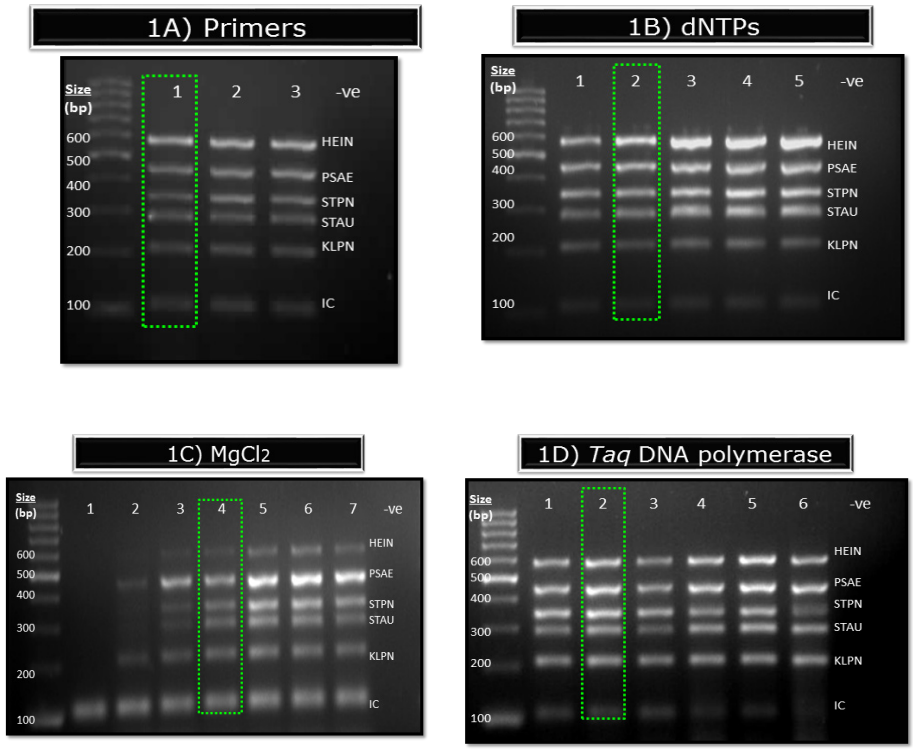

Figure 1: Optimization of PCR reagents; A) primers, B) dNTPs, C) $MgCl_2$, and D) Taq DNA polymerase, for the development of hexa-plex PCR assay.

The optimized conditions with other reagents used for the hexa-plex PCR were summarized below:

Table 3: *PCR components for a 20µl reaction used in this study*

PCR reagents	Volume (µl) used in a 20 µl reaction	Final concentration
Sense-primer mix	1.0	0.2 µM of GlmMIC-F, 0.2 µM of Mdh-F KLPN 0.2 µM of FemA2-F STAU 0.2 µM of Ply2-F STPN 0.2 µM of OprL3-F PSAE 0.5 µM of P6-F HEIN
Antisense-primer mix	1.0	0.2 µM of GlmMIC-R, 0.2 µM of Mdh-R KLPN 0.2 µM of FemA2-R STAU 0.2 µM of Ply2-R STPN 0.2 µM of OprL3-R PSAE 0.5 µM of P6-R HEIN
10X Taq buffer without $MgCl_2$	2.0	1X
$MgCl_2$	2.0	2.0 Mm
dNTPs	0.2	0.1 Mm
Taq DNA polymerase	0.15	0.75 units
PCR-grade water	11.65	-
Template DNA mix	2	As listed in Table 2.2

Discussion

In the absence of a reliable, rapid diagnostic tool, the uncertainties of the causative pathogens have urged for the use of broad-spectrum antibiotics, which indirectly results in potentially unnecessary costs, adverse medication effects, and the emergence of multidrug resistant bacteria. This shows that rapid, sensitive, and specific assays for the detection of RTIs' pathogens is needed for earlier and more accurate diagnosis, which hence could lead to better treatment. The risks of death, morbidity, microbial resistance to drugs and prolonged length of hospital stay due to ineffective therapy consequently could be minimized [3].

In this study, a pre-optimized mastermix PCR was prepared for the development of hexa-plex PCR assay to detect bacteria respiratory pathogens; *H. influenzae, K. pneumoniae, P. aeruginosa, S. aureus* and *S. pneumoniae*, together with an internal control to monitor false negative interpretation. The hexa-plex PCR targets six DNA amplicons as mentioned above with distinguishable sizes to the closest target bands in approximately between 50 to 100 base pairs.

For the initial stage, the present study has successfully developed on the pre-optimized mastermix, based on the standardized single-plex PCR conditions. PCR reagents including primers, $MgCl_2$, dNTPs and Taq DNA polymerase enzyme, were optimized one-by-one while keeping the other reagents constant. The mixture of sense- and antisense-primers was separately prepared by combining together all the respective primers from their same early concentration. The intensities of the bands were comparable except for P6 HEIN, and thus, this primer pair was optimized individually using the primer mixture. Once optimized, the sense- and antisense-primer mix consisted of 0.2 µM final concentration of each GlmMIC, Mdh KLPN, FemA2 STAU, Ply2 STPN, and OprL3 PSAE, plus 0.5 µM of P6 HEIN. Based on the series of tested concentrations, the final concentration of PCR reagents; 0.2 mM of dNTPs, 2.0 mM of $MgCl_2$ and 0.75 units of Taq DNA polymerase, were selected for the preparation of pre-optimized master mix. Lane 2 (Figure 1D) represented the hexa-plex amplicons corresponding to the overall optimized conditions.

As a pre-optimized mastermix PCR, this assay would enable the users to directly run PCR by just adding the recommended volume of water and DNA sample (up to 20 µl per reaction), without the need of calculations and tedious pipetting steps. Hence, this will reduce contaminations that usually occur during a PCR set up, besides providing advantages on its convenience and rapidity. Using the pre-optimized conditions, this hexa-plex PCR assay will further be evaluated for its specificity, sensitivity and also performance of detection by using clinical specimens.

Conclusion

The pre-optimized mastermix hexa-plex PCR, containing final concentrations of 0.2 µM of each primer pairs GlmM IC, Mdh KLPN, FemA2 STAU, Ply2 STPN and OprL3 PSAE, 0.5 µM of P6 HEIN primer pairs, 2.0 mM $MgCl_2$, 0.1 mM dNTPs and 0.75 units Taq DNA polymerase, was successfully developed in this study to simultaneously amplify five targeted respiratory bacteria pathogens along with an IC.

Acknowledgement

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DEVELOPMENT AND OPTIMIZATION OF MULTIPLEX PCR FOR DETECTION OF *Klebsiella pneumoniae* AND *Haemophilus influenzae*

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Abstract

Klebsiella pneumoniae and *Haemophilus influenzae* are two common pathogens associated with respiratory tract infections which can be found in human especially at the respiratory tract system and urinary tract system. These bacteria can cause severe disease such as meningitis, bronchitis, and pneumonia. Culture method and polymerase chain reaction are the current methods that was used to detect bacterial pathogens. However, both methods are time consuming. Thus, development of multiplex Polymerase Chain Reaction (PCR) to detect both bacteria becomes crucial due to the emergence of invasive disease caused by these bacteria. The aim of this study is to develop multiplex PCR (mPCR) for detection of *Klebsiella pneumoniae* and Haemophilus influenzae. The two sets of primers were designed to detect mdh gene of *Klebsiella pneumoniae* and p6 gene of *Haemophilus influenzae*. Commercialized (Qiagen Kits, USA) was used to extract genomic DNA from both bacteria. The target genes produced species-specific amplicons for both bacteria *Klebsiella pneumoniae* (204bp) and *Haemophilus influenzae* (584bp) in which the optimized concentration for mdh gene (0.2µM) and p6 gene (1µM). Moreover, other parameters that were optimized in this study are $MgCl_2$ (2.0mM), dNTP (0.2mM), Taq polymerase (0.75 units) and annealing temperature (58 °C). The multiplex PCR for rapid and simultaneous detection of *Klebsiella pneumoniae* and *Haemophilus influenzae* was successfully developed in this study.

Keywords: Klebsiella pneumoniae, Haemophilus influenzae, Multiplex PCR, Development, Optimization

Introduction

Klebsiella pneumoniae and *Haemophilus influenzae* are two common pathogens associated with respiratory tract infections which can be found in human especially at the respiratory tract system and urinary tract system. These bacteria can cause severe disease such as meningitis, bronchitis, and pneumonia. Based on several research,

Haemophilus influenzae have polysaccharide capsule that is associated with the cause of severe disease like meningitis, septic arthritis, and bacteremia [4]. The most virulent and serious invasive disease of this type is serotypes B which usually can lead to the death. Several studies have been reported that more than 95% of invasive *H. influenza* disease was caused by Hib meanwhile there is few cases on the other serotypes [1]. On the other hand, *Klebsiella pneumonia* is a common pathogen that usually associated with the nosocomial infection particularly respiratory tract system and urinary tract systems. There are some studies that have been proved that nosocomial infection caused by these bacteria has been increased over year [6].

Early detection and identification of *Haemophilus influenzae* and *Klebsiella pneumoniae* is important so that appropriate and early antimicrobial therapy can be administered. The gold standard of identification is by using culture method. However, the major problems of this method are tedious and time consuming. In order to overcome this problem, we develop a multiplex PCR for simultaneous detection of *H. influenza* and *K. pneumonia* in a single assay.

The present study describes the optimization of the primer concentration, magnesium chloride, dNTP, Taq Polymerase and also annealing temperature in multiplex PCR assay in order to develop a rapid and reliable assay for the simultaneous detection of *Klebsiella pneumoniae* and *Haemophilus influenzae*.

Objectives

To develop and validate multiplex PCR (mPCR) for detection of *Klebsiella pneumoniae* and *Haemophilus influenzae.*

Material and method

[a] Bacterial strains and growth condition

Haemophilus influenzae and Klebsiella pneumoniae strains used in this study were obtained from Microbiology and Parasitology Lab, University of Science Malaysia. Both bacteria were cultured in different media such as chocolate agar *(Haemophilus influenzae)* and blood agar *(Klebsiella pneumoniae) and stored at -20 °C*.

[b] Primer design

Two specific sets of primers were designed based on known sequence for P6 gene (584bp) and mdh gene (204bp) for *Haemophilus influenza* and *Klebsiella pneumonia.* The conserved regions of both genes were identified and primers were designed by using Primer BLAST. A set of internal control (100bp) was designed and incorporated into both primers as detection of inhibitor in PCR during amplification stage. All primers were submitted and compared in Genbank to validate the forward and reverse primers.

No	Organism Name	Gene	Forward primer	Reverse primer	Size(bp)
1	*Klebsiella pneumoniae*	mdh	CCG ACC TGT TTA ATG TGA AT	AAG GTA TTG GAG CGG ATA AT	204
2	*Haemophilus influenzae*	p6	TAG CTG CAT TAG CGG CTT GT	AAC GAC CCA CTG TGT TGA TGA	584

[c] Multiplex PCR assay

Multiplex PCR assay was standardized using genomic DNA which was extracted from *Haemophilus influenzae* (ATCC 49247) and *Klebsiella pneumoniae* (ATCC 1706). PCR amplification cycles were performed using a Gradient Cycler MJ Research (PT 200) with one initial denaturation at 95°C for 5min, 30 cycles of denaturation at 95°C for 30secs, annealing for 30secs at 60°C, and extension at 72°C for 30s, followed by an extra annealing temperature at 60°C for 30s and final extension at 72°C for 3min. The PCR product were resolved by 2.0% agarose gel electrophoresis, stained with FloroSafe (1st Base) and visualized under UV illuminator using image analyzer. Negative control (distilled water) and positive control was included in every run of PCR. Few parameters of PCR were optimized starting with primers, incorporated with internal control, optimization of $MgCl_2$, dNTP, Taq Polymerase and annealing temperature. Primer of *Klebsiella pneumoniae* was optimized starting with 1pmol to 0.1 pmol meanwhile primer of *Haemophilus influenzae* was kept constant. Internal control was then incorporated into every reaction mixture including negative control. The magnesium chloride was varied between 0.5mM to 4.0 mM, dNTP was optimized between 0.16mM to 0.5mM, and for Taq Polymerase was adjusted from 0.75unit/µl tp 5.00unit/µl. Lastly, annealing temperature was optimized starting from 54.1°C to 65.8°C.

Result

[a] Optimization of primer

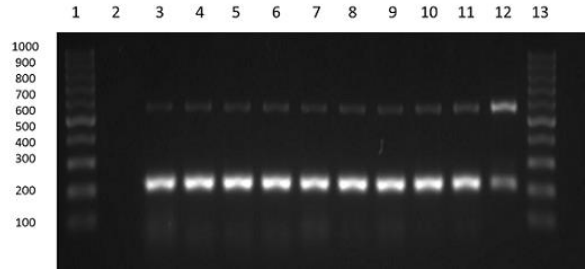

Figure 1. PCR products from multiplex PCR shown on agarose gel. Lane 1 and lane 13, were molecular weight marker (100bp). Lane 2 was negative control, lane 3 was 1pmol/µl of Klebsiella pneumoniae and Haemophilus influenzae, lane 4 was 0.9pmol/µl, lane 5 was 0.8pmol/µl, lane 6 was 0.7pmol/µl, lane 7 was 0.6pmol/µl, lane 8 was 0.5pmol/µl, lane 9 was 0.4pmol/ul, lane 10 was 0.3pmol/ul, lane 11 was 0.2pmol/µl and lane 12 was 0.1pmol/µl while concentration for Haemophilus influenza was kept constant.

[b] Incorporated of internal control

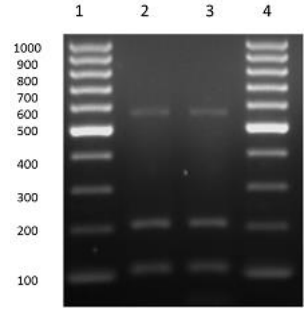

Figure 2. PCR products from multiplex PCR shown on agarose gel. Lane 1 and lane 4, were molecular weight marker (100bp). Lane 2 and lane 3 was duplicate in which internal control at 100bp, Klebsiella pneumoniae at 204bp and Haemophilus influenzae at 584 bp.

[c] Optimization of $MgCl_2$

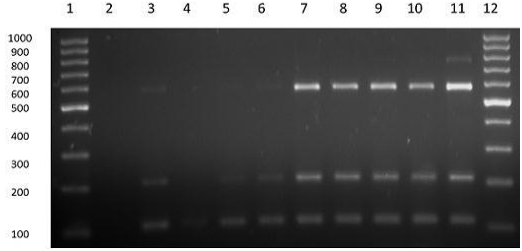

Figure 3. PCR products from multiplex PCR shown on agarose gel. Lane 1 and lane 12, were molecular weight marker (100bp). Lane 2 was negative control, lane 3 was positive control which consist of 0.2pmol/µl of Klebsiella pneumonia, 1pmol/ul of Haemophilus influenza and 1.25pmol/µl of internal control, lane 4 was 0.5mM $MgCl_2$, lane 5 was 1.0mM$MgCl_2$, lane 6 was 1.5mM $MgCl_2$, lane 7 was 2.00mM $MgCl_2$, lane 8 was 2.50mM $MgCl_2$, lane 9 was 3.00mM $MgCl_2$, lane 10 was 3.50mM $MgCl_2$, and lane 11 was 4.00mM $MgCl_2$ while other parameter were kept constant except for $MgCl_2$.

[d] Optimization of dNTP

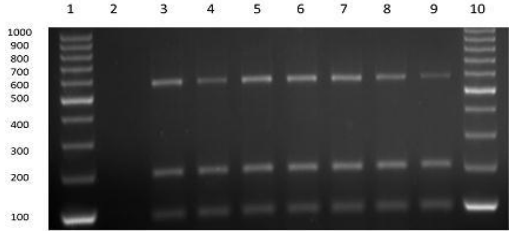

Figure 4. PCR products from multiplex PCR shown on agarose gel. Lane 1 and lane 10, were molecular weight marker (100bp). Lane 2 was negative control, lane3 was positive control which consist of Klebsiella pneumonia, Haemophilus influenza and internal control, lane 4 was 0.10mM dNTP, lane 5 was 0.16mM dNTP, lane 6 was 0.20mM dNTP, lane 7 was 0.30mM dNTP,lane 8 was 0.40mM dNTP, and lane 9 was 0.50mM dNTP, while other parameter were kept constant except for dNTP.

Optimization of Taq Polymerase

[e] Optimization of Taq Polymerase

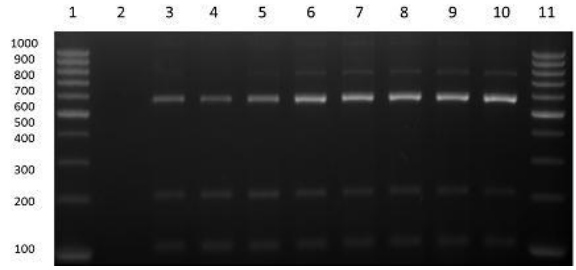

Figure 5. PCR products from multiplex PCR shown on agarose gel. Lane 1 and lane 11, were molecular weight marker (100bp). Lane 2 was negative control, lane3 was positive control which consist of Klebsiella pneumonia, Haemophilus influenza and internal control, lane 4 was 0.50 unit/µl, lane 5 was 0.75 unit/µl, lane 6 was 1.00 unit/µl, lane 7 was 2.00 unit/µl, lane 8 was 3.00 unit/µl, lane 9 was 4.00 unit/µl and lane 10 was 5.00 unit/µl while other parameter were kept constant except for Taq Polymerase.

[f] Optimization of annealing temperature

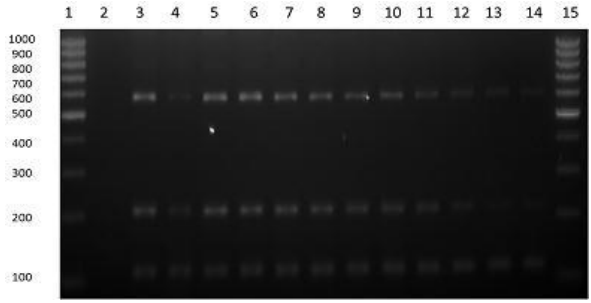

Figure 6. PCR products from multiplex PCR shown on agarose gel. Lane 1 and lane 15, were molecular weight marker (100bp). Lane 2 was negative control, lane3 was 54.1 °C, lane 4 was 54.6°C, lane 5 was 55.5°C, lane 6 was 56.7°C , lane 7 was 58.1°C, lane 8 was 59.5°C, lane 9 was 60.9°C, lane 10 was 62.3°C, lane 11 was 63.5°C, lane 12 was 64.4°C, lane 13 was 64.4°C, and lane 14 was 65.8°C while other parameter were kept constant.

Discussion

Multiplex PCR has been proved to be a reliable method for the detection and evaluation of *H. influenzae* and *K. pneumonia*. Classical method such as culture method for determining the presence of bacteria in general are time consuming, labor intensive and have low sensitivity [5]. In order to overcome this problem, multiplex PCR has been develop to improve the ability to detect bacteria and diagnose disease in fast time since the infection with both bacteria may lead to severe disease including pneumonia and meningitis.

In our study, primers were designed based on specific gene that used to detect both bacteria at the genus and species level. Malate dehydrogenase (mdh) gene has been select-

ed for identification of *Klebsiella pneumonia* due to its function as an essential enzyme in the tricarboxylic acid (TCA) cycle as well as the noncyclic anaplerotic pathway of *Klebsiella pneumoniae* [3]. On the other hand, outer membrane protein P6 has been chosen for because this gene is present in all the strains of *Haemophilus influenzae* and is highly conserved antigen in outer membrane of this bacteria [2].

All parameters such as primer concentration, $MgCl_2$, dNTPs, Taq Polymerase, and annealing temperature were optimized in order to obtain good result. Positive control was included in every run to ensure band of interest are amplified at the correct sizes. Primer concentration was optimized to ensure there is no inhibition factor between primer pairs in multiplex PCR. In this study, only mdh gene was tested with various concentrations from 1pmol/µl to 0.1pmol/µl, meanwhile, p6 gene was kept constant. The best intensities for primer concentration can be seen at 0.2pmol/µl. Incorporation of internal control was used for validation of PCR from inhibiting factor which may lead to false negative result. The combination of primers has been successfully developed for this study.

The optimum concentration of $MgCl_2$ is vital because higher concentration may lead to nonspecific product meanwhile low concentration will reduce amplicon yield. The optimum concentration of $MgCl_2$ can be seen at 2.0mM $MgCl_2$. The concentration of $MgCl_2$ was kept constant during optimization of dNTP. The optimum concentration of dNTP (0.16mM) was chosen based on the constant amplicon intensities obtained for two genes. Both $MgCl_2$ and dNTP was very important to make sure Taq polymerase work at optimal condition.

Then, various concentration of Taq Polymerase was tested and carried out starting from 0.5 unit/µl to 5.0 unit/µl. Taq polymerase is important for the efficacy and specificity of amplification process of PCR. The optimum concentration of this study can be seen at 0.75 unit/µl. Highest concentrations of Taq Polymerase lead to nonspecific product as we can see at Figure 5. Lastly, annealing temperature was been tested by using different temperature. Higher temperature minimized the chances of having nonspecific product, however reducing the intensities of certain target gene. The optimal annealing temperature for this multiplex PCR which has constant intensities is at 58.1°C.

Conclusion

The multiplex PCR for rapid and simultaneous detection of *Klebsiella pneumoniae* and *Haemophilus influenzae* was successfully developed in this study. Moreover, the incorporated internal control prevented false negative in the study.

Acknowledgement

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EFFECTS OF REIKI THERAPEUTIC NURSING PROGRAM ON POST CESAREAN SECTION COMFORT

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Abstract

Objective: To compare the effective of reiki therapeutic nursing program (RTNP) on the comfort of postpartum mother who attained RTNP and standard care who had cesarean section.

Design: Quasi-experimental research.

Methods: Subjects were purposively selected and assigned to either experimental (n=25) or control group (n=25). The experimental group was assigned to attend reiki therapeutic nursing program (RTNP), started at gestational age of 28-30 weeks. Reiki attunement was done, and a first researcher encouraged pregnant women to continue practicing self-reiki for 6 to 8 weeks.

Result: The result showed higher comfort level ($p < 0.05$) at every survey time (12^{th}, 24^{th}, 48^{th}, and 72^{nd} hour) after the postoperative periods.

Conclusion: The finding confirms that RTNP improves body-mind balance and increases inner strength to deal with mental and physical discomfort after a cesarean section. RTNP proved useful as an effective nursing intervention because it increased comfort after post cesarean section. This research suggests that it would be beneficial to train staff nurses in antenatal clinics and postpartum wards in the use of reiki.

Keywords: Comfort, Reiki, Cesarean Section, Therapeutic Nursing Program

Research Purpose

The aim of this study was to compare postoperative comfort level in women who had undergone a reiki therapeutic nursing program and standard care.

Background

Postoperative comfort is an important factor during the recovery after cesarean section because it is referred for to mother and baby's well-being. This care should be provided by nursing. However, if nurse taught patients suitable techniques they will be able to administer self-care, [6] because discomfort after cesarean section causes recovery difficulty and can delay the mother's contact hampering bonding for activities such as breastfeeding, daily hygiene care and establishing a regular sleep pattern which are important in establishing the bond between mother and child. Effective postoperative care after a cesarean section is important for the mother and baby's quality of life too. Balance of the body, mind, environment and society are relevant to holistic comfort and are needed for relief, ease, and transcendence, [10,11]. Thus it is essential to heal the whole person when they are afflicted by illness [5,12].

An analysis of the effects of reiki therapeutic nursing program on postoperative cesarean section comfort requires evaluating body, mind, society and environment level and their impacts on the holistic comfort. A balanced concept of holistic care body and mind is important [2,10,11,14]. Comfort is a holistic outcome because it designates a dynamic and multifaceted state of persons, based on the perception of all the aspects taken together at one time, because the effects in one carry-over effects on other aspects.[12] Nurses should identify comfort need of the patients by taking part in health seeking behaviors. As a result of being comforted in this manner the patients and nurse will be more satisfied with care [20].

The reiki therapy is explained by psychoneuroimmunology concept in the interaction between psychological process in the nervous and immune system of the human body. Reiki practitioners use hands-on contact and energy to transmit change so as to shift the basic DNA structure of our genetic makeup. It is also able to achieve balance of the parasympathetic and autonomic nervous system which is affected by the hypothalamus and pituitary glands to increase endorphins hormone [7, 13, 18,19,24,25].

Hypothesis

Women who gave birth by cesarean section experience greater levels of comfort after receiving RTNP than women who receive standard postoperative care.

Objective

To compare the effective of reiki therapeutic nursing program (RTNP) on the comfort of postpartum mother who attained RTNP and standard care among patient with cesarean section.

Method

Definition

Standard care: Standard care involves the post cesarean mother remaining in routine

nursing care for 12 to 72 hours and quietly receiving pain management for 24 hours, vital were noted sign at time series, and retained fowley catheter etc.

RTNP: The RTNP was provided with reiki treatment by a reiki master who is a registered nurse experienced in reiki therapy for patients.

The experimental group practiced reiki with a researcher and by themselves for 6-8 weeks. The researcher encouraged to use reiki after surgery every 12 hours for 72 hours.

Setting

The study was conducted in the antenatal care unit and postpartum wards of a tertiary university hospital in the province of Songkhla, Thailand between January 1st and June 1st, 2014. The data were analyzed by using frequency, percentage, mean, standard deviation, and *t*-test .

Subjects

During a six month study, fifty cesarean section patients in the antenatal care unit were selected using the purposive sampling method and divided into two groups. Each group-consisted of 25 people. Experimental group attained the reiki therapeutic nursing program and control group receiverd standard treatment. The inclusion criteria were as follows: to be able to read and write Thai, those with gestational age between 28 to 30 weeks and had previously undergone a cesarean section, age between 20-35 years. In addition, those with no infertility problems, those who had been scheduled for a cesarean section at Prince of Songklanakarind hospital, and those who didn't have medical disease, normal pregnancy with previous cesarean section.

Reiki Therapeutic Nursing Program Framework for the study of comfort

Reiki is a balance energy system healing method that can be used as an alternative or complementary therapy for the healing of body-mind-spirit unity in human beings. It involves direct hand-on contact with patients.[3,17,19] The energy from the hands of reiki practitioners emit an energy field of approximately 2-4 MHz that change the biofield of people who recieved reiki treatment.[1,15] Reiki practitioners use hands-on contact and the energy they transmit is able to shift the basic DNA structure of our genetic makeup.[19,25] It is also able to achieve balance of the parasympathetic and autonomic nervous systems [7,13] Relaxation is affected by the hypothalamus and pituitary glands that increases endorphins.[18,24] Reiki practitioners who practice reiki frequently will have better balanced health as a result of being able to recharge, realign, and rebalance their own body.[23] The research used a modification reiki therapeutic nursing program (RTNP) by Keawpimon, 2008.[9]

The steps of the program (RTNP program) include holistic assessment, introduce and providing reiki experience, training and coaching, empowering for continuous use,

observing and reflecting, and fostering to be used after the post-operative period. The six main steps of this framework were conducted by researchers.

Instruments

The demographic questionnaire: The researcher constructed questionnaire to obtain data on age, marital status, educational level, religion, patient with previous cesarean section, tubal resection, gravidity, and pain management 24 hours after cesarean section. The instrument provides a stable assessment of individual differences among postpartum mothers. As previously stated, it was used at the beginning of the study to assess personal data attributes that may have confounded the outcome variables.

Reiki patient record book: Participants continuously used reiki record book for instruction and also allowed women to record their observations and reflections.

The maternal post cesarean section comfort questionnair:

The researcher modified the General Comfort Questionnaire version. [11] It was completed by each respondent at 12^{th}, 24^{th}, 48^{th} and 72^{nd} hour of post cesarean section, to indicate her level of comfort. This 34-items questionnaire consists of both positively and negatively stated items that have been generated from a four-dimensional grid of physical, psycho-spiritual, social and environmental aspects and the three states of relief, ease, and transcendence, as described by the theory of holistic comfort.[11] Items were scored on 5 point Likertscale ranging from "very comfortable" to "uncomfortable." Content validity index was approved by three experts. Reliability was examined with a good result (Cronbach's Coefficient Alpha was 0.84).

Intervention

Participants were assigned by purposive selection to the experimental group or to the control group. All of the pregnant women were screened to ensure that they were happy to take part in the research. Before the research began all patients signed consent form and their confidentiality was protected at all times. Participants in the control group received standard care and met the researcher at the antenatal care unit and then again the day before their cesarean section operation. The experimental group received standard care plus reiki at a gestational ageof 28-30 weeks, 30-32 weeks, and 37 weeks. The experimental group were introduced to the concept of reiki and provided reiki experience by reiki master who was a registered nurse. The researcher encouraged the pregnant women to practice self-reiki on themselves for 6 to 8 weeks during gestational age at 28-37 weeks. The researcher visited the experimental group at antenatal care unit and asked them to provide feedback about practicing self-reiki. The researcher checked each women's reiki record book to confirm that the women had practiced reiki continuously. After surgery the patients were given comfort questionnaires and encouraged to practice reiki every 12 hours for 72 hours (using hand positions of their own choice on the same pattern as pregnancy period.

Results

Demographic Characteristics

The information obtained was analyzed using the SPSS 16.0 software for Windows. The final sample consisted of 50 participants, 25 in the experimental group and 25 in the control group. Ages ranged from 20 to 35 years (M=31; SD= 3.47) all participants were married; 62% had tertiary education, 38% had secondary school education; 30% worked for the government; 42% in the control group were Buddhist, 34% in the experimental group were Buddhist, 8% in the control group were Muslim; 64% had previously undergone cesarean section operation; 68% of women underwent a cesarean section at gestational age of 38 week; 56% underwent a tubal resection procedure, and 84% received an analgesic drug within 24 hours to reduce pain after cesarean section.

Post cesarean section comfort during 12, 24, 48 and 72 hour

See table 1, for comparison of mean score between the two groups of maternal post cesarean section comfort. The experimental group demonstrated significantly higher comfort than did the control group over the 12th, 24th, 48th and 72nd hours after cesarean section.

Table 1 *Comparison of maternal comfort post cesarean section in different period (N=50)*

Times	Experimental group (n=25)		Control group (n=25)		*"t"*	*P*
	M	SD	M	SD		
12 hrs	105.96	11.83	94.40	10.96	-3.582	0.001***
24 hrs	79.68	9.94	68.00	11.69	-3.805	0.000****
48 hrs	112.8	15.75	95.36	14.17	-4.115	0.000****
72 hrs	113.48	11.16	100.36	15.67	-3.408	0.001***

*p< 0.05,**p< 0.01,***p≤ 0.001,****p≤ 0.000

The reports of discomfort between the two study groups were significant forfor 12th, 24th, 48th and 72nd hours (p <0.05). There were significant increase in comfort at the 12th, 24th, 48th and 72nd hour postoperatively. This was similar between the groups when measured with statistic Independent simple *t*-test.

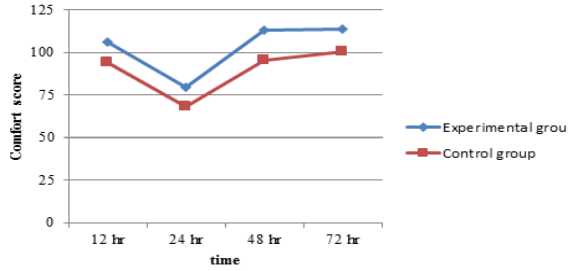

Figure 1. Comfort score of maternal post cesarean section in different period

This figure present that the experimental group shows higher mean score, over the four assessment times after post cesarean section compared to the control group.

Discussion

In this study, the effect of reiki therapeutic nursing program on postoperative comfort was investigated and we have found that it significantly increased postoperative comfort.

Postcesarean section comfort is important issue in maternal care. Several studies have shown the importance of adequate postoperative decreased pain. However, maternal comfort was related to body, mind, society and environment, to achieve well-being, bonding between the mother and newborn, mobilization as well as rehabilitation during post cesarean recovery. The effect of using reiki therapeutic nursing program during pregnancy enhanced maternal comfort and increasing endorphin, and improving comfort after post cesarean section. Those who practiced reiki frequently their body change to a state of relaxation, presumably by down regulating autonomic nervous system tone and will relieves tension and anxiety. According to Richeson, *et.al*, 2010.[14] when implementing reiki to the recipient need for 8 weeks with 45 minutes per week of self-reiki, practitioner developed the sense of autonomy. In this study, there are twenty one participants practicing self-reiki for 6-7 weeks and four participants had practiced self-reiki more than 7 weeks. This enhanced participant's relaxation.[22] Furthermore, self-reiki practice period induced concentration, it helps the brain to function properly. The regular practice of reiki enhanced meditation like state which strengthen the mind of induced individual to be ready for challenging situation.[8] Then participant in this study had developed the confident to face discomfort experienced during the post operative periods.

Conclusion

The study finding suggested that 20-30 minutes of reiki healing practice at least 3-5 times per week for 6-7 weeks, and also continuing self-reiki at every 12 hours post cesarean section is an effective reiki self-care for facilitating maternal comfort. Therefore, the results provide evidence of the benefit using reiki as complementary nursing intervention to improve the nursing quality of post cesarean section.

Limitations

This study indicated that the comfort questionnaire was a researcher-modified method used for the at first time new version of the general comfort questionnaire that is not equivalent of items, which was a consistency in four dimensions.

Acknowledgments

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PRELIMINARY STUDY : THE EFFECT OF APITHERAPY APPLICATION IN LOCALLY ADVANCED CERVICAL CANCER PATIENT

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Abstract

Introduction: Cervical cancer is the second most common cancer among women. When the cancer grow they induce pain in the adjacent pelvic structures, hydronephrosis, discharge and bleeding. There have been great interests in the anticancer properties of natural compounds because they are believed to be relatively non-toxic.

Objectives: To see the apitherapy response through in-vivo test for cervical cancer.

Methodology: Patients were recruited alternatively to either study or control arm. Age 25-75 years old and the tumor stage distribution as FIGO-IB2-IVA. Both groups of patients received similar concurrent chemoradiotherapy policy used in our institution, and apitherapy were suplemented to the patients in study arm during the chemoradiotherapy session.

Results: Twenty three patients were recruited, twelve of them were appointed for the study arm (with apitherapy), and the rest were appointed for the control arm (without apitherapy). Eight patients (66.7%) from twelve patients of study arm showed a complete response after the the treatment, compared with the control arm which only showed 4 patients (36.4%) of complete response out of eleven patients. No differences were detected in toxicities related to concurrent chemoradiotherapy or apitherapy treatment.

Conclusions: This is the first study that used the topical application of apiproducts in addition to standard concurrent chemoradiotherapy for the treatment of locally advanced cervical cancer. Although it is still too early to tell the efficacy of the treatment, but the current results are very convincing.

Key words: Cervical Cancer, Radiotherapy, Chemotherapy, Propolis, Honey.

Introduction

Carcinoma of the uterine cervix is the second most common cancer amongst women in the developing countries. Large bulky tumor and late stage at presentation are quite common in clinical practice. When the cancer grow, they cause pain in the adjacent pelvic structures, hydronephrosis, discharge, and bleeding. Lately there have been great interests in the anticancer properties of natural compounds, because they are believed to be relatively non-toxic such as honey. More than 25% of drugs used during the last 20 years were directly derived from plants, while another 25% were chemically altered natural products [11]. Due to their potential anticancer, antimicrobial and anti-inflammatory properties, natural compounds such as thymoquinone (bioactive constituent of black seed) [3], ginger (*Zingiber officinale* Rosc.) [8] and chrysin (bioactive constituent extracted from honey) [12] have promoted extensive studies on natural products. Induction of apoptosis is one of the key mechanisms of anticancer agents. Apoptosis is a gene-directed programmed cell death characterized by cell shrinkage, blebbing of the plasma membrane, chromosomal DNA fragmentation and other morphological alterations. Failure to regulate apoptosis can lead to pathologies such as cancer and autoimmune disorders [7]. Agent that has strong antioxidant property may have the potential to prevent the development of cancer as free radicals and oxidative stress play a significant role in inducing the formation of cancers [10]. Phytochemicals available in honey could be narrowed down into phenolic acids and polyphenols. Variants of polyphenols in honey were reported to have antiproliferative property against several types of cancer [5].

Methodology

Patients were recruited alternatively to either study or control arm. For the Inclusion criteria, patients were diagnosed of cervical cancer with the histopathological proof of squamous cell or adenocarcinoma. The age distribution are between 25-75 years old and the tumor stage distribution as FIGO-IB2-IVA with adequate renal function. Both groups of patients received similar concurrent chemoradiotherapy policy used in our institution (Nuclear Medicine, Radiotherapy & Oncology Clinic, Hospital Universiti Sains Malaysia, Malaysia) and the apitherapy were suplemented to the patients in study arm during the chemoradiotherapy session. The radiotherapy schedule as follows; external radiotherapy 45 Gy in 20 fractions over 4-week period treating 5-days a week using 6-MV linear accelerator. Supplemental intracavitary brachytherapy were applied twice. Standard brachytherapy were inserted and ICRU-38 recommendation were followed for dosimetry. A radiation dose of 9 Gy was prescribed to Manchester point-A (paracervical point). Concurrent chemotherapy consists of cisplatin 40mg/m2 every week for 4-such on the 1st or 2nd day of the week.

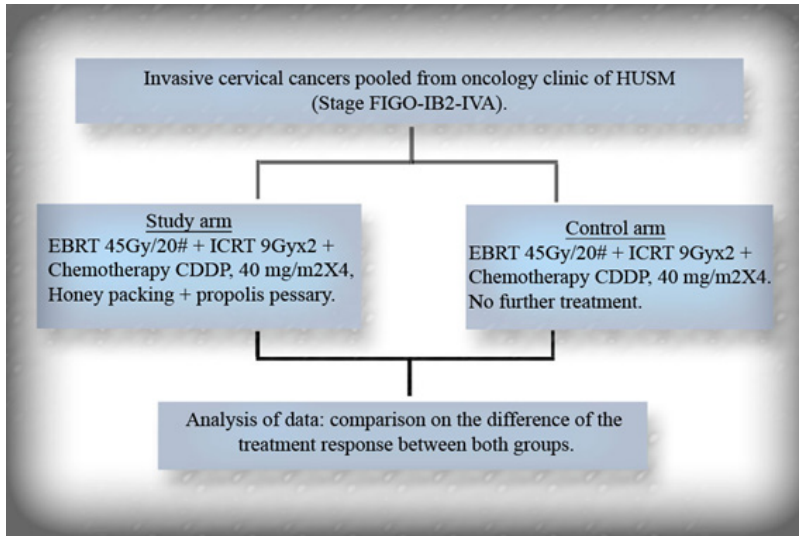

Figure 1 : Study design.

Figure 2 : Tualang honey from FAMA and propolis suppository used in study arm.

Results & Discussion

Table 1 :*The Association of Group Type and Treatment Response*

Group Type	Treatment Response		*p*-Value*
	CR	PR	
T	8 (66.7%)	4 (33.3)	0.146
C	4 (36.4)	(63.6)	

* Pearson Chi-Square

CR = Complete response
PR = Partial residual disease
T = Treatment

Twenty three patients were recruited, twelve of them were appointed for the study arm (with apitherapy), and the rest were appointed for the control arm (without apitherapy). Eight patients (66.7%) from the twelve patients of study arm showed a complete response after the treatment, compared with the control arm which only showed 4 patients (36.4%) of complete response out of eleven patients. Although the *p*-value was not significant (0.146) but the percentage showed the opposite, and one of the factors that affect this significancy was the number of sample size which was too low for each group due to patients recruitment limitations. No differences were detected in toxicities related to concurrent chemoradiotherapy or apitherapy treatment. From this results, probably Tualang honey and propolis enhanced the treatment by inducing the apoptosis of the cervical cancer cells. In one of the studies by [1], they evaluated that the cytotoxic effects of Tualang honey as well as its ability to induce apoptosis in human breast and cervical cancer cell lines. From this outline, chemoradiotherapy still acts to suppress the cancer cells, but not enough to give a full role to suppress the tumor, and also because of the patient complications itself, it might affect the efficacy of the chemoradiotherapy. But with the help of supplemented Tualang honey and propolis pessary, it could enhance this treatment due to their medicinal and health promoting properties like its natural anticancer, natural antibacterial, natural immune booster, natural anti-inflammatory agent and more [6]. Honey may provide the basis for the development of novel therapeutics for patients with cancer and can correlate tumours. Jungle honey fragments were shown to have chemotactic induction for neutrophils and reactive oxygen species (ROS), proving its antitumor activity [2]. Recent studies on human breast [1], cervical [1], oral [4], and osteosarcoma [4] cancer cell lines using Malaysian jungle honey showed significant anticancer activity. Honey has been shown to have antineoplastic activity in an experimental bladder model in *vivo* and in *vitro* [9].

Conclusions:

This is the first study that used the topical application of apiproducts in addition to standard concurrent chemoradiotherapy for the treatment of locally advanced cervical cancer. Intravaginal honey packing and propolis pessary during concurrent chemoradiotherapy were well tolerated in this interim analysis. Although it is still too early to confirm the efficacy of the treatment, but the current results are very convincing. This is a preliminary data with low number of patients and needs further studies to validate. Further patients recruitment and data collection is necessary to determine actual difference in the treatment response.

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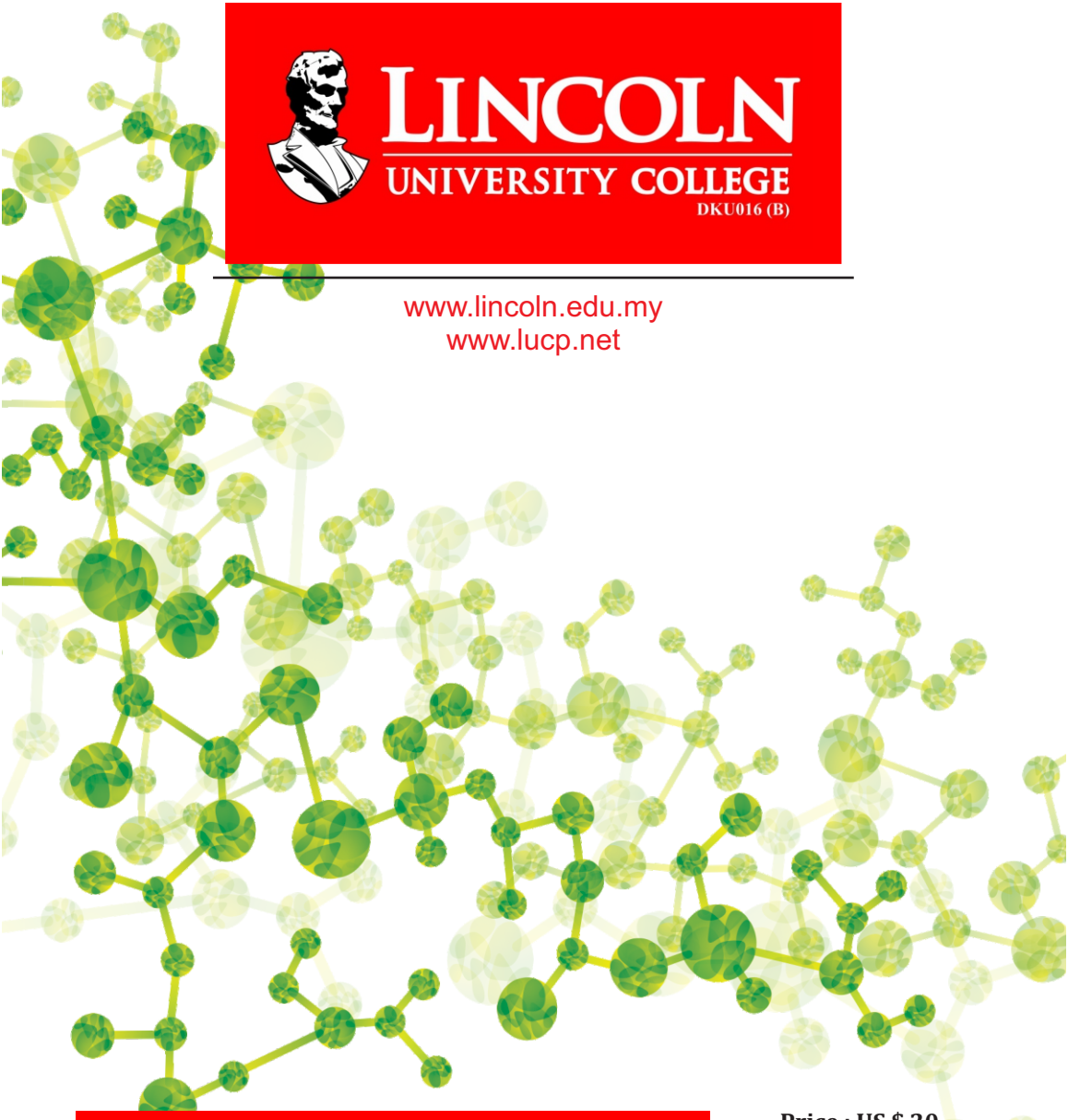

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