LIVING DANGEROUSLY IN A VIRUS WORLD
ARE WE AT THE
LOSING END?

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Professor Dr. Zamberi Sekawi

INAUGURAL LECTURE SERIES

Professor Dr.
Zamberi Sekawi

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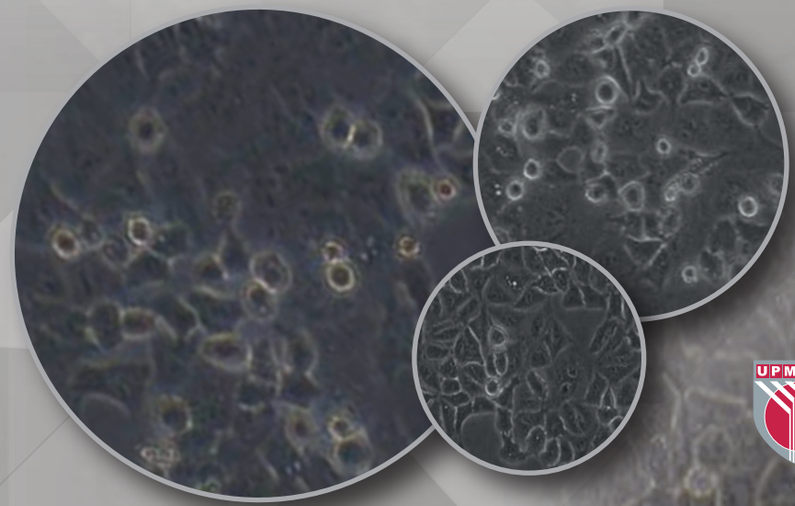

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BSc (Med), MD (UKM), MPath (Microbiol)

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ABSTRACT

Infectious diseases still contribute to significant morbidity and mortality in many countries around the world, which includes Malaysia. Human viruses are among the important causative pathogens that pose serious threats to the world population. It has been demonstrated in over the past centuries that viruses can kill millions through outbreaks, especially infections that involve the respiratory system, such as the influenza pandemic and SARS coronavirus outbreak. These kinds of infections can spread rapidly across the world. Malaysia has had its fair share of fatal outbreaks such as the enterovirus 71 and Nipah virus outbreaks. Four important issues will be highlighted in this paper: the molecular epidemiology of respiratory viruses, prevention of the hand-foot-and-mouth disease, beyond childhood hepatitis B immunisation and adult immunisation. Respiratory viruses are the cause of the most common infections among humans. The vast majority of the infections are asymptomatic and mild in nature, and they are also mostly self-limiting. Molecular methods offer increased sensitivity and specificity in detecting these respiratory viruses. Such methods can also be used to detect other uncommon but important viruses. Molecular epidemiology is a useful and increasingly valuable tool. It can be used to explain viral evolution, its geographical transmission and hospital infection transmission. From these data, we can design strategies and interventions to counteract and prevent infections. The use of single interfering RNA as an anti-RSV agent will also be highlighted. Hand-foot-and-mouth disease used to be a mild and self-limiting childhood disease, but in recent years an increasing number of deaths have been reported due to complications involving the central nervous system particularly involving the related enterovirus 71. This clearly shows that the virus has evolved and mutated and increased in pathogenicity. To counteract such

infections, in addition to maintaining a high level of hygiene, an effective vaccine against the virus is also badly needed. The development of a vaccine for the enterovirus 71 will be highlighted, where it shows good potential, through the use of a combination of inter-buccal and intra-dermal routes, to achieve maximum protection. This combination was shown to elicit the production of both IgA and IgG. Further, the childhood hepatitis B immunisation program has been successful in reducing the prevalence of hepatitis B and hepatocellular carcinoma in Malaysia. However, beyond this childhood programme, we are now increasingly facing the issue of hepatitis B virus mutants and occult hepatitis B infections. This poses a threat of transmissions, particularly during blood transfusion and also organ transplants. What is worrying is that this condition has been found to occur more among vaccinees than unvaccinated individuals. The impact of these mutants and occult infections is also explored. Adult immunisation is thus of growing concern particularly because of its low uptake despite the availability of effective vaccines. Adults need to be vaccinated just like children as the effectiveness of childhood vaccines wanes with aging. Majority of adults, particularly the elderly, have increasing morbidity due to the onset of chronic diseases and thus it is vital for them to get themselves protected via immunisation. Health care professionals should also be immunised against specific diseases to protect themselves and to prevent transmission to their patients. Unfortunately, the uptake of such has been inconsistent, and thus strategies and interventions should be carried out to remedy the situation. We face constant threats from viral infections which have the potential to affect mankind adversely, as we have experienced before. It would therefore be prudent to be at the forefront in tackling these threats.

INTRODUCTION

The mere mention of virus infection is typically bad news for patients. Viral infections can vary from a mild infection to fatal ones. Fortunately, the vast majority of viral infections are asymptomatic. Most viral infections are self-limiting which means that the patients will recover regardless of whether they are treated. Humans and in fact all living things have been constantly interacting with viruses. Whether or not the humans will end up with an infection will depend on the ability of their immune systems to counteract the invasion of viruses.

It has been proven throughout human history that before the advent of vaccines viruses posed a serious threat to mankind. It had the potential to wipe out the human population. The influenza epidemics and pandemics are good examples. Epidemics (or outbreaks) occur on a regular basis, especially that caused by highly contagious viruses, such as respiratory viruses and the measles virus. Measles epidemics, for example, are still a serious threat in Africa, even though an excellent vaccine is available. This shows that the threat from viruses is still relevant and remains dangerous to mankind.

Pandemics occur when an epidemic reaches beyond a few continents by which time a large population of the world would be at risk, predominantly facilitated by the ease of modern day travel. An excellent example is where in 2009 the H1N1 pandemic influenza A reached the entire world within a span of a few months. It has now evolved to become part of the seasonal human influenza. Though the pandemic was for the most part brought under control, it is still prevalent in parts of India, making us aware that it is not easy to contain a virus when its transmission involves a respiratory route.

In Malaysia, we have our fair share of viral infection threats, namely, dengue, Nipah virus encephalitis and enterovirus 71 infections. Additionally there is also the serious threat of blood-borne infections such as the human immunodeficiency virus (HIV), hepatitis B and hepatitis C. The issue of increase in vaccine-preventable diseases (VPD) is of particular importance in the wake of anti-vaccine lobby groups' claims that current vaccines are harmful to children. Dengue epidemics can also be considered to be beyond control as we are dealing with an aggressive and 'perfect' mosquito, *Aedes* sp, where its eggs are able to survive harsh weather, and are pre-loaded with viruses. The mosquito typically takes multiple small bites to achieve a full meal (interrupted meals) and thus is able to transfer the virus to multiple people per full meal.

One of the first major fatal virus outbreaks that occurred in Malaysia was the Nipah virus outbreak. It lasted for nine months beginning from late 1998 and resulted in 265 cases of acute encephalitis with 105 deaths. Humans contracted the infections through close contact with infected pigs. The pigs acquired the Nipah virus from a natural reservoir host, the flying foxes, *Pteropus hypomelanus* and *Pteropus vampyrus* (Looi & Chua, 2007). The outbreak was halted after the culling of over one million pigs which almost caused the billion-dollar pig farming industry to collapse.

Malaysia is currently vigilant of epidemics happening elsewhere, as in the case of the Ebola virus and Zika virus. Contact surveillance is definitely necessary whenever an imported case is reported in Malaysia. The threats of coronaviruses, especially the SARS coronavirus and MERS coronavirus, are even more serious and the impacts are even greater if the infections spread among the population in Malaysia. The threats of the spread of deadly infectious diseases are real and we must thus be ready to deal with them.

Viruses are nanosized 'microorganisms' that are found in abundance on this planet, together with other microorganisms such as bacteria and fungus. Unlike bacteria or fungi, viruses are too tiny to have their own replication mechanism and therefore, utilise the hosts' cell proteins and enzymes for replication. This means that they have to depend on getting entry into cells and 'hijack' the cellular mechanisms in order to survive. In doing so, the host cells are forced to replicate and generate millions of the virus progeny. This may have an impact on the cells themselves. Some cells will be damaged, either mildly or severely, while some would not be damaged. The destruction of the cells will eventually manifest as clinical disease.

Our immune system plays an important role in preventing these viruses from invading more cells. The lymphocytes play a central role in such control. There is thus a complex interplay between the virus and our immune system which will determine the outcome and severity of the infection.

If there is no prior exposure to the virus, the infection can be severe, especially if the virus is one known to cause significant cellular destruction. A good example is the Ebola virus, where it affects the endothelial cells and causes severe haemorrhage, which is the effect of the Ebola viral disease. This also applies to HIV where the virus slowly destroys human lymphocytes rendering them helpless against infections from other microorganisms, even though such infections would be 'trivial' infections in a healthy human. On the other hand, an 'over-reacting' immune system will also create problems for humans and manifest as a clinical disease.

In infections due to the severe acute respiratory syndrome (SARS), patients die due to massive release of cytokines (known as a cytokine storm) involving interferon gamma (Huang *et al,* 2005). This storm destroys the alveoli and makes breathing difficult for

the patients. A similar situation occurred in 2009, during the swine influenza epidemic, where the infection was found to be more deadly among the young than it was among the elderly.

Apart from infections, it is now proven that viruses can cause cancer. Since the virus enter cells for replication, some viruses are able to manipulate the host cells' genes, particularly, the tumour suppressor genes. The tumour suppressor gene is a gene that protects a cell from transforming into a cancer cell. The viruses can also induce mutation of these genes causing a loss or reduction in function, making it progress to cancer, usually in combination with other genetic changes. This leads to uncontrolled growth of the cells which eventually become cancerous. Another mechanism is direct destruction of the cells, as in the case of hepatitis B and hepatitis C. Due to constant destruction and repair, the liver, which has the ability to regenerate naturally, will present with hepatocellular cancer after 10 to 20 years.

Virus survival and ability to sustain infectivity in hosts are attributed to its ability to mutate or modify genes. The mutations are outcomes of replication in the hosts where inefficiency in proofreading the genetic copies result in more mutations. Thus, DNA viruses, which have gene-proofreading mechanisms, have a slower mutation rate compared to RNA viruses. The advantage of mutation is that the virus is able to evade the immune responses of the host resulting from prior exposure, by changing protein configurations for attachment to the host receptors (also called antigenic determinants). Thus, humans can be infected several times by the same virus, which has mutated.

Viruses are thus, amazing creations and have the potential to create havoc among mankind and great potential to wipe out the entire human species.

As a clinical virologist and an academic, I play the role of educating both future doctors and future specialists in the country to be competent in recognising viral infections and in knowing what to do to control, overcome or prevent infections. In UPM we have established, recognised and accredited undergraduate and postgraduate programmes to accomplish this objective. The vital activity is research, which leads to discovery of new and relevant knowledge and consequently better strategies in our fight against infectious diseases in general, and specifically, viral infections.

In this book, I will highlight my research involving four important issues: molecular epidemiology of respiratory viruses, prevention of hand-foot-and-mouth disease, beyond childhood hepatitis B immunisation and adult immunisation. These studies have contributed to new knowledge pertaining to the local context. Some of these studies were fundamental and can be used as a foundation for future studies.

RESPIRATORY VIRUSES

Respiratory infection is one of the most common infections in humans where the majority are caused by viruses. In fact, there are more than 200 viruses that cause respiratory infections. Unlike bacteria, which can be treated with antibiotics, the treatment of respiratory infections is largely symptomatic (except for influenza and respiratory syncytial virus infections). This means that we are totally dependent on our immune system to counteract and prevent the infections.

LOCAL EPIDEMIOLOGY OF RESPIRATORY INFECTIONS

The epidemiology of respiratory viral infections is fairly well established worldwide. However, in Malaysia, related data is scant. Our first study on respiratory viruses was done in year 1999, where we attempted to determine the epidemiology of respiratory viruses among paediatric patients hospitalised with respiratory infections, especially pneumonia, bronchiolitis and laryngotracheobronchitis, through techniques normally available at reference laboratories (Zamberi *et al*, 2003). The viruses implicated were the respiratory syncytial virus (RSV), adenovirus, influenza viruses and parainfluenza viruses. The methods used were direct antigen detection method, shell vial culture and the conventional culture method. The study spanned one year and a total of 222 specimens were received. It showed a dual peak pattern, in the months of April and December. The mean age of the patients was 13 months. Pneumonia (77.9%) was the most common clinical diagnosis followed by bronchiolitis (19.4%) and croup (2.7%). Viral aetiologies were confirmed in 23.4% of the patients. The most common respiratory virus isolated or detected was the RSV, followed by parainfluenza viruses, influenza viruses and adenovirus (Figure 1). This study provided an insight on the common viruses that caused serious respiratory infections that led to hospitalisation among paediatric patients in Malaysia. The epidemiology was almost similar to that in other studies carried out in other countries, where RSV was the predominant pathogen. Doctors should thus be vigilant especially when managing high risk patients, such as children with congenital heart disease, underlying respiratory conditions and premature babies.

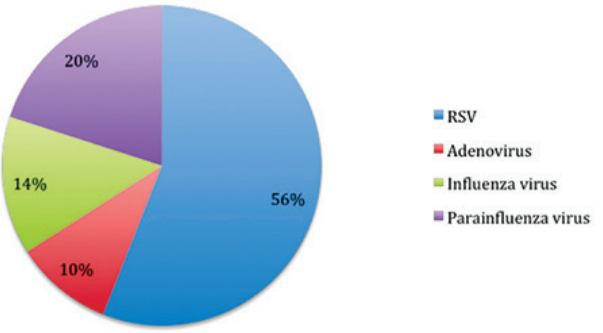

Figure 1 Distribution of respiratory viruses

GENETIC DIVERSITY OF RSV STRAINS

The respiratory syncytial virus (RSV) is a common virus that causes respiratory tract infections. It is a major cause of lower respiratory tract infections, namely bronchiolitis and pneumonia, in children. RSV can cause serious respiratory infections in high-risk paediatric patients, especially those with congenital respiratory or cardiac diseases, and also premature infants.

The RSV genome consists of approximately 15,200 nucleotides, which are transcribed into 10 transcripts encoding 11 different proteins, nine structural and two non-structural proteins. Among these the G protein involves the attachment of the virus to the cell while the F protein assists in viral penetration, fusion and syncytium formation.

RSV is divided into two antigenic subgroups, A and B, on the basis of their reactivity with monoclonal antibodies against G and F proteins. Subtype A tends to cause more severe clinical illness and predominates in most outbreaks. Subtype B is known for its asymptomatic infections and infects a majority of the population. Despite its pathogenic importance there is currently no effective

anti-viral agent or vaccine for RSV. Thus far Ribavirin has been used to treat serious cases but the outcomes have been variable.

Ten years after our previous study, in 2009, we studied the genetic diversity of RSV strains among children below five years of age hospitalised with acute lower respiratory tract infections (ALRTI) at Hospital Serdang (Etemadi *et al*, 2013a). A total of 165 nasopharyngeal aspirate (NPA) samples were obtained and tested for the presence of RSV and other respiratory viruses. RSV was found to be positive in 83 (50%) of the samples, using reverse transcription polymerase chain reaction (RT-PCR). Further classification of 67 RSV strains showed that co-circulation of subgroups A and B, comprised 11/67 (16.4%) and 56/67 (83.6%) of the strains, respectively. There appeared to be a predominance of subgroup B. Further, phylogenetic analysis of 32 sequenced samples showed that all 9 RSV-A strains were clustered within the NA1 genotype while the remaining 23 strains of the RSV-B subgroup could be grouped into a clade consisting of strains with 60-nucleotide duplication region. They were further classified into newly discovered BA10 and BA9 genotypes (Figures 2 and 3). The finding suggested the emergence of RSV genotypes of NA1 and BA. This study further supported the view of there being widespread dissemination of the BA genotypes in the community.

This was the first documentation of the phylogenetic relationship and genetic diversity of RSV strains among hospitalised children diagnosed with ALRTI in a local hospital. Understanding the epidemiology and evolution of the strains will help in designing effective vaccines in future.

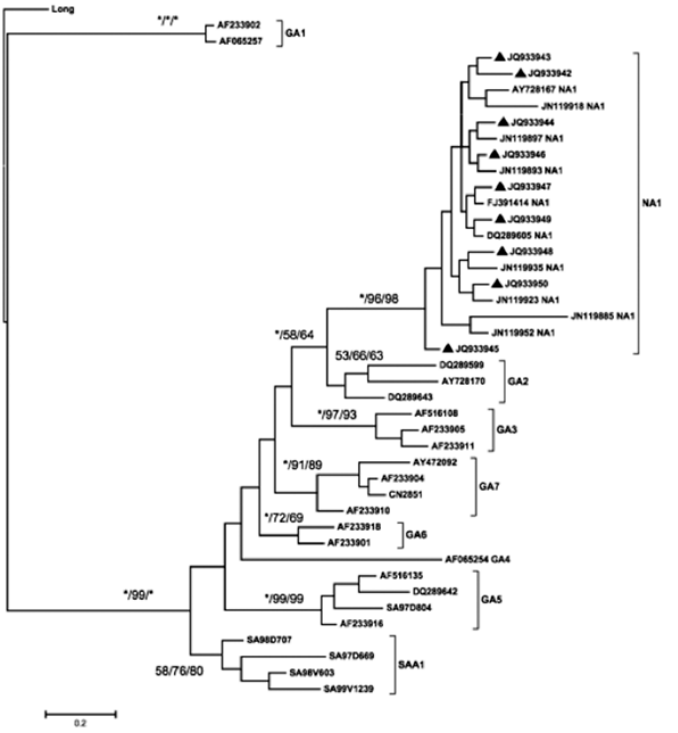

Figure 2 Phylogenetic tree for RSV group A nucleotide sequences based on the second variable region of the G protein (270 bp) constructed by the Bayesian analysis method using MrBayes 3.0 software.

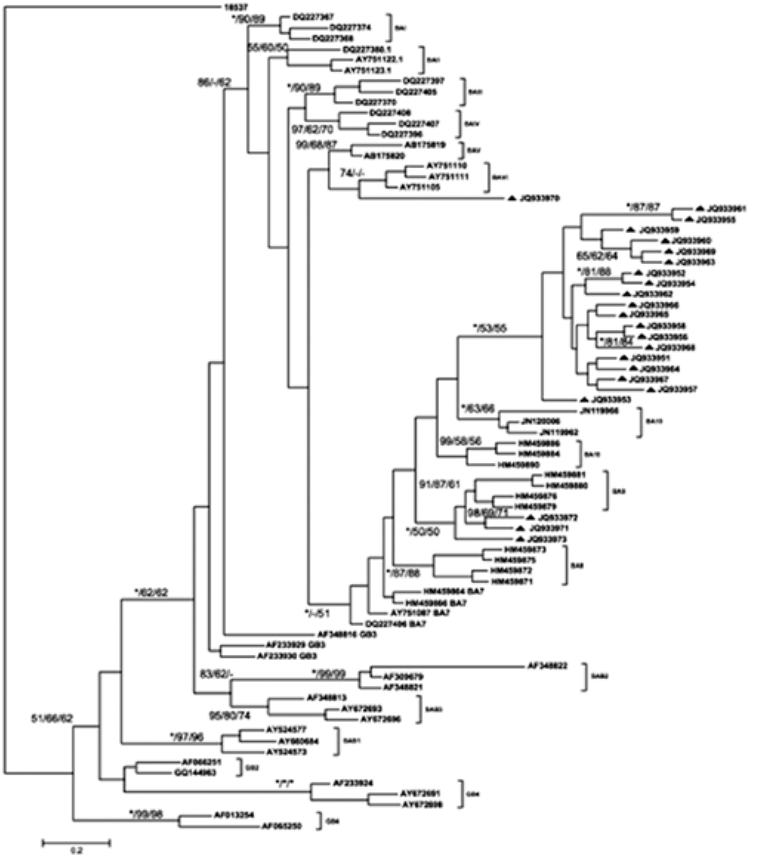

Figure 3 Phylogenetic tree for RSV group B nucleotide sequences based on the second variable region of the G protein (270 and 330 bp) constructed by the Bayesian analysis method using MrBayes 3.0 software.

THE SEARCH FOR AN ANTI-RSV AGENT

The current treatment of RSV is very selective on severe cases, as in the case of severe RSV-induced bronchiolitis. A broad-spectrum antiviral agent known as ribavirin has been used for many years to treat such infections. It is a nucleoside analogue agent, which interferes with RNA metabolism required for viral replication. It is mainly administered in aerosolised form but oral and intravenous formulations have also been used. The problem however is that the outcomes have not been consistent and there are also concerns over toxicity for this age group, especially in terms of respiratory deterioration. Ribavirin is teratogenic and hence, should be avoided for female staff. The oral and intravenous formulations are also associated with haematological toxicity and are contraindicated in renal impairment (Brendish & Clark, 2017).

The search for effective anti-viral agents against RSV is currently on going. Many potential candidates have undergone various phases of clinical trials but unfortunately, to date, there is still no concrete positive outcome.

Our research team thus embarked on an innovative method to target the respiratory syncytial virus. The method involved the use of small interfering RNA or siRNA. It is a class of double-stranded RNA molecules, usually 20 to 25 base pairs in length and operating within the RNA interference (RNAi) pathway. It prevents translation of genes by interfering with the expression of specific genes with complementary nucleotide sequences. This process will degrade the mRNA after transcription. In principle, siRNA can knock down any gene and hence, this powerful characteristic can be used to inactivate viruses.

Apart from the G and F proteins, RSV has other proteins which are involved in the replication process. The small hydrophobic (SH), matrix (M) and M2 proteins are virus transcription factors while

the nucleoprotein (N), phosphoprotein (P) and the large (L) protein are RNA dependent RNA polymerase present in the nucleocapsid of the virus.

The M2-2 protein of respiratory syncytial virus is particularly important in the regulation of viral RNA transcription and replication. It provides an initial high level of mRNA leading to RNA synthesis in the form of a regulatory 'switch' to facilitate virion assembly. This switching is then followed by a shift in favour of genomic viral RNA assembly and the lack of this M2-2 gene can effectively attenuate virus growth.

The M2-2 protein could be a potential anti-viral candidate against RSV infection. In our study, we designed and validated siRNAs that specifically target the RSV M2-2 gene (Chin *et al,* 2016). Four siRNAs targeting different regions of the M2-2 gene were designed using a web tool. *In-vitro* evaluation of the siRNAs silencing effect was performed by using an RSV-infected Vero cell line. Viral M2-2-linked GFP recombinant plasmid was co-transfected with non-targeted siRNA, pooled siRNA, siRNA 1, siRNA 2, siRNA 3 and siRNA 4 using synthetic cationic polymer. The silencing effect on the M2-2 gene at the protein level was measured both qualitatively and quantitatively using fluorescence microscopy and flow cytometry.

The inhibition of the RSV M2-2 gene was further investigated at the mRNA level by transfecting siRNAs with viable RSV. The effectiveness of siRNA in silencing the gene at the mRNA level was assessed using quantitative real time PCR.

This study showed that all the four siRNAs designed could effectively and efficiently silence the M2-2 gene. siRNA 2 showed the highest (98%) silencing effect at the protein level and siRNA 4 with 83.1% at the mRNA level (Figure 4)

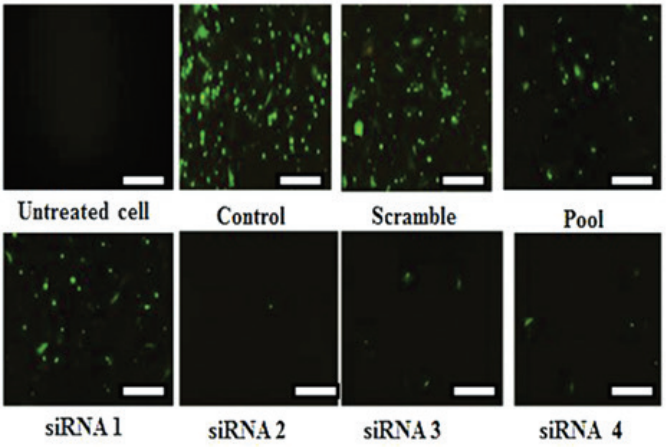

Figure 4 Fluorescence microscopy view of co-transfection of viral M2-2 linked GFP recombinant plasmid with 5 different siRNAs (siRNA1, siRNA2, siRNA3, siRNA4 and pooled siRNA) at 50 nM. Low intensity of green fluorescence emission when compared to the control indicates that viral M2-2 linked GFP recombinant plasmid strongly reduced GFP expression and vice versa. Scramble siRNA served as a negative control. Observation was made 48 h post infection. Scale bars indicate 10 μm.

Viral assay was performed to validate this finding. It showed no cytopathic effects 6 days post-infection with the siRNAs being compared to the control. These experiments confirmed that the M2-2 gene can be successfully silenced by siRNA, leading to successful inhibition of RNA replication and transcription.

In conclusion, this study showed the effectiveness of siRNA in silencing the M2-2 gene both at the protein and mRNA levels, and thus it could potentially be used as a novel therapeutic agent in the treatment of RSV infections. However, further study is warranted to investigate the M2-2 protein silencing effect and subsequent inhibition of RSV infection.

THE IMPACT OF THE HUMAN RHINOVIRUS ON RESPIRATORY INFECTIONS

Human rhinovirus (HRV) is one of the most common viruses that infect man. It belongs to the Picornaviridae family of viruses and comprises three species, HRV-A, HRV-B and HRV-C. In terms of size, it is also one of the smallest viruses, about 30 nm in diameter. It predominantly causes the common cold and other upper respiratory tract infections, such as sinusitis and otitis media. The environment in the nose, with temperature lower than that of body temperature, between 33 to 35^{o}C, facilitates its growth. There are currently 160 serotypes of this virus which pose a challenge in terms of vaccine development.

Human rhinoviruses spread through aerosols of respiratory droplets and contaminated surfaces (or fomites). HRV infection has traditionally been associated with upper respiratory infections. When common cold infections occur, typical symptoms such as sore throat, runny nose, nasal congestion, sneezing and cough will appear. While these symptoms appear to be mild, there are accumulating evidences that HRV has increasingly become an important pathogen in causing ALRTI. In relation to this there are reported cases of bronchiolitis and pneumonia among young children, exacerbations of asthma in older children and exacerbations of chronic obstructive pulmonary disease and pneumonia in older age groups (Etemadi *et al* 2017).

We thus went on to investigate the role of the human rhinoviruses in causing ALRTI in the local context.

We investigated the molecular epidemiology, demographic and clinical characteristics of HRV among children hospitalised with ALRTI in Hospital Serdang (Etemadi *et al,* 2013b). Nasopharyngeal aspirates were collected from 165 children of less than five years of

age. The samples were subjected to reverse transcriptase-PCR for HRV. Phylogenetic analysis of the VP4/VP2 and 5'-NCR regions was used to further characterise the HRV. Other respiratory viruses such as RSV and influenza A virus (IFV-A) were also investigated using semi-nested multiplex RT-PCR assay. Clinical parameters were analysed to differentiate between HRV, RSV and influenza A virus (IFV-A) mono-infections and between HRV species.

We found that RSV (49/165, 29.7%) was still the most common cause of ALRTI in these patients. This is followed by HRV (36/165, 21.8%) and IFV-A (10/165, 6.1%). It was noted that there were 18 cases of co-infections especially with HRV, 14 were dual infections and four were triple infections (Table 1). The most prevalent multiple infection was HRV-RSV co-infection (11/18, 61%).

Table 1 HRV co-infections with other respiratory viruses

Pathogens	No.	%
Total	18	100
HRV + RSV	11	61.1
HRV + HMPV	2	11.1
HRV + PIV-2	1	5.5
HRV + RSV + HAdV	1	5.5
HRV + RSV + HBoV	1	5.5
HRV + FluA + HAdV	2	11.1

Altogether, 54 of the 165 (32.7%) samples were positive for HRV. Majority of the HRV serotypes (61.1%) were identified as HRV-A. No HRV-B was detected. We were the first group to detect the newly discovered HRV-C in Malaysian patients.

The mean age of the HRV-infected patients was 11.8 months as compared to RSV (9 months) and IFV-A (12.7 months). Interspecies comparison revealed that the mean age of HRV-C-infected patients was significantly higher than the HRV-A-infected ones (16.3 vs 7.1

months, p=0.047). The majority of the HRV-A infections were found in children of 6 to 11 months of age, while HRV-C infections were among those of 12 to 23 months of age.

The clinical features of the children infected with the three common viruses (HRV, RSV and IFV-A) were compared. Children infected with HRV were admitted significantly earlier than those infected with RSV and IFV-A. In terms of the duration of hospitalisation, children infected with HRV had the shortest duration (1.9 days, $p<0.001$) compared to those infected with RSV (4.0 days) and IFV-A (4.8 days). There were however, no distinctive differences between the three infections. It was noted that the occurrence of fever with HRV infections was less than that in the RSV and IFV-A infections. Disease severity characterised by the need of oxygen, admission to intensive care unit and prolonged hospital stay however did not differ among the different viral infections. It was reported that diarrhoea was significantly higher in the IFV-A-infected patients (40%, p=0.031) than in the HRV- (8.3%) and RSV-infected (14.3%) ones.

In HRV-infected patients, pneumonia was the most common discharge diagnosis followed by bronchiolitis and post-viral wheeze. In comparing HRV-A and HRV-C infections, vomiting occurred almost two times more with HRV-C infections. However, the HRV-C patients were more likely to present rhonchi and post-viral wheeze than the HRV-A patients. There was no difference in disease severity.

Phylogenetic analysis of HRV strains revealed a very large variation in concomitantly circulating strains (Figure 5 and Figure 6) and was related to other strains from other geographical areas. This indicated that repeated HRV infection by various strains is a norm, as these strains do not confer cross-immunity against one another.

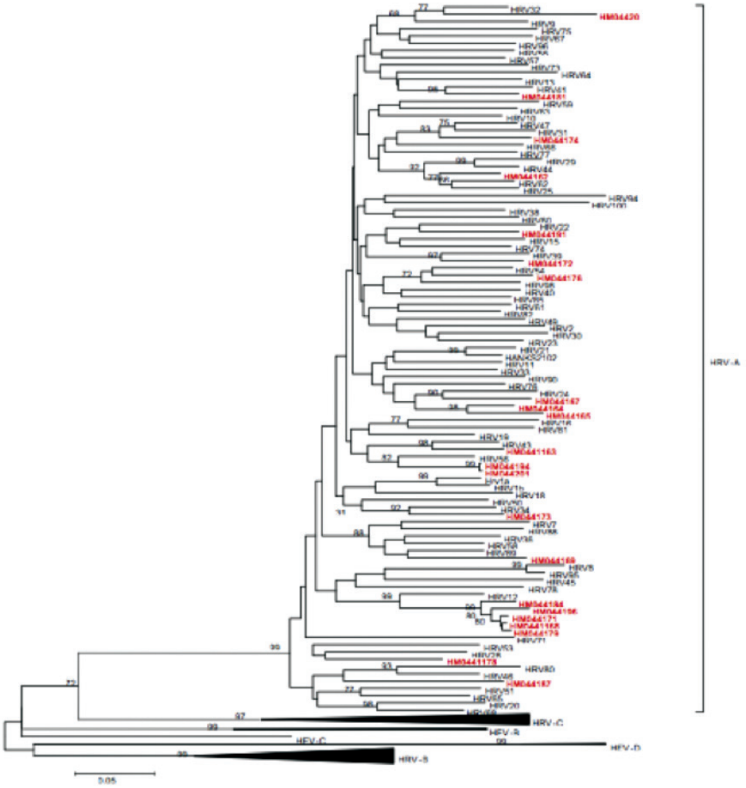

Figure 5 Neighbor-joining dendrograms depicting phylogenetic relationships of human rhinovirus species A strains in the 420-nucleotide region in VP4/VP2. Red type indicate strains of this study while reference sequences are shown in black. Branches showing >70% bootstrap support are indicated.

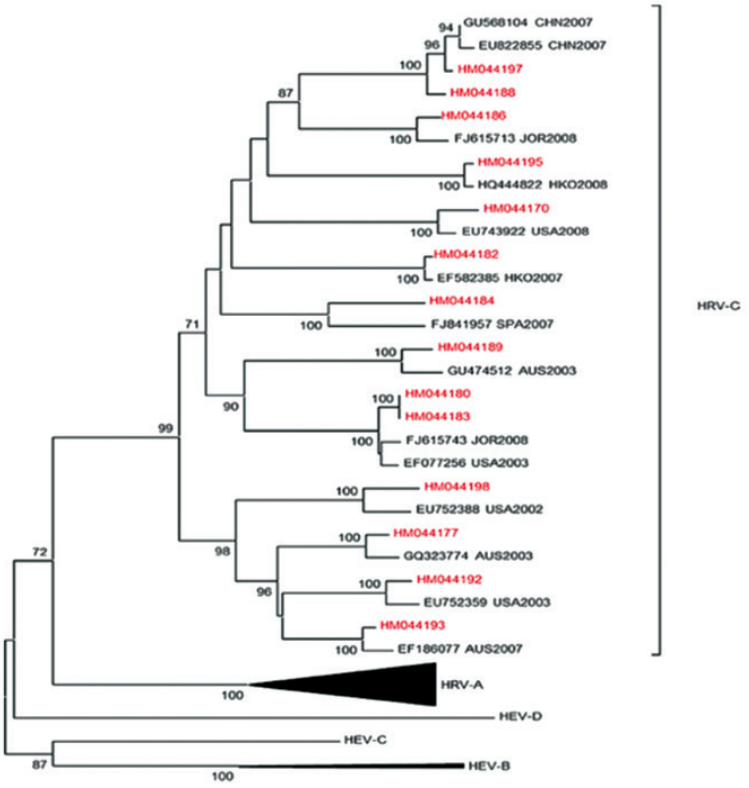

Figure 6 Neighbor-joining dendrograms depicting phylogenetic relationships of human rhinovirus species C strains in the 420-nucleotide region in VP4/VP2. Red type indicate strains of this study while reference sequences are shown in unbold type. Branches showing >70% bootstrap support are indicated.

This study highlighted a couple of important points. Firstly, it emphasised the fact that HRV can cause ALRTI, and therefore, it is important to seriously consider this virus as an important cause of children being hospitalised with respiratory infections. The HRV-A strain seemed to be the most prevalent. The absence of HRV-B indicates that it plays a minor role in disease severity. Further, the detection of the newly discovered HRV-C revealed that it has the potential to pose a major respiratory disease burden. This study also revealed some differences in terms of clinical features between HRV-A and HRV-C. Secondly, the study revealed that the use of molecular-based techniques such as polymerase chain reaction (PCR) increased the chances of virus detection, which will give more value in the clinical management of these patients.

We went on further to investigate the host-virus responses in HRV infections (Etemadi *et al,* 2017). We wanted to find out the molecular mechanisms of the host's response towards HRV infection. This can serve as a platform to develop effective anti-HRV therapeutic strategies. We chose HRV-B as virus model and A549 cells (human adenocarcinoma alveolar basal epithelial cells) as the host model. The virus and the cells were maintained and standardised in terms of quantity. After determining the appropriate multiplicity of infection (MOI), the A549 cells were infected with HRV-B.

HRV-B induced specific cytopathic effects (CPE), characterised by shrinkage and rounding-off as well as detachment and destruction of cell layers, as observed by light microscopy (Figure 7). CPE started to appear at 12 hours post-infection (hpi) on A549 cells and continued to increase up to 48 hpi. Analysis of the virus propagation in cell culture supernatant confirmed the time-dependent increase of viral RNA up to 48 hpi.

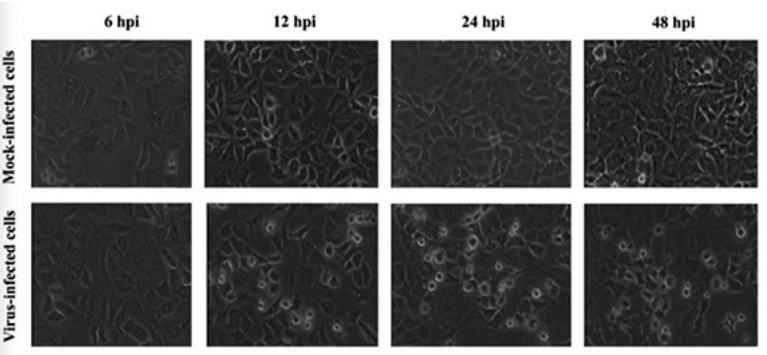

Figure 7 Infection of A549 cells with HRV72. Representative microphotographs of A549 cells either mock-infected (upper) or infected with HRV72 (below) at various time points. The cells were visualised using a phase contrast microscope at 200x magnification.

RNA was extracted from the cells using standard techniques. After ensuring that there was appropriate RNA amounts as indicated by RNA Integrity Number, microarray hybridisation was performed using Affymetrix Genechip technology, covering more than 20,000 genes. Representative of differentially expressed genes from different functional groups including chemokines (CXCL8, CCL20, CXCL3), anti-apoptotics (BCL2A1), transcription factors (FOSL1, JUN, and EGR1) and signal transduction mediators (DUSP6 and GNB4) were selected and validated by qRT-PCR. The expression profiles of the IFN genes including IFN-β, Il29 (IFN-λ1) and IL28 (IFN-λ2/λ3) were also further evaluated using qRT-PCR.

It was found that HRV infection modified a large number of genes in a timely manner. The transcriptional response to HRV infection was characterised by significant up- and down-regulation of the genes. A comparative analysis of three independent experiments between HRV-infected and mock-infected cells identified collectively 991 differentially expressed (DE) genes across all time

points, from which 459 (46%) were up-regulated and 532 (54%) were down-regulated. It appeared that the down-regulated genes surpassed the up-regulated genes. DE genes at 6 hpi (187 genes up-regulated vs. 156 down-regulated) were significantly represented by gene ontologies related to the chemokines and inflammatory molecules exhibiting the characteristics of viral infection. The 75 up-regulated genes surpassed the down-regulated genes (35) at 12 hpi and their enriched ontologies fell into discrete functional entities, such as regulation of apoptosis, anti-apoptosis and wound healing. At later time points of 24 and 48 hpi, predominated down-regulated genes were enriched for extracellular matrix proteins and airway remodeling events (Figure 8). The overlaps of the DE genes across all-time points are displayed using Venn diagrams in Figure 9.

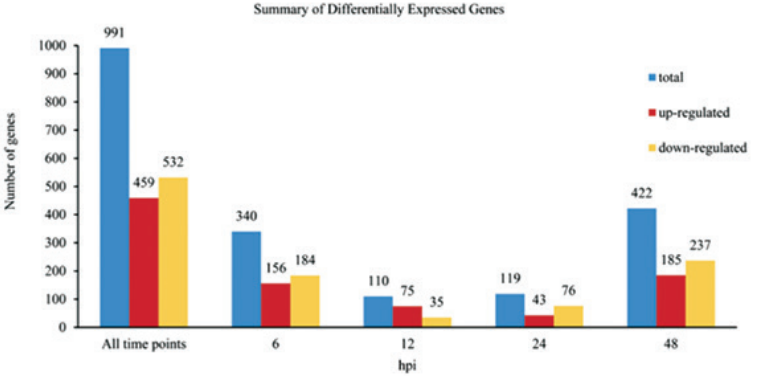

Figure 8 Summary of DE genes induced by HRV72. The DE genes were determined at four time points by filtering of the genes at *p*-value 0.05 and with a fold change of 1.5.

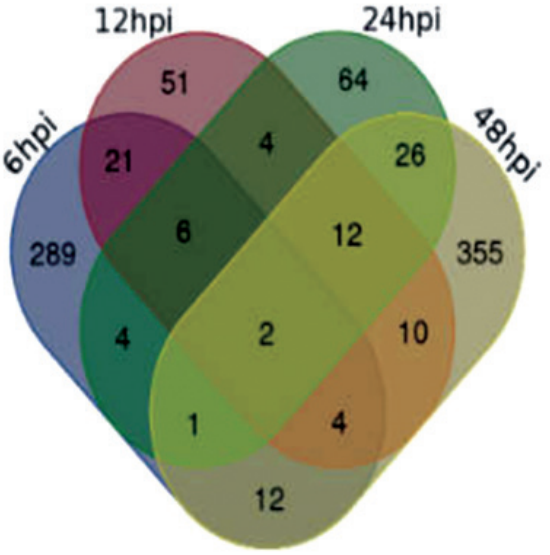

Figure 9 Venn diagram showing overlap of DE genes in HRV-infected cells compared with mock-infected A459 cells with 1.5-fold (p-value 0.05).

From analysis of the cellular gene expression of HRV infection based on four time points using an *in vitro* system, this study revealed the comprehensive gene expression profile of epithelial cells' response to HRV infection. This study thus aids the foundation for further investigations on the genes and their pathways that might be involved in the pathogenesis of the HRV infection. Ultimately, this fundamental knowledge can lead to the development of possible targets for treatment and even prevention.

FIRST MALAYSIAN REPORT OF BOCAVIRUS INFECTION

In 2012, our group became the first in Malaysia to report the presence of human bocavirus in Malaysian patients (Etemadi *et al,* 2012). It was originally reported by Allander and co-workers in 2005, in respiratory secretions from children with respiratory infections in Sweden (Allander *et al* 2005).

Human bocavirus (HBoV) is classified under a family of small viruses called Parvoviridae. Human bocavirus is a non-enveloped and icosahedral virus. It is very tiny, with a diameter of 18 to 26 nm and a genome size of 5.3 kb. Since its discovery, it has been reported worldwide in young children with respiratory or gastrointestinal symptoms. HBoV has four different genotypes, HBoV 1 to 4.

The patient in whom the virus was discovered was a 13-month-old baby with a history of asthma who had been admitted to Hospital Serdang. He was admitted due to wheezing after experiencing fever and cough for a few days. Physical examinations revealed a tachypnoeic child with bilateral coarse crepitations. His nasopharyngeal aspirate (NPA) was sent for testing. No bacterial infection was detected and direct immunofluorescence assay was negative for influenza A virus, influenza B virus, parainfluenza types 1, 2 and 3, adenovirus and respiratory syncytial virus. We then carried out our own in-house molecular testing, utilising both multiplex and regular PCR methods, for several other viruses. They were negative for human metapneumovirus, coronaviruses OC43, 229E, HKU11 and NL63 and rhinovirus. Eventually, it tested positive for human bocavirus, which was confirmed by PCR (Figure 10).

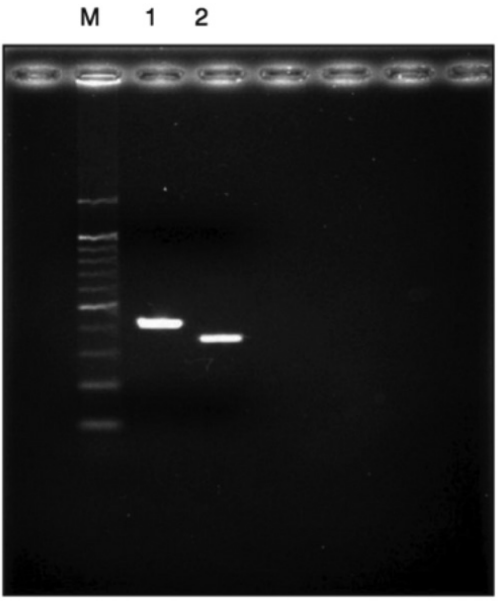

Figure 10 Agarose gel electrophoresis of human bocavirus PCR product.
Line M: Marker,
Line 1: using confirmation primer set (420 bp)
Line 2: using screening primer set (354 bp)

This study emphasises the fact that newly emerging viruses can cause substantial infections in a susceptible population. Advanced diagnostic techniques are thus needed to detect such viruses. The addition of such techniques in routine diagnostic laboratories can assist doctors in making informed decisions in the clinical management of their patients.

FIRST MALAYSIAN REPORT OF HKU1 CORONAVIRUS INFECTION

Coronavirus is an enveloped RNA virus with a genome size of approximately 26 to 32 kb, the largest for an RNA virus. It primarily infects the upper respiratory and gastrointestinal tract of mammals and birds. There are currently six strains of coronaviruses that infect humans: HCoV-229E, HCoV-OC43, HCoV-NL63, HCoV-HKU1, SARS-CoV and MERS-CoV. Human coronaviruses predominantly cause the common cold, second to rhinovirus infections. It can also cause pneumonia and severe pneumonia, especially the SARS-CoV and MERS-CoV.

Human coronavirus HKU1 was first discovered in 2005 in Hong Kong and had never been reported in our country. In 2012, we reported the first Malaysian case of HCoV-HKU1 infection (Amini *et al* 2012). It was a case of acute pharyngitis in a 3-year-old patient where the patient's asthma was exacerbated. She presented acute respiratory symptoms and asthma wheeze. NPA was collected and sent to our laboratory for analysis. Bacterial infections were excluded through the tests performed. Molecular methods, using a highly sensitive commercial multiplex (Seegene, Korea), were then employed for the detection of 15 respiratory viruses, which include influenza A and B viruses, human respiratory syncytial virus A and B, parainfluenza 1, 2, 3 and 4 viruses, human bocavirus, human metapneumovirus, adenovirus, rhinovirus, enterovirus and coronaviruses OC43/HKU1 and NL-63/229E. It yielded positive results for coronavirus OC43/HKU1. To differentiate between the two coronaviruses, we designed our own in-house PCR test which was positive for the HKU1 virus, confirmed by nucleotide sequencing (Figure 11).

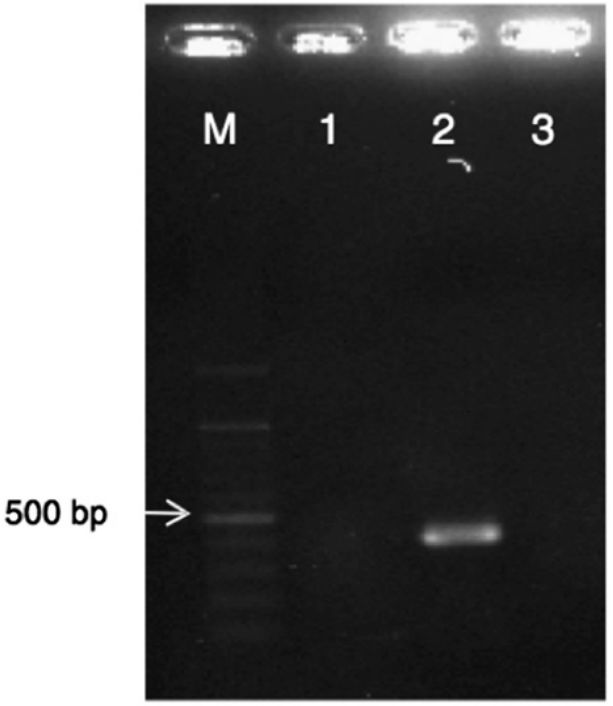

Figure 11 Ethidium bromide staining of 2% agarose gel showing RT-PCR product (443 bp) of human coronavirus HKU1–specific primers. Lane M, size marker (100 bp); Lane 1, Negative control RT- PCR mix; Lane 2, HKU1; Lane 3, OC43.

This study emphasises the need to be vigilant in considering emerging viruses when diagnosing patients in Malaysia.

HAND, FOOT AND MOUTH DISEASE

Hand, foot and mouth disease (HFMD) is one of infections that affect children. The common causative agents are predominantly enterovirus 71 (EV71) and Coxsackievirus A16 (CA16). Other enteroviruses such as CA6 and echovirus can also cause the disease.

Enterovirus 71 and Coxsackievirus A16 are RNA viruses from the same genus, Enterovirus in the Picornaviridae family. They are predominantly spread through the faecal-oral route and also through contact with an infected person's saliva, faeces, respiratory aerosols or contaminated water. They can remain in faecal matter for up to 11 weeks. Colonisation occurs in the intestinal tract and then spreads into the bloodstream and eventually to the organs.

HFMD patients are commonly children and typically present symptoms of fever, mouth ulcers (also known as herpangina) and vesicles on the palms and soles. It is usually mild in nature. Further, in children less than two years of age, atypical rashes are frequently seen. In adults the viruses can cause upper respiratory tract infections. Although HFMD is a self-limiting disease, the EV71 can cause severe and potentially fatal neurological diseases such as encephalitis (usually in the brainstem), acute flaccid paralysis and meningitis. These symptoms can develop in a matter of hours or days. CA16, on the other hand, is associated with mild HFMD.

In the 1990s, epidemics of EV71 infections were observed in Europe and eventually spread to Asia. In 1997, Malaysia was also affected where outbreaks started in Sarawak and then spread to Peninsular Malaysia, with reports of fatalities among children (AbuBakar *et al*, 1999). A total of 35 children died in the outbreaks. CA16 as the cause of HFMD has also been reported in Malaysia since 2000 (Podin *et al,* 2006). It was seen during the outbreak or inter-outbreak of EV71. Outbreaks of CA6 have also been reported in many other countries, such as Singapore, Taiwan and Japan.

We embarked on a small-scale study to look at the epidemiology of HFMD among children below twelve years of age in Seri Kembangan, Selangor (Beh *et al,* 2014) during a non-outbreak period. A total of six general practitioner clinics were involved in the study. The specimens collected were throat swab, vesicles swabs and rectum swabs. The specimens were subjected to semi-nested reverse-transcription polymerase chain reaction (snRT-PCR) and positive specimens were sent for sequence analysis. Phylogenetic analysis was then carried out.

A total of 28 cases of HFMD were detected and only twelve (42.9%) tested positive for viruses by snRT-PCR. There was no significant gender or age difference among those infected. The mean age of the patients with positive results was 3.6 years old. The breakdown of causative viruses identified were seven (58.3%) CA6, two (16.7%) CA16 and three (25%) EV71. Phylogenetic trees of the three viruses were then constructed based on the partial VP1 sequences.

The CA6 strains were shown to be closely related to the Taiwan and Japan strains (Figure 12). Further, the CA16 were identified as being from genotypes B2b and B2c. The subgenotype B2b was closely related to China strains isolated in 2011. However, subgenotype B2c was similar to Malaysian strains isolated in 2007. During the period 1997 to 2003, subgenotype B2a was dominant while after 2005, subgenotype B2c was also detected (Chan *et al,* 2012). Other subgenotypes such as subgenotype B1 had also been reported in Sarawak, from 1998 and 2000.

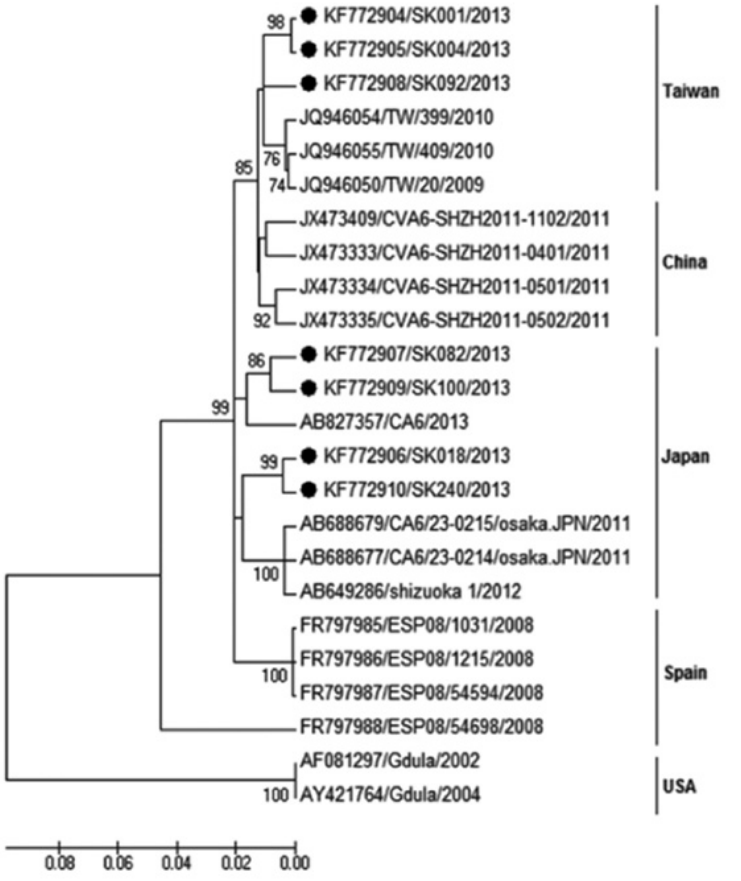

Figure 12 Phylogenetic tree of CVA6 VP1 was constructed using partial VP1 sequences (nt 2627-3357) of the detected isolates. CVA6 strains from other countries were included to show the relationships between the detected CVA6 strains. The dots indicate CVA6 strains.

The EV71 strains detected in this study were from genotype B5 and closely related to isolates from Selangor and Johore. This has been the dominant subtype circulating in Malaysia (Chan *et al,* 2012).

This small study illustrated the importance of molecular epidemiology in HFMD surveillance, as it will enhance intervention in the event of outbreaks and also fatal cases. Clinical diagnosis alone is not adequate and the simple serological tests are not reliable and accurate. On the other hand, molecular epidemiology will provide an accurate picture of the prevailing viruses in circulation and the public health authorities can then be vigilant in monitoring related cases and prevent outbreaks.

THE DEVELOPMENT OF THE ENTEROVIRUS 71 VACCINE

Looking at the potential threat of EV71 and in the absence of an effective treatment, it is worthwhile to develop a vaccine which can potentially protect millions of life globally. Over the years there have been several types of EV71 vaccine candidates, including attenuated strains, inactivated whole-virus, virus-like particles (VLP), recombinant proteins, recombinant vectors and peptide vaccines (Yi *et al,* 2017).

Thus far, the inactivated whole virus technology seems to be the most effective strategy in producing the vaccine. There are currently two approved inactivated whole virus vaccines available for commercial use in China. Immunisation with the formalin inactivated EV71 strain can elicit high levels of virus-specific antibodies and the important characteristic of cross-neutralisation activity. Further, clinical trial results have indicated that is results in 90% prevention against EV71-associated HFMD or herpangina and

80% of other EV71-associated disease symptoms. However, these approved vaccines are specific for EV71 and do not protect against other HFMD-causing viruses, such as CA16. It is thus worthwhile to note that a bivalent formalin-inactivated EV71/CA16 vaccine is currently under development (Yi *et al,* 2017).

Immunisation with recombinant VP1 protein of EV71, expressed in *Escherichia coli*, yeast or the baculovirus system, can induce high levels of EV71 VP1-specific IgG antibodies but the titre produced is still lower than that of the inactivated whole virus. Other technologies such as virus-like particles, DNA vaccines and recombinant vector vaccines are currently still under development.

Many years ago, I was involved in the development of a vaccine against EV71 using DNA vaccine technology. The objectives of the study were to design and construct a DNA vaccine using the viral capsid protein (VP1) gene of EV71. This vaccine candidate was tested *in vitro* for the expression of the VP1 protein in a mammalian cell culture followed by *in vivo* testing on its ability to express protein and to elicit immune responses in mice (Wong *et al,* 2007).

The VP1 genes of EV71 from two local outbreak isolates were amplified using PCR and then inserted into a eukaryotic expression vector, pVAX1. The 3.9 kb recombinant constructs were transformed into competent *Escherichia coli* cells and the positive clones were screened and selected using PCR analysis, restriction digestion analysis and DNA sequencing. The constructs were also tested for protein expression in Vero cells. The VP1 protein was successfully expressed in the mammalian cell line and detected using RT-PCR, Indirect Immunofluorescence Assay (IFA) and western blotting.

In *in vivo* studies, female Balb/c mice were immunised with the DNA vaccine constructs through intramuscular injection. ELISA and virus neutralising assay were performed to detect the presence

of anti-VP1 IgG in the mice and its neutralising effects against EV71.

Following DNA immunisation, the antigenic viral antigens expressed from plasmids seemed to be expressed, folded and assembled in the same manner as when they are expressed during a natural viral infection.

The anti-VP1 IgG levels in the mice immunised with the DNA vaccine constructs increased after the first booster but declined following the second booster, probably due to a shift towards cellular immune response. The anti-VP1 IgG in these mice exhibited neutralising activity against EV71. This study demonstrated promising results on the use of the vaccine against EV71.

The route of entry of the EV71 infection is through the oral route but the vast majority of vaccine administration is via the intramuscular route, which in this case is less ideal. While the intramuscular delivery system has been fairly well established, it still requires injection through a needle, which can be painful and may be subject to adverse events. In the case of EV71 infections, it does not mimic a natural route of infection. We thus embarked on developing a candidate EV71 vaccine using the oral route (Saeed *et al,* 2014a). An oral mucosal vaccine seems to be a good alternative because it provides a medium that is as close to natural infection as possible. The duration of exposure to the vaccine is also longer, triggering a longer interaction with the antigen presenting cells and the lymphocytes.

Despite the mentioned advantages, an oral vaccine is inherently problematic because the vaccine will be subjected to digestion in the gastrointestinal tract, particularly in the stomach, due to increased acidity and gastric digestive enzymes. Thus, this approach will render the vaccine less useful due to degradation.

Administrating the vaccine via the buccal route thus seems to be the more ideal choice. The exposure of the antigen to the surrounding immune cells will generate a different type of antibody, which is immunoglobulin A (IgA). IgA is a type of antibody that plays a crucial role in the immune function of the mucous membranes. Its dimeric form is the most prevalent and is called secretory IgA (sIgA). It is found in mucous secretions in the body, such as tears, saliva, sweat and secretions from various organs. The secretory component of sIgA protects the immunoglobulin from a harsh environment such as that in the gastrointestinal tract.

The challenge of this route of administration is to maintain the vaccine in the oropharyngeal area for as long as possible to ensure maximum interaction with the immune cells. This requires a carrier that is able to adhere to the mucosal surface and generate a sustained release of antigens. The carrier also needs to protect the peptides, protein, epitopes or antigen of interest against the adverse environment and to deliver them to the intended location without degradation or change in conformation. The choice of a good delivery system is thus very important, ideally a system that can offer controlled drug release (Shen *et al,* 2013). These include chitosan, alginate, starch, polyoethylene glycol and calcium carbonate.

Chitosan is safe and biodegradable (Saeed *et al,* 2015a). Its other favourable properties include thermostability, low swelling, extended release, highly adhesive and viscous, as well as providing smooth reversible encapsulation (Bowmab & Leong, 2006). Chitosan could enhance mucosal delivery of drugs due to its muco-adhesiveness. It is no surprise that chitosan is also used in therapeutics, where it slows down the release of loaded drugs. Chitosan is expected to protect antigen epitopes in a controlled release manner.

Alginate is another medium that is worthy of consideration. It is a naturally occurring polymer typically obtained from brown seaweed. It has numerous applications in biomedical science and engineering. Its favourable properties include biocompatibility, low toxicity, relatively low cost and ease of gelation. It can be made into hydrogels, which has wide applications including delivery of antigens as in the case of vaccine design.

In the choice between chitosan and alginate as the most suitable delivery system, this study looked at calcium phosphate released under artificial conditions mimicking gastric juices of pH 2 and body temperature of 37°C. The results showed chitosan to be superior to alginate in terms of size, formulation stability, swelling and crosslink reversibility. Hence, chitosan was chosen as the delivery system for this study (Saeed *et al,* 2014b).

The next step was to select the most appropriate adjuvant. Adjuvants work by binding to vaccine epitope, peptide or antigen, increasing its molecular weight, delaying its clearance from the circulatory system by the phagocytic cells, improving its antigenic uptake by macrophages, and extending its release to the immune cells. These characteristics lead to the control of adjuvant release, which could prolong antigen delivery, presentation and activation of a measurable number of lymphocyte clones. Ultimately, the main outcome is an elevated vaccine response and enhanced immune protection.

In this study, calcium phosphate was chosen as the adjuvant. It has long been used by researchers to promote both systemic and mucosal immunity and is a non-toxic, biodegradable and non-antigenic adjuvant (Saeed *et al,* 2014a).

One of the objectives of this study was to determine the appropriate size of the calcium phosphate, either nano or microsizes, and the appropriate concentration of chitosan. Both calcium

phosphate sizes were tested against a combination of several chitosan concentrations. The combinations were tested for safety and their ability to release calcium over a period of 96 hours.

The delivery system was tested against human liver cells Hep-G2 to confirm its safety. The results showed that calcium phosphate of nanosizes could be released faster than that of microsizes. In terms of sustained release, chitosan of above 1% concentration showed extended delivery of adjuvants. This information is very useful in designing vaccine delivery. In order to have a rapid but sustained delivery of the antigen of interest, the size is thus preferably nanosize and enveloped in a slightly higher concentration of chitosan.

This study then proceeded to determine the best mode of vaccine administration (Saeed *et al,* 2015b). Inactivated EV71 were absorbed to nanosize and microsize calcium phosphate adjuvants. Both adjuvants were then administered through intradermal and intramuscular routes. The objective was to find the best antigen-chitosan-calcium phosphate size and administration route combinations.

This study resulted in two important findings. Firstly, the use of nanosize adjuvant in the vaccine delivered through intradermal administration (0.1 ml) induced the highest level of virus specific antibodies compared to that of the microsize adjuvant. This is an indicator of the importance of using nanosized antigen for increased release to the immune cells. Secondly, the use of intradermal administration requires one-tenth of the vaccine dose compared to intramuscular administration, to achieve the same level of antibodies production.

The study went on to determine the best vaccine delivery strategy - buccal, intradermal or both routes. The results showed that combined vaccine delivery by both buccal and intradermal routes

elicited the best immune response with enhanced production of both IgG and IgA antibodies. This contributes to achieving both systemic and mucosal protection, providing the ultimate protection against EV71 infections. Absorbing the inactivated EV71 into the nanosize adjuvant coated with chitosan deployed an improved immune response of specific IgA, even with a single buccal administration.

In conclusion, the development of an effective enterovirus 71 vaccine still has a long way to go. Though at the moment we have two EV71 vaccines available (only in China), which comprise inactivated virus, its effectiveness is limited solely to EV71-related diseases. Our study attempted to develop a vaccine that is able to develop as close as possible to natural infection where the body can mount a good combination of antibodies production, particularly IgA, IgM and IgG. So far, a strategy of combined buccal exposure and intradermal injections has shown good results.

BEYOND UNIVERSAL CHILDHOOD HEPATITIS B IMMUNISATION

Chronic hepatitis B is one of the most important causes of liver failure and liver cancer worldwide. It is caused by a DNA virus called the hepatitis B virus. It is a worldwide problem with more than 350 million chronic hepatitis B carriers. In Malaysia, there are an estimated 1.1 million chronic hepatitis B carriers as of 1998 (Liaw *et al,* 2005). The estimated HBsAg prevalence in Malaysia in year 2000 was 0.77% (Ott *et al,* 2017).

The hepatitis B virus is a member of the hepadnavirus family. The virus is 30 – 42 nm in diameter and consists of an outer lipid envelope enclosing the icosahedral nucleocapsid protein. The envelope contains proteins which are involved in viral binding to susceptible cells. The nucleocapsid covers the viral DNA and an enzyme called DNA polymerase.

The virus produces in excess, surface antigens called HBsAg, which are composed of lipid and protein found on the surface of the virus. The presence of HBsAg is used as an indicator of infection.

Hepatitis B is transmitted through blood and body fluids. In high prevalence areas such as China and South East Asia the primary mode of transmission is through vertical (childbirth) and perinatal transmissions. However, in Africa, transmission is common among children, as in other moderate prevalence countries such as Eastern Europe and Japan. In low prevalence countries like the United States and Western Europe, hepatitis B is primarily transmitted through sex and injection drug use (Cluster *et al,* 2004). Other modes of transmission include surgical and dental procedures, tattooing or contact with objects contaminated with infected blood.

Hepatitis B may be either acute (and self-limiting) or chronic. In acute infections, the patients are able to clear the infection spontaneously over a span of weeks or months. The acute presentations are variable, from mild to severe. The patients present symptoms of fever, nausea and vomiting, weakness and fatigue, loss of appetite, abdominal pain, dark urine, jaundice and joint pain. In general, children are less likely to be able to clear the viral infection compared to adults.

More than 95% of infected adults and older children are able to recover and attain protective immunity for life. However, for younger children, only about 30% would be able to do so. The situation is worse for newborns who acquire the infection from their mothers, where 95% of the babies would not be able to clear the virus (Bell and Nguyen, 2009). More than 90% of children and less than 5% of adults present with chronic hepatitis B infections. Chronic hepatitis B infection is defined as the persistence of the "s" antigen for more than 6 months.

Hepatitis B virus primarily interferes with the functions of the liver. It replicates in the hepatocytes. In the attempt to clear the virus, the host's immune response causes damage to the liver cells, particularly by cytotoxic T lymphocytes. The persistent presence of this virus will result in attack of the liver cells (known as hepatocytes) causing consistent activity of necrosis and regeneration. This will result in scarring of the liver which will eventually transform into carcinogenesis leading to hepatocellular carcinoma. Depending on the host's response to the virus, where some hosts are able to mount restricted immune response, the damage occurs persistently for a long time.

Hepatitis B accounts for 60% of liver cancer in Malaysia, especially among those of Malay and Chinese ethnicity, in addition to hepatitis C (11%), cryptogenic diseases (16%) and alcoholism (6%) (Goh *et al,* 2015).

In 1992, the World Health Organization recommended the integration of the hepatitis B vaccine into the national immunisation programmes of all highly endemic countries by 1995 and all other countries by 1997. Malaysia had already embarked on this programme since 1989. The programme consists of 3 doses, starting from birth, a second dose at 1 month of age and the third dose at 5 months.

Antibodies against hepatitis B surface antigen (anti-HBs) provide protection against hepatitis B infection, and thus, vaccination with HBsAg provides adequate immunity against hepatitis B. A minimum of 10 IU/L of anti-HBs should be mounted to attain a protective level against hepatitis B.

A summary of global immune response of more than 9,000 infants from 20 countries revealed that a median of 98% infants achieved seroprotective anti-HBs after three or four doses of the hepatitis B vaccine (Shillie and Murphy, 2013). A slightly lower

median seroprotection rate of 93% was noted for infants weighing less than 2 kg. It is suggested that delaying the first dose of the vaccine until one month of life can increase the proportion of preterm infants attaining seroprotection.

In a community clinic in Malaysia, infant immune response to the vaccine was evaluated after completion of the primary immunisation series. A total of 572 infants were enrolled. About 97% of the infants attained adequate anti-HBs levels of more than 10 IU/L and no infant with HBsAg was detected among them, even among the infants born from mothers with chronic hepatitis B. This indicates that the 3-dose vaccine series is able to provide infants the necessary protection against hepatitis B (Cheang *et al,* 2013).

The implementation of the hepatitis B immunisation program has thus been a success (Ng *et al,* 2005). A cross-sectional seroprevalence study of hepatitis B in 190,077 schoolchildren in Malaysia, aged 7 – 12 years of age, showed a decline in HBsAg prevalence rates from 2.5% (1985) to 0.4% (1996). This suggests that the immunisation program has been the most effective measure in preventing vertical transmissions of the disease in Malaysia.

However, the question still remains of how long the immunity will last and whether a booster dose is necessary.

A study was done in 2013 investigating HBV infections among new students in a local university (Ng *et al,* 2013). The overall prevalence of HBsAg among 2,923 of the students was 0.62%. The prevalence among those born before 1989 was 1.08% and for those born on or after 1989 it was 0.2%. What is interesting is that only about 34% of the vaccinated cohort had adequate anti-HBs. Six students (0.4%) from the vaccinated cohort were found to have anti-HBc, meaning that they had acquired hepatitis B infections before, while another three students were HBsAg positive. This study suggests that the universal and voluntary vaccination programme

in Malaysia has been effective in preventing hepatitis B and also that a significant proportion of vaccinated individuals did not have adequate seroprotection levels of anti-HBs.

Our study aimed to evaluate the level of hepatitis B immunity among Malaysian undergraduate students 23 years after commencement of the nationwide hepatitis B childhood immunisation programme in Malaysia (Hudu *et al,* 2013a). A total of 402 serum samples obtained from volunteer undergraduate students were screened for the presence of hepatitis B surface antibodies using qualitative ELISA.

The results showed that 62.7% of the subjects had protective anti-hepatitis B surface antigens (>10 IU/L). The estimated post-vaccination immunity was found to be at least 20 years, indicating persistent immunity against hepatitis B. Subjects with antibody levels of less than 10 IU/L were given a booster dose of hepatitis B vaccine and anamnestic response was 94.0% when they were re-evaluated a month later. This clearly shows that the immunisation program has been successful and important in bringing down the prevalence of hepatitis B in the country.

Despite waning antibody responses, hepatitis B vaccination is effective in preventing chronic HBV infections years after primary vaccination.

It is believed that the immune memory cells play a very important role in the prevention of hepatitis B. They are likely to be present even after anti-HBs are no longer measurable. The immune memory cells accelerate anti-HBs response upon re-exposure to HBsAg. This is called a booster.

The critical issue about universal HBV vaccination is not whether it is effective in protecting children from HBV infections, but whether its protection can endure till adulthood. Booster vaccine dose is not recommended as a routine practice if there is adequate

seroprotection post-immunisation. However, it is recommended for adolescents and young adults with increased risk of HBV infection.

OCCULT HEPATITIS B INFECTION

Occult hepatitis B infection (OBI) is defined as the presence of HBV DNA in the blood or liver, absence of HBsAg, with or without anti-HBc (Brechot *et al,* 2001). OBI with positive anti-HBc is known as seropositive OBI. In OBI infections the amount of HBV DNA in the blood is typically very low, therefore a sensitive detection method, such as PCR or real time PCR, is needed.

OBI has been found in patients with hepatocellular carcinoma, past hepatitis B infection, chronic hepatitis C or even in individuals without serological markers. In other words, OBI is not just restricted to patients with liver disease but may also be observed in individuals with normal liver parameters such as blood donors.

Occult hepatitis B infection is postulated to be a result of various clinical conditions, which include the incubation period of acute infections, the tail-end stage of chronic hepatitis B, low-level viral replication after recovery from hepatitis and escape mutants not detected by current HBsAg tests.

OBI attributed to mutations in the virus surface protein, HBsAg, is called surface mutation (Hudu *et al,* 2013b).

It is associated with flare ups of liver disease in hepatitis C patients who do not exhibit changes in HCV RNA levels and even reduces the response rate to interferon therapy. It is frequently detected in cryptogenic liver diseases and autoimmune hepatitis. Hepatitis B virus reactivation is a well-known complication in patients with occult infection under immune suppression, such as anti-cancer therapy and human immunodeficiency infection. It also increases the risk of hepatitis B virus transmission through blood transfusion.

The mutations on the surface protein of the hepatitis B virus pose challenges to available diagnostic tests. Detection of OBI thus requires highly sensitive tests such as PCR or real time PCR. The fact that a vast majority of available tests are not designed or able to detect OBI has implications for blood transfusion and organ transplants, where there can be potential transmission of the virus to the recipients.

Another circumstance of concern is vertical transmission of the mutated virus, which potentially occurs in countries where vertical transmission is the most common mode of infection. The concomitant administration of hepatitis B vaccine and hepatitis B immunoglobulin (HBIg) at birth may also pose problems whereby both active and passive immunisations may exert evolutional pressures to select mutants (Wu *et al*, 2010). Thus, vaccinated children are more likely to harbour mutations than unvaccinated children.

The neutralising epitope that is of importance is a region called the "a" determinant, situated at amino acids 124 – 147. Antigenic response to this epitope will mount neutralising antibodies, anti-HBs, which are vital to inactivate the virus and thus prevent the infection. Mutations in this region could lead to a conformational change of this epitope and therefore can affect the antigenicity (Ma and Wang, 2012). Several notable mutations, such as T/I126S, Q129H, G130N, S143L, D144A, G145A and G145R, result in diagnostic failure and they are thus able to escape from being neutralised by vaccine-induced antibodies. The exact mechanism of the mutation is as yet poorly understood. It is speculated that the hepatitis B viral DNA polymerase enzyme lacks proofreading ability and this makes it highly susceptible to nucleotide misincorporation during reverse transcription or replication.

In Malaysia, mutations were observed in 11% of patients receiving antiviral therapy, of which, 8% carried mutants previously described as vaccine-associated escape mutants and another seven strains carried other types of mutants (Meldal *et al,* 2011). Although the introduction of a hepatitis B vaccination program has been effective in many countries, the emergence of anti-HBc in vaccinated individuals suggests the possibility of contact with hepatitis B virus with mutant strains subsequent to vaccination. These surface mutants are of enormous importance because they are capable of infecting both unvaccinated and vaccinated individuals. In other words, the whole population is at risk.

From a clinical point of view, establishing diagnosis through routine test methods will result in a significant number of cases not being detected. These undiagnosed cases will progress undetected leading to occult chronic infection, liver failure and subsequently, hepatocellular carcinoma.

We were trying to assess the OBI situation in Malaysia. An earlier study had reported that ten Malaysian blood donors were identified as having OBI (Candotti *et al,* 2012) but this study did not look into the extent of the disease. Our study aimed instead to detect OBI and characterise the hepatitis B virus among blood donors and hepatitis B vaccinees (Hudu *et al,* 2015). The phylogenetic analysis of the vaccine-escape hepatitis B strains would give an insight into the prevalence of the strains as well as help in the design of diagnostic assays to detect these mutants.

One thousand serum samples were collected for this study, where 500 samples were from blood donors from the National Blood Centre and another 500 samples from volunteer undergraduate students at Universiti Putra Malaysia. The samples were then tested for the presence of Hepatitis B surface antigen, antibodies and core antibodies using the ELISA method. DNA detection was performed

via nested PCR, and the S gene was sequenced and analysed using bioinformatics.

Of the 1,000 samples that were screened, 5.5% (55/1,000) were found to be HBsAg-negative and anti-HBc-positive. All 55 samples registered positive for HBV DNA by nested PCR. A total of 84.8% of the samples were anti-HBs positive indicating some degree of protection against the virus. Of the 55 samples that were positive for HBV DNA, 48 samples were positive for anti-HBs. None of the samples were positive for HBsAg.

It is interesting to note that anti-HBc can be used as a surrogate marker for occult hepatitis B infection. This may be useful in countries of limited resources where sensitive nucleic acid testing is expensive and not affordable. Furthermore, detection of OBI needs highly sensitive nucleic acid testing (NAT) as the DNA copies can be really low. Thus, anti-HBc can also be used as a complementary test to enhance the safety of blood transfusion procedures (Hudu *et al,* 2016a)

All of the aforementioned 55 isolates were found to belong to genotype B, which is also the predominant genotype reported in Malaysia (Figure 13). Several mutations were found across all the sequences, both synonymous and non-synonymous mutations, with the most nucleotide mutations occurring at position 342, where adenine was replaced by guanine and cytosine at position 46 replaced by adenine in 96.4% and 98.0% of the isolates, respectively (Table 2).

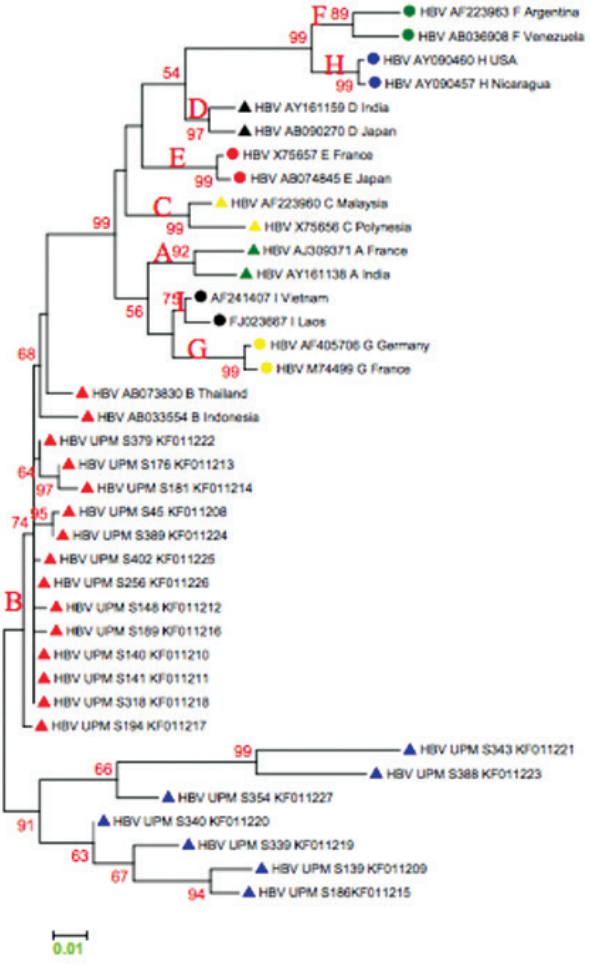

Figure 13 Phylogeny of the S gene of 35 isolates from Malaysian blood donors and reference strains from established genotypes (A to H) from the NCBI GenBank. The blue triangles indicate an aberrant genotype. The evolutionary relationship was inferred using the ML method based on the Kimura model. Malaysian isolates are indicated by HBV UPM D followed by the isolate number and all belong to genotype B, with the exception of the aberrant strains.

Table 2 Distribution of amino acid mutations within different regions of the surface protein.

a Determinant region (124–147 aa)	MHR (100–160 aa)	Outside MHR
T125K	V106F	Q16K, G18V, G18R, G18M, L95Stop
D144G	S117R	T37N, T37I, S61Stop, F19L, F20L, W35R, W35G, W36C, W36R, G50A, G50D, G50V, H60P, H60S, R73L, I82L, V168L
T125A	C121W	T23I, V47G, C48G, C48V, L49I, Q54H
T125I	T123N	I25N, S55P, S55G, L12Q, L12P, L12R
S132C	T148A	V14L, V14G, Q16R, V180G, N52S, D99G
M133L	S155X	P29L, L13F, P11T, Q56T, S58Y, S132T
	A157D	165Stop, L15Stop, I28T, I28E, S59R
	T125K	S59N, S64F, S64G, I68V, C69F, C90F
	D144G	C90R, C90S, F93V, F93L, L94M, I92H
	T125A	I92L, Q51Stop, F80L, C85G, C85F, C85L
	T125I	I86F, I86V, T63S, L88M, T27R, Q30R
	S132C	D33N, D33S, N40D, P62H, P62Q, R78W
	M133L	L89R, W74R, P66L, L39I, L39T, K24Q
	P151L	I57T, S53Y, Q54Stop
	L109P	
Total: 6	15	90

Aligned deduced amino acid sequence showed corresponding mutations at various points of the amino acid sequences of the surface protein, including substitutions of deletions. The mutation at amino acid 16 was common to all Malaysian isolates, resulting in the substitution of glutamine (Q) with lysine (K) in 53 of the isolates, while the remaining two isolates contained an arginine residue or a stop codon. A total of 105 amino acid mutations were found in all 55 Malaysian isolates, 85.7% (90/105) outside the major hydrophilic region (MHR) and 14.3 % (15/105) within the MHR, of which 40% (6/150) were within the "a" determinant region.

Most of the sequences were found to exhibit a close relationship with the reference strain from Panama, which belonged to genotype B, serotype adw[2]. Other isolates were closely related to reference isolates from Canada, the Philippines and Indonesia. Conversely,

sequences from vaccinated Malaysian individuals were separated into two clusters, the majority of which were found to have a close evolutionary relationship with reference isolates from China, Taiwan and Japan. However, some sequences from vaccinated individuals were distantly related to other sequences belonging to different clades and sub-clades, as a result of frequent mutations within the *S* gene.

The risk of transmitting occult hepatitis B infection depends on the presence of the HBV DNA copied in the plasma and the volume of the plasma transfused. Hence, the higher the viral load in the transfused blood, the higher the chance of infectivity. The presence of high anti-HBs in the donors' blood also influences the rate of infectivity.

The immune status of recipients and donors also play an important role in determining infectivity. Recent infectivity data indicate a transmission rate of 3.8% (Seed and Kiely, 2013), but the rate is higher in unvaccinated recipients of occult hepatitis B blood or blood products.

The only reliable detection method is the detection of the HBV DNA by nested PCR or real-time PCR. This is because these two methods are highly sensitive in detecting minute amounts of hepatitis B DNA. A viral load of less than 200 IU/mL has been defined for OBI diagnosis. In more than 90% of OBI patients, the viral load in serum was around 20 IU/mL. A serum with more than 200 IU/mL should be interpreted as an infection caused by escape mutants and not an OBI (Sara *et al*, 2011).

Most OBI cases are asymptomatic and clinically not well defined. These cases are associated with the risk of developing cirrhosis and hepatocellular carcinoma. Immunosuppression may also lead to HBV reactivation.

Hepatitis B core antibodies (anti-HBc) are detected in almost every patient with previous exposure to the hepatitis B virus. However, one cannot understand the activity of the disease based on this marker alone. Asymptomatic individuals with immunity, as a result of previous natural infection or vaccination, with the presence of anti-HBs are referred to as isolated anti-HBc. In our study, all individuals in this group of isolated anti-HBc had HBV DNA detected by molecular method. We are however concerned about the degree of protection available to individuals where the neutralising antibody, anti-HBs, were either undetectable or lower than the recommended limit of 10 IU/L. Therefore, our study aimed to identify the implication of an isolated hepatitis B core antibody and evaluate the effect of hepatitis B vaccine boosters in isolated anti-HBc cases, among adults who had received the HBV vaccine as infants.

A total of 408 undergraduate students who had received hepatitis B vaccination as infants volunteered for this study. They were born after 1989 when the hepatitis B childhood vaccination programme had been implemented and thus it was assumed that all of them had been given the full dose of the vaccine, as the coverage of hepatitis B childhood immunisation is almost 100%.

They were tested for the presence of Hepatitis B surface antigen, surface antibodies (anti-HBs) and core antibodies using ELISA. Molecular detection of hepatitis B viral DNA was also performed using nested polymerase chain reaction (Hudu *et al,* 2013c).

Slightly less than two-thirds of the volunteers (62.5%) were found to have adequate hepatitis B protection (anti-HBs more than 10 IU/L). The prevalence of isolated anti-HBc was found to be 4.9% (20 of 408 individuals), out of which 80% had hepatitis B surface antibodies titre higher than 10 IU/L. All the 20 anti-HBc positive individuals had detectable hepatitis B viral DNA in their serum.

The subjects with isolated anti-HBc with undesirable protective levels of anti-HBs were given a single booster dose of recombinant hepatitis B vaccine. It was good to note that there was 100% anamnestic response from these individuals, attaining high concentrations of anti-HBs a month after the booster dose had been administered. An absence of anamnestic response in those with isolated anti-HBc could indicate low-level carriers with immunological tolerance to surface antigens that are incapable of producing antibodies. However, before such a conclusion is drawn vaccine failure owing to improper handling and storage must first be ruled out.

In Australia, a study involving anti-HBc positive patients revealed a prevalence of 0.69% OBI (Martinex *et al,* 2015). They identified eight nucleotide changes in the small S protein and polymerase gene which were believed to have caused altered antigenicity and low viral replication which resulted in undetectable HBsAg and extremely low levels of HBV DNA in the serum.

An interesting study was carried out in China, looking at the prevalence of occult hepatitis B virus infection among individuals with a family history of chronic hepatitis B virus infection (Zhang *et al*, 2015). Currently, the impact of OBI in individuals living with family members with the infection is still not clear. Serum samples were collected from 747 HBsAg-negative people with a family history of HBV infection and 579 HBsAg-negative volunteer blood donors (which acted as the control group). The prevalence of OBI was 8.0% (60/747) among the HBsAg-negative individuals with a family history of chronic HBV infection, compared to 2.6% (15/579) among the blood donors ($p < 0.05$). The prevalence of HBV genotype B infection was lower in the OBI group than in the control group ($p = 0.031$). The substitution rates in the major hydrophilic region and the "a" determinant seemed to be higher

in the OBI group, and stop codon mutations more frequent in the OBI sequences. This indicates that the S region mutations and the escape mechanism were not likely to be the major causes of increased prevalence of OBI.

In a cross-sectional study in Iran, the prevalence of OBI among selected high-risk groups of children born to HBsAg-positive mothers was determined (Shahmoradi *et al,* 2012). A total of 75 HBsAg negative children who had received full prophylactic coverage (vaccine and HBIG) against HBV participated in the study. Their sera were examined for the presence of HBV DNA. The sequences of the HBV genome isolated from the patients with OBI were then analysed and compared with the wild type HBV genome prevalent in Iran.

Twenty-eight percent of the patients were found to harbour OBI in the presence of adequate levels of anti-HBs, of whom only four showed serological evidence of past HBV exposure, as indicated by the presence of anti-HBc. The HBV sequences obtained from the analysis of the sera of the HBsAg seronegative carriers showed point mutations, deletions and splicing alternatives associated with OBI. Ten (71%) of the 14 HBV surface-positive isolates carried G145R, which confirmed the presence of vaccine-escaped mutants in the children despite full immunisation against HBV.

The children with OBI were further evaluated for persistence of the HBV DNA. Follow up was carried out for an additional 36 months (Sadeghi *et al*, 2015). In the follow up tests all the children still tested negative for HBsAg, HBeAg and anti-HBe. Their liver function tests were also within the normal range. Real-time PCR showed that all of them were negative for HBV DNA except for one child.

The only still OBI-positive patient had an HBV DNA level of 50 copies/ml. The amino acid alignment of the whole surface protein showed the presence of the G145R mutation, similar to that seen previously. His initial and latest anti-HBs levels were above the protective levels. He also persistently tested negative for anti-HBc. His mother, who was positive for HBsAg, had no mutations in the 'a' determinant region. On follow-up, 18 months later (54 months after initial sampling), his samples showed that HBV DNA was undetectable.

This study clearly showed that in children, HBV DNA was cleared in the presence of adequate levels anti-HBs. It can be postulated that in high-risk children, over time, adequate levels of anti-HBs after vaccine and HBIg immunoprophylaxis after birth could eventually clear the virus. However, periodic monitoring of the OBI status is highly recommended.

The transmission of any diseases through blood transfusion is not acceptable by today's standards and this issue has always been taken seriously. To enhance the safety of blood transfusions, rigorous screening methods are implemented. The current standard method of screening blood for hepatitis B is by nucleic acid testing for HBV DNA, which is highly sensitive. HBsAg mutants however pose diagnostic challenges, as they may not be detected using standard detection methods due to the alteration on the hepatitis B surface antigen.

The prevalence of occult hepatitis B infection in blood donors was estimated to be 8.55 per 1 million donations, according to a 2008 international survey (Dong *et al*, 2015).

The clinical outcome of occult HBV transmission primarily depends on the recipient's immune status and the number of HBV DNA copies present in the blood products. The presence of donor

anti-HBs reduces the risk of HBV infection by approximately five-folds. The risk of HBV transmission may be lower in endemic areas than in non-endemic areas, because most recipients would have already been exposed to HBV. Blood safety for HBV, including OBI, has substantially improved, but the possibility for OBI transmission still remains (Dong *et al*, 2015).

There are important clinical concerns regarding OBI (Yuen *et al*, 2017). Firstly, OBI can be transmitted through blood products and organ transplants from donors with OBI. The risk of transmission thus needs to be ascertained. Secondly, we also need to understand the effect of OBI on the measurable parameters for liver disease, such as liver function, liver histologic features and long-term complications, including liver cirrhosis and hepatocellular carcinoma. Thirdly, it is also important to understand whether the presence of OBI in patients with other chronic hepatitis diseases, e.g., chronic hepatitis C infection and alcoholic liver disease, would have additive or synergistic harmful effects on the development of hepatocellular carcinoma. Finally, we need to define the risk of HBV reactivation in OBI patients who undergo immunosuppressive therapy.

The risk of HBV chronicity depends on the age of the subjects when they first contract the virus. The risks of HBV chronicity are 90%, 30% and 2% for those whose ages are <1 year, 1 to 5 years and >5 years old, respectively (Lai *et al*, 2003). This means that in more than 95% of adults who contract the virus it would not have resulted in HBV chronicity. The majority of these subjects display no symptoms, and develop anti-HBs and anti-HBc, during and upon resolution of acute infections. Some would have a typical history and symptoms of acute infections such as malaise, loss of appetite, nausea and/ or tea-coloured urine. The sequela of "acute" hepatitis

B is not as simple as previously believed. It is proven that HBV DNA is present in the serum and peripheral blood mononuclear cells of patients who have recovered from acute HBV infections. In such situations, the liver inflammation is usually mild.

EMERGING INFECTIONS OF HEPATITIS E

The Hepatitis E virus is a tiny non-enveloped virus, 27 – 34 nm in diameter, with a single-stranded RNA genome. The virus belongs to the genus, Orthohepevirus, which is a member of the family Hepeviridae. It has four species (A – D) and eight genotypes (1 – 8). Based on seroprevalence data, it is estimated that one-third of the world's population has been infected with HEV.

HEV is excreted in the stool of infected persons and is transmitted by the following routes: faecal-oral; through potable water contamination; ingested food, from infected animals (raw or uncooked pig, wild boar or deer meat); zoonosis, from human exposure to body fluids of infected animals; transfusion of contaminated blood products; vertical transmission (maternal-foetal); and transplantations with HEV-infected grafts. Of these, the most common is through contaminated water.

The four prevalent genotypes are genotypes 1 through 4. Genotypes 1 and 2 cause outbreaks of hepatitis (Guerra *et al*, 2017), where genotype 1 is present in Asia and North Africa, and genotype 2 in Mexico and Central Africa. They are transmitted mainly through contaminated water and the faecal-oral route. Genotypes 3 and 4 are endemic, and are considered swine viruses, capable of infecting humans as accidental hosts. It is good to note that despite the distinct genotypes, cross-immunity occurs which has good implications for vaccine development.

The incubation period of this virus is 2 to 6 weeks. The most common age group infected is between 15 to 40 years of age. The common symptoms of hepatitis E are fever, nausea, abdominal pain, vomiting, anorexia, malaise, jaundice and hepatomegaly. In most cases, it is a self-limiting disease lasting from a few days to weeks. In developed countries, it can cause chronic disease with progression to cirrhosis. However, fulminant hepatic failure can also result in morbidity and mortality among high-risk groups, such as, pregnant women, immunocompromised individuals, elderly people and patients with pre-existing liver diseases such as chronic hepatitis B or C.

In Japan, HEV cases were found to be directly associated with eating raw or undercooked deer meat, while several cases of HEV were epidemiologically linked to consumption of poorly cooked wild boar meat or pork.

In our study, we looked at the issue of hepatitis E virus in chronic hepatitis B patients. We characterised HEV by comparative genomic analysis to relate it to a zoonotic origin.

A total of 82 chronic hepatitis B patients were recruited for this study which spanned from May 2015 to May 2016. Serological and molecular investigations of HEV were conducted among these patients. The detected HEV were sequenced and the genomes and deduced amino acids were characterised using molecular evolutionary genetic analysis software.

Of the 82 chronic hepatitis B patients that were tested, 9.8% (8/82) were found to be HEV positive. Phylogenetic analysis of the eight HEV RNA sequences showed that they were of genotype 4 (Figure 14). Homology of the Malaysian sequences, in terms of geographical relatedness to other Asian countries, revealed that most of the Malaysian isolates were closely related to reference isolates

from Laos, India and Thailand and one of the Malaysian isolates was related to isolates from Japan, Nepal and China.

Amino acid sequence analysis of our isolates which was compared to a reference swine HEV from China exhibited 99% similarity and showed that our isolates had 3 to 11 amino acid variations from the swine HEV at different positions of the gene. This study highlighted the fact that comparative analysis of the hepatitis E virus detected among the chronic hepatitis B patients suggests a zoonotic origin.

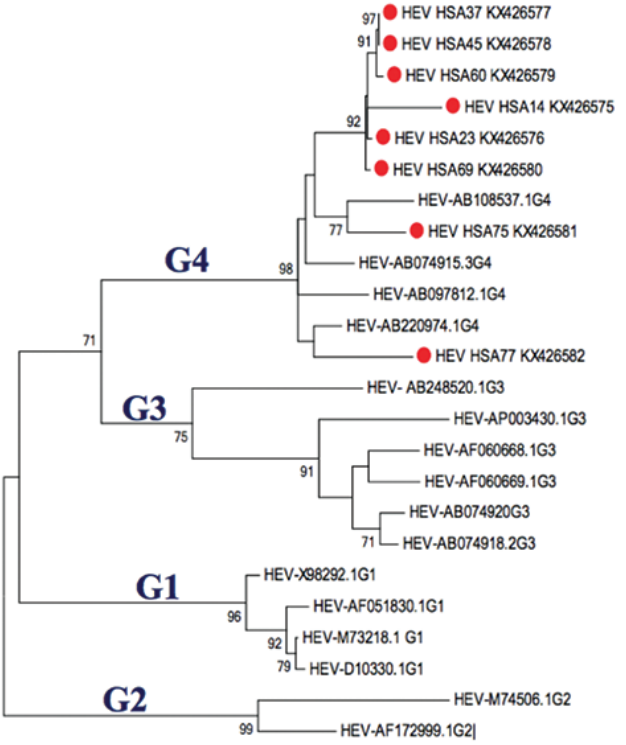

Figure 14 Hepatitis E virus genotyping by molecular phylogenetic analysis. The evolutionary relationship was inferred using maximum likelihood. The red dots indicate Malaysian isolates belonging to genotype 4. G1, G2, G3 and G4 indicate genotype 1, 2, 3 and 4, respectively.

THE IMPORTANCE OF ADULT IMMUNISATION

Immunisation is recognised as one of the greatest public health achievements. There have been huge successes among the paediatric population in the prevention of potentially fatal infectious diseases, such as measles, tetanus and whooping cough. However, this is not

the case for adults. Adults need protection from infectious diseases too and there are many vaccines available to protect them.

Adults need vaccines just like children. As we age, influenza poses significant threats of morbidity and mortality and this can be prevented through annual influenza vaccinations. As a result of the low number of vaccinees, vaccine-preventable diseases among adults result in a significant economic burden. Our immunity also wanes with age. Antibodies produced as a result of childhood immunisation reduce significantly over time and will no longer be able to protect us against the intended infectious diseases. Booster vaccines are thus required to maintain the antibodies at optimum levels.

Low vaccine uptake means that preventable infectious diseases can result in costs to individuals and society in terms of deaths, disabilities and economic losses from doctor visits, hospitalisations and loss of income.

In the United States, in 2015, it was estimated, based on the prevailing vaccination uptake rates, that vaccine-preventable diseases cost individuals and society $9 billion (~ RM40 billion) annually, through direct costs and productivity losses, $7.1 billion (~ RM30 billion) of which occurs among the unvaccinated (Ozawa *et al,* 2016). This illustrates that increasing adult immunisation rates can potentially reduce the annual economic burden. In 2015 the coverage of all vaccines for adults remained low (Williams *et al,* 2017). The vaccination coverage for adults aged ≥19 years for influenza, pneumococcal and Tdap vaccines were 44.8%, 23.0% and 24.7%, respectively. However, the vaccine coverage for adults aged ≥65 years was significantly higher (influenza, 73.5% and pneumococcal, 63.6%).

Adults might choose not to receive a vaccination for various reasons, including belief that a healthy person does not need any vaccines, concern about side effects, belief that the vaccine can cause illness, poor attitudes toward vaccines and lack of comfort or distrust in the government and health care system.

A study was done to identify the barriers to and perceptions of immunisations in adults of all ages (Sevin *et al*, 2016). The top five factors likely to affect the decision to receive an immunisation among the 304 respondents in the study were: "doctor's recommendation" (80.6 %), "knowing why I should get a vaccine" (78.2 %), "knowing which vaccines I need" (75.5 %), "cost" (54.2 %), and "concern about getting sick if I get a vaccine" (54.0 %). Significant differences in factors influencing the immunisation decision exist among the respondents, based on ethnicity and education level. For those participants with self-identified diabetes, heart disease or asthma, less than half were aware that certain immunisations could reduce the risk of complications associated with their diseases.

Understanding adult patients' attitudes could assist in creating a more effective design and tailoring of interventions to improve adult vaccination uptake. In a study determining patient perspectives of adult vaccines, patients expressed positive attitudes towards vaccines in general and were in favour of receiving reminders/ recall notices for adult vaccines (Albright *et al,* 2017).

In the United States, a programme called the 4 Pillars Practice Transformation Program, (also known as the 4 Pillars Toolkit) is a primary care practice improvement aid programme focused on changing behaviour using evidence-based strategies organised into four domains. The pillars are convenient vaccination services, communication with patients about the importance of immunisation and the availability of vaccines, enhanced office systems to facilitate

immunisation and motivation through an office immunisation champion who monitors progress and encourages adherence to vaccination-promoting office procedures to improve vaccine uptake. The Toolkit has been tested in several trials and was found to be moderately effective in increasing immunisation rates in adults and children. Implementing an intervention based on the 4 Pillars Practice Transformation Program is a cost-effective undertaking in primary care practices for individuals aged 65 and older, with predicted public health benefits (Smith *et al,* 2017).

Despite guidelines for adult vaccination, there are substantial gaps in knowledge, attitudes and beliefs among both the public and healthcare providers that lead to low vaccine coverage. A systematic approach that involves education, elimination of barriers and establishing and improving infrastructure for adult immunisation is therefore required.

IMMUNISATIONS AMONG HEALTH CARE PROFESSIONALS

Health care professionals (HCP) are in constant contact with their patients and therefore are at risk of exposure and possible transmission of vaccine-preventable diseases (VCP). HCPs do not only refer to doctors, nurses and those working in hospitals but also includes students of these professions, volunteers and administrative staff. To protect themselves and prevent the risk of transmission of hospital infections to patients, it is essential for HCPs to maintain immunity against VCPs. There should thus be a comprehensive policy, at all medical or health facilities, that requires all HCPs to get themselves protected via immunisations.

Transmission of nosocomial influenza from health care professionals (HCP) has been identified as an important cause of morbidity and mortality among patients, especially in high-risk groups such as the elderly, children and the immunocompromised.

Influenza vaccination is thus one of the highly recommended vaccines for HCPs to be taken annually. The aim is to reduce the risks of contracting influenza and influenza-like illnesses, minimise absenteeism, and prevention of hospital-acquired influenza and its associated morbidity and mortality among their patients.

We carried out a cross-sectional survey at three hospitals in Kuala Lumpur and Selangor, to ascertain the rate of influenza vaccination uptake, the knowledge and attitude of HCPs regarding the influenza vaccine as well as the employers' policy on encouraging their workers' influenza vaccination uptake. This study is the first of its kind in Malaysia (Hudu *et al,* 2016b).

This survey included HCPs from both government and private hospitals in the Klang Valley. A total of 690 questionnaires were distributed and received a reasonably good response rate of 76.4% (527/690). Nurses and midwives constituted the majority of the respondents (49.3%), followed by technologists (16.7%) and clinicians (10.8%). Majority (80.5%) were involved in the direct care of patients, of which 53.1% had received the flu vaccination within a period of one year.

Of the 527 HCPs that participated in this study, 271 (51.4%) had been vaccinated. The highest vaccine uptakes in terms of specialty were internal medicine and emergency medicine. The results of this study also showed that a high proportion of HCPs concurred that influenza poses a serious threat to their health. Those HCPs who were vaccinated did so to protect themselves against influenza. A significant proportion of the HCPs had good knowledge about the transmission of the influenza virus as well as the signs and

symptoms of infection. Having said that, 49.5% believed that they would definitely get vaccinated the following year,

This study also assessed employer policies on flu vaccination among their employees. In this study, 31.9% of the HCPs said that their employers provided them with free or subsidised flu vaccine. It was also found that 61.9% of those that were offered influenza vaccination on site received the vaccine.

This study highlighted the fact that even though HCPs had good knowledge on the need for the influenza vaccine and some employers provided the vaccine, the uptake was still low. It is worth looking into the reasons why this is happening.

There are several potential strategies to increase vaccination rates among HCPs that are worth considering (Ozisik *et al,* 2017). Different groups of HCPs tend to have different perceptions and expectations of adult vaccination and therefore have different motivations. Some of the motivations that can be encouraged are motivations of self-protection, to protect patients, free and accessible vaccines, previous vaccination and model colleagues.

Another strategy to improve vaccination rates among HCPs is to educate them on adult vaccination and also improvements in health literacy. Institutional leadership and implementation of policies play major roles in pushing this strategy where strong commitment and support are definitely needed.

CONCLUSION

Human viruses pose a constant threat to humans. Looking at the day-to-day morbidity and mortality rates that they have caused in clinics and hospitals, tackling the infections will always be challenging. This is further worsened by the infectious nature of the diseases they spread. We have experienced deadly infectious diseases in the past, which spread rapidly across many countries in a short period

of time. It is thus prudent and important for doctors, scientists and researchers to be at the forefront to counteract these threats. A proper surveillance system to monitor the progress of these diseases should be given priority. As a whole, research is a very important tool and mechanism to ensure that our interventions and actions are indeed on the right track. To add impact the research should ideally be collaborative and comprehensive in nature. Through such efforts at the global level, the threat from human viruses can be contained in a systematic and methodical manner.

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BIOGRAPHY

Zamberi Sekawi was born in 1969 in Kuching, Sarawak. His father worked as a police officer while his late mother was a housewife. His early education was at Sekolah Rendah Bantuan St Joseph, Kuching (1976 to 1981) and Sekolah Menengah Bantuan St Joseph, Kuching (1982 to 1986). He was subsequently accepted to study medicine at Universiti Kebangsaan Malaysia and graduated with an MD in 1994.

In the same year, Zamberi applied to work in his hometown, Kuching, as a house officer but was instead posted to Hospital Kuala Lumpur. It involved a lot of hard work requiring high physical, emotional and spiritual strength. He was posted to various sections which included medicine, surgery and obstetrics and gynaecology postings. Upon completion, he joined the Department of Pathology at Hospital Kuala Lumpur. There he was mainly attached to the Forensic Unit, which exposed him to many medicolegal and forensic cases. He developed an interest in forensics and decided that he wanted to become a forensic pathologist. He thus enrolled in Universiti Kebangsaan Malaysia to pursue a Masters of Pathology degree in 1997. By the end of the first year, he had developed a deep interest in clinical virology and so decided to switch his speciality from Forensic Pathology to Clinical Microbiology.

Inspired by his lecturers, Zamberi wanted to become an academician. He therefore joined Universiti Putra Malaysia in 1999 as a trainee medical lecturer. He graduated with a Master of Pathology in Medical Microbiology in 2001. As a young lecturer he took charge of the teaching of medical microbiology and was eventually appointed as Phase 1 coordinator to oversee the running of the pre-clinical curriculum. During his tenure as coordinator he was heavily involved in the improvement of curriculum content and delivery. He was also one of the key members involved in the

establishment of the Master of Pathology programme, a specialist-training programme.

Further, under the mentorship of Professor Dr Mariana Nor Shamsudin, he was actively involved in research, initially focusing on respiratory viruses and resistant organisms. His current research interests are on respiratory viruses, hepatitis viruses and immunisation. Over the years, he successfully acquired many research grants amounting to more than RM6 million. He has published more than 150 journal articles and co-authored five books. His current h-index (15 for Scopus, 22 for Google Scholar) is an indication of the high quality of his research work. Zamberi actively supervises both local and international postgraduate students. Thus far, six PhD and eight Master of Science graduates have graduated under his supervision. Many of his former students now hold key positions locally and internationally.

Due to his expertise, in 2010, Zamberi briefly joined the World Health Organization and was based in Fiji as a Technical Officer. He was tasked to design policy and implementation strategies to strengthen laboratory services among WHO member countries in the South Pacific. During his six months' tenure, he carried out his duties diligently and completed his mission successfully. He was subsequently invited to be a consultant for other countries such as Cambodia, Papua New Guinea and the Solomon Islands.

Zamberi has been the Deputy Dean for Academic (Medicine) since 2016, an administrative post that he previously held from 2007 – 2010. He was also the Deputy Dean for Research and Internationalisation from 2011 to 2016. During his tenure taking charge of the research portfolio, the faculty research improved immensely. The faculty acquired many research grants, published more than 400 journal articles annually and produced a significant number of patents or copyrights. In his current portfolio, he heads

the Medical Education Research and Innovation Unit, where he champions teaching-learning activities by encouraging innovative teaching methods, consolidating students' professional and personal development and strengthening assessment methods.

Zamberi is very active in his professional society, the Malaysian Society of Infectious Diseases and Chemotherapy. He is the current president of the society. Under his leadership the society has organised many national and international conferences and spearheaded national issues such as adult immunisation, antibiotic resistance and others.

LIST OF INAUGURAL LECTURES

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2. Prof. Ir. Abang Abdullah Abang Ali
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3. Prof. Dr. Abdul Rahman Abdul Razak
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4. Prof. Dr. Mohamed Suleiman
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5. Prof. Dr. Mohd. Ariff Hussein
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6. Prof. Dr. Mohd. Ismail Ahmad
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7. Prof. Dr. Mohamed Mahyuddin Mohd. Dahan
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18. Prof. Dr. Mohamed Shariff Mohamed Din
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19. Prof. Dr. Tengku Azmi Tengku Ibrahim
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20. Prof. Dr. Abdul Hamid Mahmood
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23. Prof. Dr. Ang Kok Jee
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24. Prof. Dr. Sharifuddin Haji Abdul Hamid
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27. Prof. Dr. Mohamed Ismail Abdul Karim
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