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EDITORIAL

Zika on Board

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The Olympics and Paralympic games bring joy to millions of people worldwide. This year it was held in Rio de Janeiro, Brazil amidst the Zika virus epidemic. On 1 February 2016, six months leading to the Olympics, the excitement was dampened by the World Health Organization declaration of the unprecedented vector-borne Zika virus (ZIKV) infection as a public health emergency of international concern [1]. At that point in time, people in Malaysia also felt the heat but had the consolation that the epidemic occurred across the Atlantic on the other side of the world. On 27 August 2016, Singapore reported the first local confirmed case of ZIKV infection in the city-state [2]. It was only a matter of time that Malaysia reported its first confirmed case of ZIKV infection on 2 September 2016 [3]. A 58-year old woman from Klang, Selangor was diagnosed as the first ZIKV case, who had earlier visited her daughter in Singapore who was infected by the ZIKV. As of 22 September 2016, the number of confirmed ZIKV cases in Malaysia has mounted to six with both Polynesia and Micronesia strains [4]. The occurrence of the disease in our continent brings to light how rapidly globalization and free movement of population across geographical borders can accelerate the arbovirus threat across the globe.

The ZIKV is spread through the bite of infected female *Aedes* mosquitoes and evidence has shown it can also spread via sexual and blood transmission [5]. Most of the cases are asymptomatic or subclinical while the symptomatic cases are self-limiting. Other manifestations include neurological (Guillain-Barré syndrome and meningoencephalitis) and autoimmune (thrombocytopenic purpura and leukopenia) complications. More alarming is the association of the virus with infants born with microcephaly as a result of pregnant mothers infected with ZIKV with the risk of microcephaly ranges between 1-13% [6, 7], though the direct causal relationship is still under investigation.

In line with the international response, Malaysia has intensified the surveillance and management activities to control its ZIKV epidemic. These include clinical surveillance, laboratory surveillance, microcephaly and Guillain-Barre Syndrome case detection as well as preparedness and response at all ports of entry [8]. The public health delivery system in Malaysia has improved tremendously over the years following lessons learned from the emerging and re-emerging infectious diseases that affected the country over the recent years. Apart from those commendable measures; in light of this ZIKV outbreak, there are looming questions even though may appear elementary are nevertheless pertinent which the healthcare fraternity should address. Where do we go from here? What more do we need to know and do to help us manage and control this outbreak more efficiently and effectively? These questions would certainly pose a challenge to our public health especially when this arbovirus shares the same transmission vector with dengue and chikungunya i.e., *Aedes* mosquito where the authorities in Malaysia are still struggling to achieve a satisfactory control level in the country.

What shall we do? We need a paradigm shift. We need to look at the re-emergence of ZIKV in a bigger perspective and manage it accordingly. Thus, instead of reacting merely to the notified ZIKV cases, we need to start strategizing how the transmission dynamics of the arbovirus family can be altered; this possibly means to look out of the box for the solutions. The conventional measures for prevention and control should also be complemented with two other elements

which are often neglected and underestimated in most outbreak response i.e., effective communication and social mobilization [9]. We must actively engage in communication with the public to hasten the containment by using all available forms of social medium. With tons of information about the virus in the media, the message should emphasize more on health education; empowering community on the case reports, transmission routes and infection complications rather than general statements on impact and world reaction towards ZIKV [10]. Only then social mobilization, which is commonly underutilized, may help mitigate the social and economic impact during an outbreak. An informed public understands the limitation and the need for the community. Only then we will appreciate the ripple effects - they will bring the community on board, educate the community to actively participate in the outbreak management, and share the responsibility as well as the outcome. Even when the community is faced with great anxiety, an informed public would be able to understand and support any move or decision made by the authorities concerned.

Of late, the controversy which surrounded ZIKV in Malaysia involved the ethical issue in dealing with termination of pregnancy in women with possible ZIKV-related fetal brain abnormalities. This was following a statement made by the Mufti of the Federal Territory, saying that Muslim women could abort their pregnancies if they were infected by the Zika virus to avoid the adverse effect on the lives of their families or the baby itself [11]. In Malaysia, the current law does not provide for abortion for pregnant mothers infected with Zika unless the pregnancy poses a threat to the mother's life [12]. The recent Centres for Disease Control guideline does not include pregnancy termination as an option in managing suspected or confirmed Zika infection. It advocates monitoring the pregnancy with serial fetal ultrasounds in suspected or inconclusive cases and retest for ZIKV when ultrasound suggests abnormalities consistent with Zika infection and fall short in mentioning the alternative path of termination of pregnancy [13]. On the other hand, World Health Organization guideline mentions subtly on the discontinuation of pregnancy as a possible next step in the management of pregnancies with the likelihood of foetal brain abnormalities and states that women who wish to discontinue their pregnancy should receive accurate information about their options to the full extent of the law [14]. The failure to include guidelines on the option of safe, legal termination of pregnancy in Zika-response strategies is not only an issue of reproductive rights but also an issue of reproductive justice [15]. At the time of writing, it is learned that the Ministry of Health of Malaysia will hold a discussion on the matter with the National Fatwa Council regarding termination of pregnancy for women infected by the Zika virus in order to reach a consensus. Irrespective of the outcome of the *fatwa*, we are in the opinion that whether a woman who wishes to carry her pregnancy to term or discontinue the pregnancy should be offered appropriate counselling so that she, together with her partner, will be able to make a fully informed choice on the next step of action.

Despite being a re-emerging disease, there is still much evidence required to effectively manage and control the ZIKV outbreak. The disease behaviour remains dynamic, and a concerted effort by the health authorities and policy makers in implementing the appropriate dynamic alignment to meet the challenges is imperative. It also requires heightened public awareness of personal responsibility which is of paramount importance. The public health preventive strategies remain the cornerstone in the control of this mosquito-borne disease.

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EDITORIAL

Herd Immunity or Heard Not of Immunity?

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INTRODUCTION

The Lancet published this early report by Andrew Wakefield et al on February 28th, 1998; "*12 children (mean age 6 years [range 3–10], 11 boys) were referred to a paediatric gastroenterology unit with history of normal development followed by loss of acquired skills, including language, together with diarrhoea and abdominal pain….Onset of behavioural symptoms was associated, by the parents, with measles, mumps, and rubella vaccination in eight of the 12 children*" [1].

This article flipped the concrete evidence-based success story of vaccination into an emotionally charged and debatable topic of the century. It was only after a decade of much larger studies which failed to replicate their findings that it became evident that there was no association between Measles, Mumps and Rubella (MMR) vaccination and autism. While it is well known that scientific investigations can be wrong but what is unacceptable here is the fraudulent research practice, in this case, the presentation of wrong data, and the lead author's overwhelming undeclared conflict of interest. The aftermath could not be more devastating, Lancet withdrew the paper fully and the loss of his license to practice medicine in the UK in 2010.

THE WIDER IMPLICATION

The MMR-Autism link saga to the medical world meant that more research, time and money were poured to refute the study and also to expose the fraud but the repercussions however were not only confined to the medical profession. The greatest damage was the appalling tangential increase in vaccine refusal among parents worldwide which fuelled the measles outbreak across the United Kingdom (UK), United States and Canada in the year 2008 and 2009. UK for example, saw a drop in vaccination rates from 87.4 percent to 79.9 percent in the year 2000-01 and 2003-04 respectively and not surprisingly, a dramatic increase in measles cases in the UK in the year 2007-08, which was equal to the combined total measles notifications for the past decade [2].

As the news coverage on the controversy intensified, and coupled with advancement of technology in the social media network, the public perception on vaccination has suddenly changed, the most successful health revolution in the 20th century is now at stake. Seemingly increasing public distrust and confusion over the safety of vaccination were echoed and mischievously elaborated geographically, reaching out to most of the third world countries including Malaysia causing the dreaded domino effect of declining immunisation rates in many countries including our own.

THE BLIGHT ON OUR SUCCESS

We began our free national immunisation programme for Diphteria, Pertussis and Tetanus in 1958, the vaccination for Tuberculosis, Polio and Measles were gradually added into the immunization schedule between the years 1960 to 1980s. Malaysia has done very well since, based on the latest Millenium Development Goal (MDG) report in 2015, we have reached almost full coverage for one-year-old intake of the Measles, Mumps and Rubella (MMR) vaccine. The rate of intake of this vaccine was initially 70.1% in 1990, with massive improvement to 94.3% (2008) and 95.2% in 2013 [19], validated by a recent study in 2016 from a rural clinic in Sabah at 98.5% [29].

Lurking behind this success however is the rising trend of parents refusing to vaccinate their children. We now notice an increase in the number of vaccine refusal from 470 cases in 2013 to 648 in the following year and 1292 in 2015. Among the states in Malaysia, Kedah state recorded the highest number of vaccine rejection cases with steady rise from 239 cases in 2014 to 318 cases a year later.

Why is this happening? Data from the state of Kedah health statistics suggested that the major cause for the refusal was the concern regarding the vaccine contents and their religious permissibility (*halal*). This is supported by a cross-sectional study in 2013 done in Perak that showed the main reasons for parental immunisation refusal were preference to alternative treatment (75%), assumption that vaccines have no effect (37.5%) and apprehension on the vaccine contents (25%), other reasons included not being informed regarding vaccination from health practitioners, information from family members and media, religious influence, personal belief and long waiting time in the clinic [18]. In this study the refusal rate was 8 per 10,000 children per year and immunisation defaulter rate was 30 in 10,000 children per year. Vaccine refusal could also be caused by deferral which could be due to either ill infants or parents missing the schedule or appointments [17].

The number of vaccine preventable diseases has also showed steady increment for the past few years, in tandem with the decrement of immunisation rates. Measles cases in Malaysia has quadrupled from 195 cases in 2013 (6.6 cases per million population) to 794 cases up till September 2016 (34.7 per million population). This is certainly a blight on our success and it pushes us off track from the MDG target of global measles elimination by 2015.

APPREHENSION OF VACCINE CONTENT

Certain chemicals are present as ingredients in the vaccines to ensure safety and effectiveness of the final products. These substances naturally exist in the environment and only become toxic if they reach or exceed a certain threshold.

Among chemicals used in vaccine preparation include thimerosal (mercury), which is an organic compound containing ethylmercury. Its primary role is to prevent bacterial and fungal contamination and has been used as vaccine preservative since 1930's [30].

Virtually all vaccines are now mercury-free; and even if present its potential harmful effect is almost negligible as the chemical content in the vaccine is extremely low.

Aluminium is another compound used in vaccine preparation. It acts as an adjuvant to enhance the immune response to the vaccine antigen [30]. Exposure to aluminium from vaccines is well below the current minimum risk level of 2.0 mg/kg per day [30]. Interestingly, the content of aluminium is higher in breast milk compared to vaccines [31] as well in certain medications such as antacids [31].

However, another reason of apprehension that is being used as bone of contention by anti-vaccination campaigners is the permissibility (*halal*) of the vaccine contents.

THE ISLAMIC VIEWPOINT

The objectives of Islamic law (*maqasid shariah*) are the preservation of five fundamental elements in a person; religion, life, lineage, intellect and property. Correspondingly, the maxim of Islamic law (*Qawaid al Fiqh*) adheres to the principle of avoiding harm, thus taking steps towards maintenance of health and this includes vaccine administrations to prevent serious and life-threatening illnesses among children are in accordance to these principles.

As stated earlier, the main religious consternation regarding vaccination among Muslims parents revolves around the issue of permissibility (*halal*) of the vaccine contents. In this regard, many scholars in Islamic Jurisprudence have in fact issued clear ruling (fatwa) regarding the permissibility of most vaccines used as part of national immunisation programmes worldwide, including Bacillus Calmette-Guerin (BCG), Hepatitis B, Diphtheria, Tetanus, Pertussis and Rubella vaccines [20-24].

Differences of opinion however does exist among the scholars regarding vaccines that have substances derived from pork, which are forbidden (*haram*) in Islam, being used during their manufacturing process. As an example, for the production of oral polio and rotavirus vaccines, trypsin enzyme of porcine origin is used during production to

dissociate the virus from cultured cells, but it is later removed through the process of microfiltration. The use of this substance however has led the Malaysian Fatwa Committee National Council of Islamic Religious Affairs in 2008 to issue a ruling that the use of Rotavirus vaccine is forbidden, other religious considerations by the council include the availability of an alternative trypsin source and the absence of an urgent state (*darurah*) for its use. But other opinion does exist which can be considered to be more in tune with the spirit of Islam that discourages complexity in performance of religious duties, the ruling from the European Council of Fatwa & Research in 2003 led by Yusuf al-Qardhawi. He concluded that the use of oral polio vaccine was permissible based on the following reasons; the negligible amount of trypsin used in the vaccine preparation, the fact that trypsin is filtered and thus not detectable in the final vaccine, and finally what is forbidden (*haram*) is made permissible in the state of necessity. As a result of this ruling, many Muslim countries such as Saudi Arabia, Bahrain, Yemen, Qatar, Iraq, Morocco, Sudan and Pakistan [20] have incorporated Rotavirus vaccine that uses porcine trypsin in their national immunisation programmes.

THE WAY FORWARD

This requires efforts by all relevant stakeholders, government and non-government, to reverse the trend we see locally as well as worldwide. One great stride forward was the WHO approved Global Vaccine Action Plan, a framework to prevent millions of deaths by 2020 through more equitable access to existing vaccines for all peoples in all communities [30]. The aims here are to strengthen routine immunisation to meet vaccination coverage target, accelerate control of vaccine-preventable diseases as well spur research for development of new and improved vaccines [30].

Healthcare providers are undoubtedly the front liners in educating the parents and clarifying any doubts which may prohibit vaccine adherence among them. We know that counseling parents with clear information about the risks and benefits of vaccines, and taking advantage of clinical consultation visits for explanation of immunisation are among the most effective strategies suggested to achieve this [31]. In Malaysia, forums and educational talks to the general public are actively organised by the Malaysian Ministry of Health and other non-governmental organisations to reach for these parents at all levels and localities. Besides that, social media is also very effective and is a borderless educational platform to reach the community.

Finally, the history of vaccination had been a great success story of the last century, Measles vaccination alone has been estimated to have helped save 17.1 million lives in the year 2000 [27]. Lack of knowledge on the issue compounded with contradictory information in social media have led to the disruption of herd immunity that previously had been the gate keeper in protecting our children from vaccine-preventable disease. We must do all we can to ensure it remains a success.

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REFLECTIONS

Medical Education in Malaysia: The Evolving Curriculum (Part 1)

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THE TRADITIONAL CURRICULUM

Medical education in Malaysia has evolved in the past 50 years since independence. This paper highlights the various stages of curriculum development that were made to meet the needs of the developing country.

Malaya was under British rule between the 18th and the 20th Centuries. British Malaya as it was then known comprised of the Peninsular States and the Straits Settlements of Penang, Malacca and Singapore. Western medicine was introduced to the then Malaya in Singapore, with the setting up of the first medical school in 1907. It was called the Government Medical School and later became known as the King George VII College of Medicine in Singapore. In 1910 the first batch of seven male Medical graduates received their Licentiate in Medicine and Surgery (LMS) [1].

In 1949, the University of Malaya was established, based in Singapore, with a branch set up in Kuala Lumpur in 1959. In 1961, both governments of Singapore and Malaya agreed and passed legislation in Parliament to make the Kuala Lumpur Campus an autonomous body known as the University of Malaya; with its own medical school and teaching hospital. Thus in 1962, the government approved the setting up of the University of Malaya Medical Faculty, together with its teaching hospital, in the Klang Valley. The Faculty became fully functional in 1964 with the first intake of 64 medical students. After the hospital was built, the whole complex was named the "University of Malaya Medical Centre (UMMC)", with facilities for undergraduate medical teaching, hospital services, the nursing school and other ancillary services put in place [2].

Professor Thumboo John Danaraj who was then Professor of Medicine in the Medical Faculty at the University of Singapore, was appointed as Founding Dean of the Medical Faculty, University of Malaya in Kuala Lumpur [3]. With his appointment, the process of "head hunting" and appointment of academic staff began together with the selection of potential students for the first academic session.

It was mandatory that the Faculty get relevant and competent professionals to start the ball rolling. These medical academicians came from different parts of the globe, including Sri Lanka, Canada, Singapore and the UK (Figure 1).

Front Row: Professor Winifred Danaraj, SPM (2nd from left), Professor TJ Danaraj, Dean (5th from left), Professor Loke, Biochemistry (6th from left)
Back Row: Professor Chan Kok Ewe, Pharmacology & other Professors (from left to right)

Figure 1 The founding teachers: Faculty of Medicine, University of Malaya, 1965 (Courtesy of the late Prof. TJ Danaraj).

With these academicians on board, the toiling of planning and designing of the medical curriculum started since the first batch of medical students was scheduled to enter the medical school in 1964.

Globally, the medical curriculum followed the traditional didactic teaching of basic sciences comprising of anatomy, physiology and biochemistry

in the first year of undergraduate medical course. In the second year, the subjects of pathology, pharmacology, medical microbiology and parasitology were introduced. This was interspersed with topics on communicable diseases and principles of social and preventive medicine (SPM). The thrust of the undergraduate curriculum then was in the various aspects of issues related to social and preventive medicine. This was deemed to be important because the newly formed Malaysia, for the most part, was still mostly rural.

Professor Danaraj, having had experience as an academician in Singapore, felt that the didactic teaching of "dry" basic science subjects may not be perceived as interesting and relevant by the medical students. Thus, early on in the undergraduate medical curriculum, he introduced the clinical correlation classes (CCC) with clinical cases brought to the auditorium to demonstrate the physical signs and correlate them with basic science topics that were learnt during the previous week (Figure 2). This made the preclinical students understand the importance of basic medical science subjects in order to be able to explain the symptoms and the development of physical signs when disease occurs.

Figure 2 Clinical Auditorium, University of Malaya Medical Faculty. Clinical integration with patients starts in year 1 (CCC) (1967) [Courtesy of the late Prof. TJ Danaraj].

This was perhaps the earliest change in the curriculum to facilitate the teachers to think about possibilities of making basic science "dry topics" more interesting to the students. This gradual introduction of clinical medicine into basic science "preclinical years" and *vice versa* in the clinical years was perhaps the beginning of integrated teaching and the evolution of the undergraduate medical curriculum in Malaysia in the late 1980's.

The clinical years begin from years 3 to 5 with rotations in general medicine, surgery, paediatrics orthopedics and obstetrics and gynaecology. In the clinical years, the integration of basic sciences in the form of clinico-pathological case (CPCs) discussions in the final year, sets the stage for future developments in the undergraduate curriculum. The clinical postings and the CPCs were meant to expose the students to develop their critical and analytical thinking skills during their clinical clerkships. Thus, learning to make reasonable diagnoses based on patho-physiological processes that had occurred, with minimal investigative procedures. This was meant to prepare them for their general medical service as medical officers in the rural areas, and also providing them with the basics for future career development.

NATIONAL STRATEGIES TO IMPROVE HEALTH CARE FOR THE POPULATION

Let us now look at the needs of the country then, and how the medical schools were tasked by the Government to contribute towards improving the health services in the then rural Malaysian society.

During the British Administration of the then Malaya, the legacy left behind by the British was a network of health services that extended to the really remote parts of Peninsular Malaya [4], as depicted in Figure 3.

When Malaya had her independence in 1957, the health programs were somewhat coordinated although there was a gross deficiency of doctors to run the district hospitals and the general hospitals. Healthcare then was provided at best by the hospital assistants (now known as medical assistants, MA).

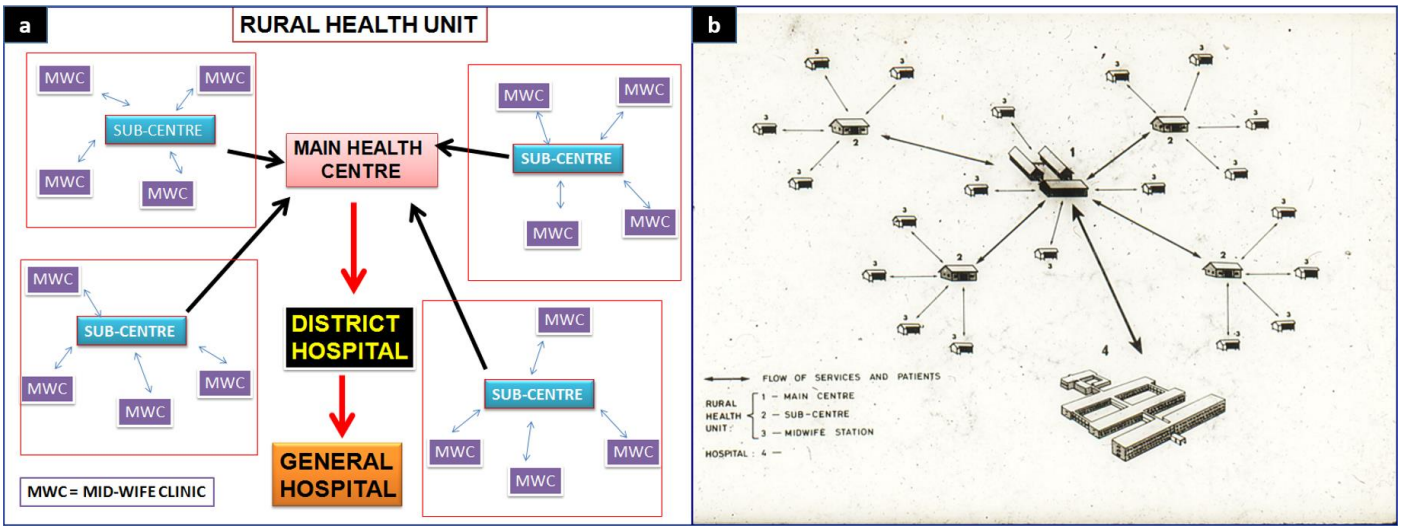

Figure 3 Network of Government run Health Care Services (Legacy from the British Rule of Malaya). a) Rural health unit. b) Replica of the original photo (Courtesy of the late Prof. TJ Danaraj).

During this period the teaching of medicine closely followed the British medical education system that was practiced in the UK. In those early days, the teaching of medicine was by apprenticeship with some knowledge of basic sciences to explain the symptoms.

Then came the didactic (traditional); and scientific discipline model. This preceptor-ship had advantages especially when there was as yet no formal structured curriculum mapping. To this day, clinical mentoring and preceptor-ship is practiced to some extent in the clinical ward rounds with bedside teaching. The concept of mentoring and development of clinical acumen was very apt in clinical practice; both during the undergraduate days and continues in the world of medical academia to this day. This is an art that is slowly dying with the advent of investigative medical practices.

While doctors have to know how to use modern investigative tools, clinical acumen is still required, to be able to make reasonable diagnosis and institute treatment; to be able to determine what investigations are appropriate and when referrals are necessary. This is so because government-sponsored medical graduates face compulsory service that may be in rural areas where there is scarce advanced investigative tools to aid them in making the diagnosis.

In the 1990's with the inevitable trend of producing more specialists, it was deemed necessary for the Ministry of Health to ensure that there will be enough primary care providers and general physicians who would approach patients in a holistic manner. This was tasked to the universities to take the lead to develop programs to train medical officers as generalists and family and primary care physicians.

Medical schools in Malaysia, in developing their medical curriculum, need to address these issues and tailor-make the curriculum to suit the healthcare needs of the country.

To be continued in Part 2: The Blended Curriculum

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ORIGINAL ARTICLE

Quintuplex PCR to Detect Antibiotic Resistance Genes in *Streptococcus pneumoniae*

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ABSTRACT

Introduction: *Streptococcus pneumoniae* is an important bacterial pathogen, causing respiratory infection. Penicillin resistance in *S. pneumoniae* is associated with alterations in the penicillin binding proteins, while resistance to macrolides is conferred either by the modification of the ribosomal target site or efflux mechanism. This study aimed to characterize *S. pneumoniae* and its antibiotic resistance genes using 2 sets of multiplex PCRs. **Methods:** A quintuplex and triplex PCR was used to characterize the *pbp*1A, *erm*B, *gyr*A, *ply*, and the *mef*E genes. Fifty-eight penicillin sensitive strains (PSSP), 36 penicillin intermediate strains (PISP) and 26 penicillin resistance strains (PRSP) were used. **Results:** Alteration in *pbp*1A was only observed in PISP and PRSP strains, while PCR amplification of the *erm*B or *mef*E was observed only in strains with reduced susceptibility to erythromycin. The assay was found to be sensitive as simulated blood cultures showed the lowest level of detection to be 10cfu. **Conclusions:** As predicted, the assay was able to differentiate penicillin susceptible from the non-susceptible strains based on the detection of the *pbp*1A gene, which correlated with the MIC value of the strains.

INTRODUCTION

Streptococcus pneumoniae is an important bacterial pathogen and is a major causative agent of respiratory infections. It also causes otitis media, pneumonia, sinusitis, meningitis, and septicemia and is frequently associated with significant morbidity and mortality [1].

During the early 1940's, isolates of *S. pneumoniae* were highly susceptible to penicillin, which was the drug of choice for suspected pneumococcal infections [2]. However, in the 1970's, penicillin-resistant and multidrug resistant pneumococcus was first reported in South Africa [3]. Since then, the rate of antibiotic resistance among pneumococci isolates to various antimicrobial agents has increased. *S. pneumoniae* resistance to penicillin is due to the alteration of the penicillin binding proteins (*pbp1A, pbp2B, pbp2X*), which reduces its binding affinity to β-lactams. Hence, cross resistance to other β-lactam drugs such as cephalosporins is not uncommon. β-lactam resistance in *S. pneumoniae* has brought a need for alternative antibiotics such as macrolides and fluoroquinolones. These are frequently used as empirical therapy in respiratory tract infection, which has resulted in increased prevalence of macrolide resistant strains of *S. pneumoniae.*

Macrolide resistance in *S. pneumoniae* is a result of modification of the ribosomal target site adenine at position 2058 [4], as the A2058 nucleotide has a key function in the binding of this antibiotic. This modification results in a reduction of macrolide binding affinity to its target or modification of the conformation of binding site. This mechanism of resistance is assigned by the determinant gene, *erm*B. Macrolides resistance is also caused by another mechanism, which is the efflux mechanism of the drug and assigned as *mef*E.

Recently, strains of *S. pneumoniae* have developed resistance to fluoroquinolones, which have a

greater intrinsic activity against this organism. Previous studies have shown that the fluoroquinolones target the type II topoisomerase, DNA gyrase and topoisomerase IV [5, 6]. Amino acid substitution within the Quinolone Resistance Determining Region (QRDR) of these enzymes has resulted in resistance to fluoroquinolones in *S. pneumoniae* [6, 7].

The increase in incidence of multi-drug resistance among *S. pneumoniae* necessitates a need for a tool to identify the presence of these genes within the strains in order to institute appropriate antimicrobial therapy. In this study, we have used 2 sets of multiplex PCRs (quintuplex and triplex PCR) assay to identify and characterize the antibiotic resistance genes, *erm*B, *pbp*1A, *gyr*A, *mef*E simultaneously with the *S. pneumoniae* species specific pneumolysin gene and the common eubacteria gene.

METHODS

Bacterial Strains

One hundred and twenty pneumococcal isolates were obtained from clinical samples processed at the Microbiology Laboratory of the University Malaya Medical Centre, Malaysia from March 1999 to December 2003. The isolates were obtained from both pediatric and adult patients. The source of the isolates included blood, nasopharyngeal secretion, tracheal secretion, sputum and bronchoalveolar lavage. Samples were grown on 5 % horse blood agar and incubated at 37° C in 5 % CO_2 for 12 -15 hours prior to other biochemical and molecular assays.

Strain Identification

The strains were identified as *S. pneumoniae* using conventional microbiological methods including susceptibility to ethylhydrocupreine disc (optochin), catalase test and bile solubility.

Susceptibility Testing

The antibiotic susceptibility of the strains was tested on Mueller Hinton Agar (Oxoid) plates containing 5 % sheep blood (Oxoid), incubated at 37° C with 5 % CO_2 using the agar dilution method as described [8]. The antimicrobial agents used were penicillin, cefotaxime, ceftriaxone, and erythromycin. The antibiotics were obtained from Sigma Aldrich (Sigma Chemical Co., St. Louis, Mo). *S. pneumoniae* ATCC 49619 was used as control. Strains were also tested against ciprofloxacin (Bayer, Germany), moxifloxacin (Bayer, Germany), gatifloxacin (Bristol-Myers Squibb, New Jersey, USA) and levofloxacin (Daiichi, Japan).

DNA Extraction

Genomic DNA was extracted from the bacterial culture using a previously described method [9]. Bacterial colonies suspended in 15 µl of dH_2O containing 50µg/mL were incubated at 37° C for 10 minutes. This was followed by addition of 10µg/mL Proteinase K and 0.1mM Tris HCL pH 7.5 and incubated at 37° C for another 10 minutes. Subsequently, the suspension was boiled for 5 minutes and finally centrifuged at 13000 rpm for 2 minutes. The supernatant obtained was used as the template in the PCR reaction. The extraction of DNA from blood samples was carried out using the standard phenol-chloroform extraction protocol [10].

PCR Amplification

The primers used in this study were extracted from previously published sequences [11]. The optimal PCR condition for a 50 µl reaction included 1X PCR buffer, 1.5mM $MgCl_2$, 0.2 mM dNTP mix, 2U Taq Polymerase (Fermentas) and 20 pmol of each primer. The PCR cycling parameters were as follows: An initial denaturation step at 94° C for 5 minutes, 15 cycles of amplification performed as follows: denaturation at 94° C for 30s, annealing temperature at 40° C for 30s and extension temperature at 72° C for 30s. Thereafter, another 20 cycles of amplification were completed as above but with an annealing temperature of 46° C and finally completed with an extension at 72° C for 2 minutes. The amplification reaction was performed in an Eppendorf Gradient Mastercycler. The PCR product was electrophoresed on a 2 % TAE agarose gel for 1 hour at 70V and the bands were analyzed using a UV trans illuminator.

Determination of Sensitivity and Specificity of the Assay

The sensitivity of the assay was determined using pure bacterial cultures and spiked blood samples. Colonies

from overnight grown pure bacterial cultures were suspended in 0.85 % NaCl and the turbidity adjusted to an OD equivalent to 0.5 McFarland standards, which is approximately 10^8 cfu / ml. Further 10 fold dilutions of the suspension were made up to 10 cfu / ml. DNA extraction was carried out on each dilution, which was later subjected to PCR amplification. The sensitivity of the assay in the clinical setting was carried out by simulating known concentration of bacterial cultures and human blood samples into blood culture bottles. This was carried out by inoculating 5 mL of healthy volunteer blood into an aerobic blood culture media (BD Biosciences, Becton Dickinson, USA), and the bottle was spiked with 100 µL of diluted culture suspension, which ranged from 10^8 to 10^3 cfu. The final concentration of the bacteria in the blood culture bottles ranged from 10^6 to 10 cfu, considering 25 mL of the culture media (25 mL) in the blood culture bottles. The blood culture bottle was incubated overnight at 37° C, prior to DNA extraction and PCR amplification. Viable bacterial count in the blood culture media was determined before and after the incubation period. The DNA from blood cultures was extracted using the method previously described [10]. The DNA extracted from each blood culture bottle with known concentration of bacterial culture was subjected to PCR. The specificity of the assay was also evaluated using DNA extracted from other gram positive bacteria such as *Streptococcus sanguis* ATCC 10556, *Staphylococcus aureus* ATCC 25923 as well as gram negative bacteria including *Acinetobacter baumannii* ATCC 15308 and *Pseudomonas* sp.

RESULTS

The pneumococcal strains were assigned into 3 groups based on their susceptibility levels to penicillin i.e. penicillin sensitive *S. pneumoniae* (PSSP) for strains with penicillin MIC of < 0.125 µg/mL, penicillin intermediate *S. pneumoniae* (PISP) for strains with penicillin MIC of ≥ 0.125 - < 1.0 µg/mL and penicillin resistant *S. pneumoniae* (PRSP) for strains with penicillin MIC of > 1.0 µg / mL. There were 58 PSSP strains, 36 PISP strains and 26 PRSP strains. Out of the 120 pneumococcal isolates that were tested, 64 strains were sensitive to erythromycin, 12 intermediate to and 44 resistant to erythromycin.

MIC of representative strains used in this study is shown in Table 1. All 120 isolates were characterized using PCR. In Table 1, strains I18, R55, R88 and R100 are representative of PRSP strains, while strains R71, R81, R77, R72 and I99 represent the PISP strains. The control strains used in the study were *S. pneumoniae* ATCC 700676 and *S. pneumoniae* ATCC 49619 representing the PSSP and PISP strains respectively.

Table 1 MICs of the representative strains used in the study

STRAINS	MIC (µg/ml)*			
	PEN	CRO	CTX	ERY
ATCC 700676	0.032 (S)	0.064 (S)	< 0.032 (S)	2.0 (R)
ATCC 49619	0.5 (I)	0.064 (S)	0.125 (S)	0.125 (S)
(I18)	2.0 (R)	1.0 (I)	2.0 (R)	128.0 (R)
(R71)	1.0 (I)	1.0 (I)	0.5 (S)	0.5 (I)
(R81)	1.0 (I)	1.0 (I)	1.0 (I)	2.0 (R)
(R77)	1.0 (I)	1.0 (I)	0.5 (S)	2.0 (R)
(R55)	2.0 (R)	2.0 (R)	2.0 (R)	1.0 (I)
(R88)	2.0 (R)	1.0 (I)	< 0.032 (S)	1.0 (I)
(R72)	1.0 (I)	1.0 (I)	0.5 (S)	> 1.0 (S)
(I99)	0.125 (I)	0.25 (S)	1.0 (I)	0.016 (S)
(R100)	2.0 (R)	1.0 (I)	1.0 (I)	2.0 (R)
(Direct Blood Culture)	S	S	Not tested	S

PEN (S: ≤ 0.06, I: 0.12 – 1.0, R: ≥ 2.0), CRO (S: ≤ 0.5, I: 1.0, R: ≥ 2.0), CTX (S: ≤ 0.5, I: 1.0, R: ≥ 2.0), ERY (S: ≤ 0.25, I: 0.5, R: ≥ 2.0)

Figure 1 illustrates the quintuplex PCR amplification assay on representative strains. The pneumolysin (*ply*) gene, which encodes the *S. pneumoniae* species specific gene, was amplified in all the *S. pneumoniae* strains and the common eubacterial gene, which acts as an internal control was also detected in all the strains. The *pbp*1A gene was not amplified in strains I18, R71, R81, R77, R55 and R100 but had positive amplification in strains R88, R72 and I99. The *erm*B gene was only detected in strains I18, R71 R77, R88, and I99. The eubacterial gene was amplified in all the strains. This confirms the presence of bacterial DNA. The pneumolysin gene (ply) was also amplified in all the *S. pneumoniae* strains which serve as S. *pneumoniae* species specific gene. Amplification of the *gyrA* gene in all the strains does not imply antibiotic resistance but acts as a housekeeping gene in this organism.

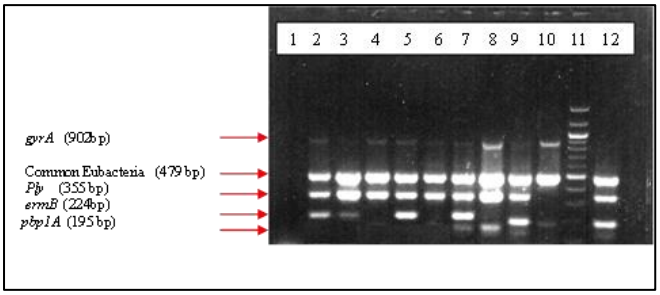

Figure 1 Screening of isolate using quintuplex PCR

Lane 1: Negative control
Lane 2: Strain I18
Lane 3: Strain R71
Lane 4: Strain R81
Lane 5: Strain R77
Lane 6: Strain R55
Lane 7: Strain R88
Lane 8: Strain R72
Lane 9: Strain I99
Lane 10: Strain R100
Lane 11: 100 bp DNA ladder
Lane 12: Direct blood culture isolate

The specificity of the quintuplex PCR was also tested against other gram negative and gram positive bacteria as shown in Figure 2. The *S. pneumoniae* species specific gene, pneumolysin (*ply)*, was only detected in the *S. pneumoniae* strain R81 whereas this gene was not amplified in the other gram positive (*Streptococcus sanguis* ATCC 10556, *Staphylococcus aureus* ATCC 25923) and gram negative bacteria (*Acinetobacter baumannii* ATCC 15308, *Pseudomonas* sp.) that were tested. The gene encoding the common region of the eubacteria was detected in all the bacterial isolates tested. Similarly, the common eubacterial gene was also observed in the blood culture bottles that were spiked with known amount of bacterial suspension.

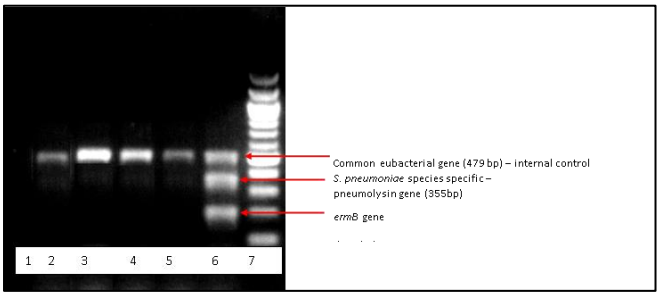

Figure 2 PCR amplification to test the specificity of the assay

Lane 1: Negative control
Lane 2: *Acinetobacter* sp.
Lane 3: *Pseudomonas* sp.
Lane 4: *Streptococcus sanguis*
Lane 5: *Staphylococcus aureus* and) were tested.
Lane 6: Lane *Streptococcus pneumoniae*
Lane 7: 100bp DNA ladder

The lower limit of detection of the assay was determined to be as low as 10 CFU as illustrated in Figure 3. This was evident as the genes were not clearly observed in blood culture bottles spiked with lowest concentration of bacteria (Figure 3).

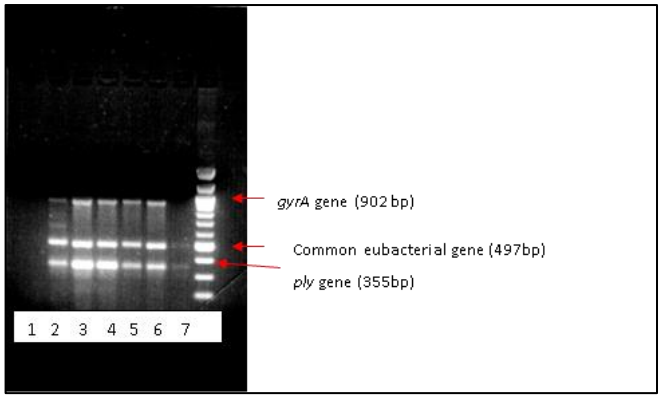

Figure 3 Amplification of the quintuplex PCR assay of simulated blood culture with known concentration of template to test the sensitivity of the assay

Lane 1: Negative control
Lane 2: Concentration of 10^6 cfu
Lane 3: Concentration of 10^5 cfu
Lane 4: Concentration of 10^4 cfu
Lane 5: Concentration of 10^3 cfu
Lane 6: Concentration of 10^2 cfu
Lane 7: Concentration of 10 cfu
Lane 8: 100 bp DNA ladder

The triplex PCR was used in addition to the quintuplex PCR to characterize the macrolide resistant strains, which did not harbour the *erm*B gene. Using the triplex PCR, only 33 of the 44 erythromycin resistant strains harboured the *erm*B gene, while 5 of 12 erythromycin intermediate strains harboured the *erm*B gene. Strains that were negative for the *ermB* gene were further tested for the presence of the *mef*E gene. Results obtained showed that the strains negative for *erm*B harboured the *mef*E gene instead. Figure 4 illustrates the PCR amplification of representative strains using the triplex PCR assay for the detection of *erm*B and *mef*E simultaneously.

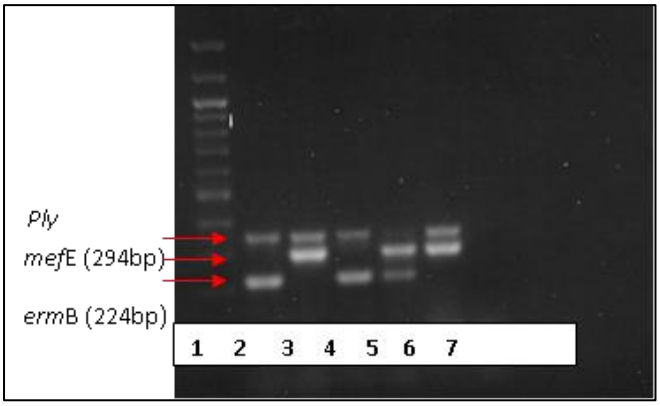

Figure 4 Triplex PCR of the macrolide resistance determinants and *S.pneumoniae* specific gene

Lane 1: 100 bp DNA ladder
Lane 2: Strain I20
Lane 3: Strain I28
Lane 4: Strain R76
Lane 5: Strain R17
Lane 6: Strain R46
Lane 7: Negative control

DISCUSSION

β-lactam antibiotics inhibit the growth of *S. pneumoniae* by inactivating the penicillin-binding proteins (PBPs), which are essential in cell wall synthesis. Resistance to β-lactams is due to alterations in the PBPs, resulting in reduced affinity of binding of the drug. In this study, the amplification of *pbp*1A gene is an indication of non-altered PBPs whereas no amplification of the *pbp*1A gene indicates alteration in the gene. Previous studies have shown that alteration in PBPs 2B, 2X and 1A confers penicillin resistance [12] but only alterations in *pbp*2X and 1A confers cefotaxime resistance [13]. However, alteration in *pbp*1A is essential to confer high-level resistance [14, 15, 16], hence the identification of *pbp*1A could be useful for detecting high-level penicillin resistance.

In this study, the *pbp*1A gene was detected by the quintuplex PCR in the 58 strains that were sensitive to penicillin but was not detected in all the 36 strains and 26 strains that were intermediate and resistant respectively to penicillin. The negative amplification of *pbp*1A gene indicates an alteration within the *pbp*1A, whereas presence of the gene indicates a susceptible strain with no alteration. An alteration in the penicillin binding proteins reduces its affinity of binding to β-lactams, hence reducing the susceptibility of the antibiotic to the organism. In our findings, amplification of the *pbp*1A gene in strains R88, R72 and I99 indicates that there was no alteration in this gene. However, there could be alteration in the other PBPs that may have caused these strains to develop resistance to penicillin.

Using the quintuplex PCR, the *ermB* gene was detected in 38 strains, of which, 5 were intermediate and 33 were resistant to erythromycin. Some strains that harboured the *erm*B gene had also possessed the *mef*E gene (triplex PCR), which confers resistance to macrolides via the efflux mechanism and some of the strains that were negative for the presence the *erm*B gene, showed positive amplification of the *mef*E gene instead.

An attempt to incorporate the *mef*E gene into the quintuplex PCR was unsuccessful as it was inhibiting the detection of the other genes simultaneously. Macrolide resistance is conferred by the presence of the *erm*B or mefE or both the genes. The *erm*B was incorporated into the multiplex because of its high prevalence. The *erm*B determinant is borne by conjugative transposons related to Tn1545, Tn1545-like elements or a Tn 917-like element, that is part of a larger composite transposon, Tn 3872 [17,18]. This accounts for horizontal transfer of the element and results in a higher prevalence of the *erm*B determinant.

The detection of the *gyr*A gene by PCR in both fluoroquinolones susceptible and non-susceptible strains indicates no significance in the presence of the gene, with regards to the development of fluoroquinolones resistance. However, this gene acts as a housekeeping gene in *S. pneumoniae*. Fluoroquinolones resistance in *S. pneumoniae* is conferred by point mutations within the Quinolone Resistance Determining Region (QRDR). The targets of fluoroquinolones are the type II and type IV topoisomerases encoded by the *gyr*A, *gyr*B, *par*C and *par*E genes. Therefore, point mutations conferring fluoroquinolone resistance may occur in either one or more of these genes. In order to detect these mutations, PCR DNA sequencing needs to be carried out on all the strains.

Isolates from direct blood cultures that were confirmed gram positive by gram staining were also characterized using the two multiplex PCRs. In this study, Streptococcus group B and Viridans group Streptococci were used. The assay showed promising

results as only the internal control gene was amplified by the assay. This shows that the assay is 100 % genus and species specific. The lower limit of detection of the quintuplex PCR of up to 10 CFU/mL would allow characterization of *S. pneumoniae* strains from cultures containing low levels of bacterial load of infection.

The characterization of *S. pneumoniae* strains using multiplex PCR has an advantage with a shorter turnaround time. Detection of *S. pneumoniae* and its antibiotic resistance genes from a bacterial culture is possible within 3-4 hours using the multiplex PCR assays. The identification of the antibiotic resistance genes allows prediction of the appropriate drug therapy. Several investigators have evaluated PCR as a tool for diagnosis of pneumococcal infection [10, 19, 20]. However, previous researchers focused solely on identifying pneumococcal infections from infants and children. Most studies concentrated on detecting the pneumolysin gene in different sources of samples, such as sputum, urine and blood. In this study, we used the multiplex PCR assay to characterize the strains and identify genes encoding antibiotic resistance, which are commonly prescribed; penicillin and macrolides. Other researchers have also detected antibiotic resistance genes via PCR, whereby, PCR has been used to detect different classes of antibiotics separately in different tubes as compared to the multiplex PCR assay, which detects 3 different classes of antibiotics in one tube and also specifically detects *S. pneumoniae* via the detection of the *S. pneumoniae* species specific genes, pneumolysin (*ply).* Therefore, we conclude that characterization of *S. pneumoniae* strains could be carried out using multiplex PCR in order to shorten the turnaround time, hence avoiding unnecessary drug prescription.

Conflict of Interest

Authors declare none.

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