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Hepatitis B immunization: long-term immunogenicity of low dose yeast-derived recombinant hepatitis B vaccine

Hepatitis B virus (HBV) infection is a common cause of morbidity and mortality in East and South-East Asia and Africa where it is endemic.[1-3] In Singapore, it accounts for about 55% of all acute viral hepatitis cases,[4] with a relatively high prevalence of hepatitis B antigen carriers among healthy individuals,[5] especially among the Chinese.[6] Even in other countries where it is not endemic, it is now becoming an important cause of concern; in the United States 200,000 to 300,000 acute viral infections occur yearly and over 1 million people are chronically infected.[7] Hence the control of this condition is highly important. Immunization initially using vaccines derived from human plasma, and later recombinant yeast-derived vaccines have been demonstrated to be effective.[8] However, long term studies are required to determine the duration of immunity that these vaccines can provide. Only a few studies of this nature in adults have been published,[9-11] as is also the case in children with one in the Gambia with follow-up for seven years,[12] with two others involving immunization starting at birth with follow-up for the first five years[13] and nine years.[14] It is highly important to determine the immunogenicity of the vaccine as well as its ability to provide continued protection to the vaccinees during the rapidly growing stage of early life when important physiological changes are occurring at a fast rate.

The dosages for adequate and optimal efficacy, respectively, as well as the minimal dosage required, has as yet to be determined. It was therefore thought that such a long-term study using various dosages would be worthwhile.

Material and methods

Children aged between 1 and 12 years old who were clinically healthy and well, were screened for the presence of hepatitis B surface antigen (HBsAg), antibody to hepatitis B core antigen (anti-HBc) and alanine aminotransferase (ALT) status. Only those free from hepatitis B infection and with normal ALT values were recruited for the study. Informed consent was obtained.

The infants were assigned sequentially to four groups, with each group receiving different doses of hepatitis B vaccine. Recombinant yeast-derived hepatitis B vaccine provided by Merck Sharp and Dohme (MSD, West Point, Pa, USA) was administered in the following manner: group 1 received 0.6µg; group 2, 1.25µg; group 3, 2.5µg; and group 4, 5.0µg. The vaccine was administered deep into the deltoid muscle to each child at 0, 1 and 5 months. The vaccinees were then monitored for soreness and/or swelling at the injection site, fever and other systemic symptoms; the parents were instructed to observe the children and monitor their daily temperature for the first week following each vaccination.

Venous blood was obtained from each case just before the first injection, at three months; i.e. two months after the first injection and one month after the second, 6 months; i.e. one month after the 3rd injection, 9 months, 1 year, 2 years and thereafter at 2 yearly intervals. The serum was stored at -20°C until serological testing.

HBsAg and anti-HBs were tested by radioimmunoassay (Austria II and Ausab, respectively) and for anti-HBc by anzyme immunoassay (Corzyme), the kits being supplied by Abbot Laboratories Inc (North Chicago III, USA); the serum ALT activity was tested using the Greiner Analyser.

The children were followed up yearly for a minimum of 11 years after study commencement to monitor their physical wellbeing and psychomotor progress as well as their hepatitis B status over the long term.

Booster doses of the vaccine were administered at 8 years to half of each group of children to determine the immunological response; the dose administered was similar to the original dose given to each respective group; blood was sampled just before the injection, one month and one year later. Subsequent blood sampling followed the usual two yearly intervals. The second half was subjected to the same immunization booster regimen at 10 years post-commencement of the study, with blood being again sampled just before, one month after the booster, and one year later. The study was approved by the Ministry of Health, Singapore, as an ongoing project to determine the appropriate vaccine dose and monitor the long-term efficacy of the vaccine, and detect any short or long term complications of the immunization.

Results

The results over the first two years have been reported previously in an earlier paper.[15] Briefly, a total of 124 children were studied, 31 being allocated sequentially to each group; some resistance to venepuncture reduced and total number completing the study during the first two years, to 31, 30, 30 and 31 in groups 1-4, respectively; all the groups were highly comparable with the ages of 4.65±2.52 (Mean ± SD), 4.53±2.64, 4.67±2.77, and 5.51±2.36years, weight 17.43±6.72,

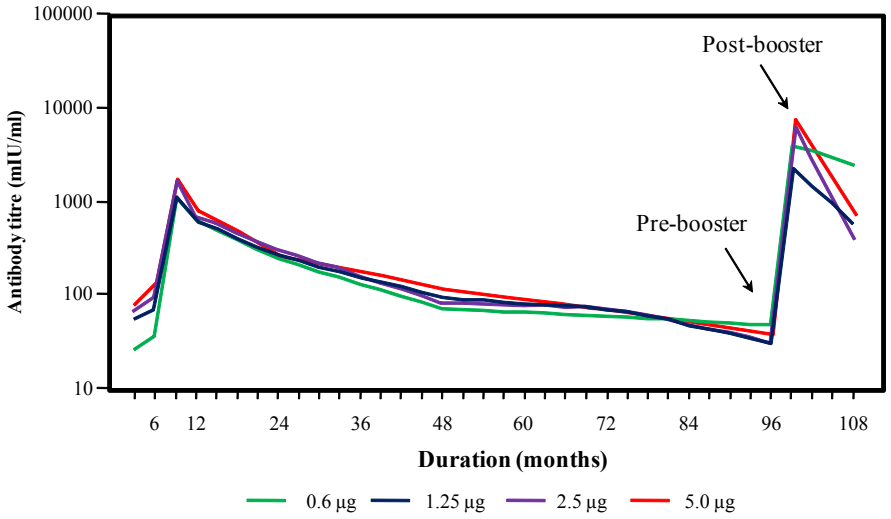
17.92±5.85, 18.61±6.92 and 19.74±6.21 kg and height 107.01±13.76, 106.36±16.68, 109.62±15.81 and 113.59±14.11cm, respectively. The children remained well throughout the duration of the study; no significant adverse reactions were observed, other than temporary soreness at the injection site in 2 infants. Physical growth and development for the whole duration of the study were comparable among the 4 groups, and within the normal range observed in the population of Singapore.

Sero-conversion with sero-protective titres (≥10 mIU/ml) at 3 months was highest in group 4 (5µg) with 96.7% and lowest in group 1 (0.6µg) with 77.4%; the sero-conversion rate improved with progress of the immunization till 9 months when they were highly comparable – sero-protective titres (≥10 mIU/ml) was 93.1%, 93.3%, 93.3%, and 100% in groups 1-4 respectively. Anti-HBc was detected in 2 children in group 1, and a further 2 in group 2. In 3 children the anti-HBc was present at only 3 months, and in the 4th (group 2) only at 6 months; though specifically looked for, anti-HBc was undetectable in all four infants subsequently. Rise in antibody titres was observed in all cases with the rise being especially marked in the 4th case. None of these children became carriers.

A dose reponse relationship was observed initially, with increasing dosage resulting in higher geometric titres (*Fig 1*) in the first six months of the study. In all groups, the peak occurred at 9 months, at which time the titres in the low dosage groups had increased significantly; they were highly comparable with that of the highest dosage group, with the dose response relationship being no longer evident.

After the peak at 9 months, a gradual decline in the titre occurred in all four groups, the rate of decline being highly comparable with GMT at four years after start of the study being 71.4mIU/ml in group 1, 95.2mIU/ml in group 2, 83.3mIU/ml in group 3 and 118.8mIU/ml in group 4; the proportion of cases

Figure 1
Antibody response in all four groups of children after immunization and effect of booster immunization in half of each group eight years after initial immunization



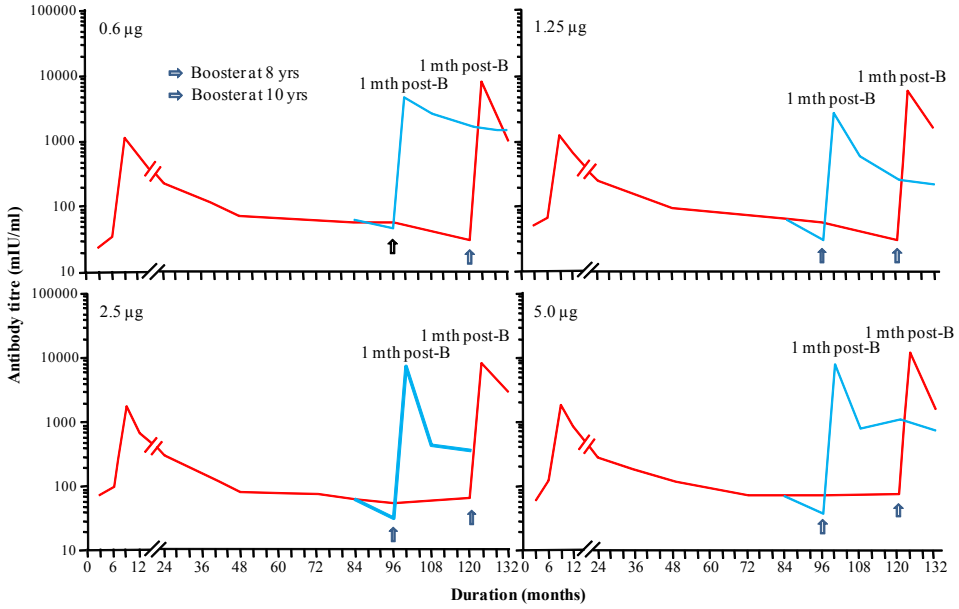
below the protective titre (<10mIU/ml) was 28%, 30%, 19% and 13%, and undetectable in 14%, 12%, 12% and 0%, respectively. Further decline occurred; at eight years, the GMT was 55.5mIU/ml. 57.7mIU/ml, 53.3mIU/ml, and 69.3mIU/ml, respectively, and the proportion below the protective titre was 33%, 29%, 20% and 22% and undetectable was 15%, 17%, 10% and 4%, respectively; the difference was not statistically significant (p>0.05). However, they remained well clinically with no evidence of clinical or serological infection (HBsAg –ve and anti-HBc –ve).

At eight years after commencement of the study, a booster given to half of each group, resulted in a remarkable response in all 4 groups one month later with a marked rise in the antibody titre; in this half of each group, the pre-booster titres were 48.4mIU/ml, 32.2mIU/ml, 33.1mIU/ml and 39.9mIU/ml, with the post-booster titres one month later of 4361.4mIU/ml, 2479.4mIU/ml, 6833.9mIU/ml, and 7723.7mIU/ml, respectively (*Fig 1*), with all cases demonstrating marked rise in antibody titres, even in those with no detectable antibody prior to the booster. The response was highly comparable and much higher than those observed at nine months after the initial immunization course in all four groups despite the great variation in the dosages used; a 100% response was observed in all four groups. The antibody titre one year later, demonstrated a decline which was again comparable in all four groups; this decline continued into the succeeding year, the pattern of decline being very similar to that after the primary course.

The same 100% response rate was again observed with the other half of each group given the booster 2 years later at 10 years after commencement of the study (*Fig 2*); pre-booster GMT titre was 30.9mIU/ml with titre <10mIU/ml in 41.7%,

Figure 2

Antibody response in all four groups of children after immunization and effect of booster immunization in the other half at 10 years after initial immunization



31.1 mIU/ml in 30.0%, 64.9mIU/ml in 22.2% and 76.4mIU/ml in 30%, respectively. A similar immediate response even in those with no detectable antibody, was observed one month later (7765.5mIU/ml, 5681.4mIU/ml, 7667.4mIU/ml, and 11052.7mIU/ml, respectively, with every case demonstrating significant antibody response), followed by a corresponding decline of antibody levels in all four groups one year later.

A follow-up rate of over 80% was obtained for the whole study period; the fall off rate being comparable in all four groups. All the vaccinees developed normally during the whole study period; no clinical infection or carrier status was observed.

Discussion

The present study on the immunogenicity of the MSD yeast-derived recombinant vaccine demonstrated the safety of this vaccine over a relatively long period of more than 10 years. Side effects were minimal and affected only a few vaccinees. The short-term immunogenicity of the vaccine was comparable to that of the plasma-derived vaccine. The interesting aspect of this study is the very low doses used in three of the four groups; the dose-response relationship observed at the initial period was probably an indication of the initial sub-optimal nature of the lower doses. However, after the 3rd dose, the peak response was highly comparable, a testimony to the ability of small 'sub-optimal' doses administered in a three-dose regimen to cumulatively achieve the same impact on antibody response as that of the 'optimal' dose many times greater. This important observation indicates that higher doses do not result in greater efficacy in a three-dose regimen in the long term, but most likely are also unnecessary at least in those with no prior exposure; this implication is especially relevant to developing countries where cost constraints are important.

The pattern of decline over the next few years presented an interesting study of immunological memory. This decline was highly comparable in all four groups despite the vastly different dosages used. Unlike the dose-response relationship of the initial response, no dose-response relationship to the decline was observed, the decline in relation to time being similar in all four groups. Of particular interest is the effect of the challenge of a booster dose given after an interval of 8 and 10 years during which period, the antibody titres have fallen to non-protective values in a significant proportion, and non detectable levels in some, presumably due to lack of exposure to hepatitis B antigen. Yet in this situation, an immediate and remarkably strong antibody response, much greater that of the initial response to the three-dose regimen, was observed in all the children following the booster using the dosages identical to the original doses, given only once to separate subgroups, at 8 and 10 years post immunization.

Evidently, the absence of antibodies is no criterion for judging immunological status of the children where initial response has been observed; presumably the cellular immunity was still very much alive, the memory of the earlier immunization enabling the body to respond rapidly and effectively to the booster. Furthermore, dose-response relationship was no longer detectable despite the dosages being so different; the response in each of the four groups was almost identical. Apparently, the dose-response relationship did not seem to apply after the first immunization course; it would appear that the initial vaccination of the infant has primed him(her) to mount a prompt response to

any subsequent similar antigenic exposure regardless of the dose. In such a situation, there should be no real indication to administer booster doses, once an immunization course has successfully elicited a significant positive response; any future exposure to the natural virus would elicit the same strong response as the booster itself.

No clinical infection or carrier status was observed despite a transient viremia as demonstrated by the presence of anti-HBc antibody with the massive rise of anti-HBs antibody in four cases; apparently the viremia enhanced the antibody response, probably like a booster, but did not cause any significant disease, as evidenced by the disappearance of the anti-HBc shortly after.

Comparison with other long-term studies is not possible because of the challenge doses given in the course of this study, a feature missing in other studies. This prompt response to a booster dose encouraged us to expect that in the event of contact with the hepatitis B virus, the prompt antibody response will prevent any infection from occurring. This was further confirmed by the enhanced antibody response of the four children with evidence of contact with the natural hepatitis B virus; they were in the lower dose groups, yet were not infected but experienced much higher antibody responses. This observation was also reported in another study[13] where sudden rises in antibody titre were observed with the appearance of anti-HBc antibody; this interesting observation indicated that even with contact with the hepatitis B virus with its subsequent circulation in the body, enhanced antibody response probably was at least partially responsible for preventing the carrier status.

The significance of this antibody response in the long-term, is however unclear and may need further evaluation; disappearances would indicate that its effect is at most only transient without any permanent impact. The need for a booster is thus unnecessary, even though Coursaget et al[12] recommended a booster dose after five years to prevent subsequent infection. The low-dose regimen was still highly effective, an observation at variance with that of another study where carrier rate rose with decrease in vaccine dose of plasma-derived vaccine,[16] that study involved neonates whose response might differ from that of older infants and children who tend to respond better to hepatitis B vaccination.[17]

The follow-up rate of over 80% in this study makes the findings representative of the whole study population. The chance of any bias created by a high drop out rate was therefore minimized. The factors favouring such a good follow-up rate are the relatively small size of the country making accessibility easier, a largely literate population understanding well the reasons for continuous monitoring, and most importantly a well motivated and persistent team of doctors and nurses who were able to case trace the vaccinees even when a change of address occurred.

Very low doses of yeast-derived MSD recombinant hepatitis B vaccine is highly immunogenic when administered in a three-dose regimen. Immune response remains still active even in the absence of detectable antibodies at 8 and 10 years after the immunization, an indication that immune memory is probably life-long. Corresponding efficacy is highly likely as no carrier status occurred over the duration of the study.

(Reported by Tan KL, Goh KT, Chan SH, Oon CJ, Scientific Committee on Hepatitis and Related Disorders, Ministry of Health)

Editorial comments

When the hepatitis B vaccine first became commercially available, the cost was very high. A number of clinical trials were conducted by the Scientific Committee on Hepatitis and Related Disorders, Ministry of Health, Singapore, to determine the immunogenicity of reduced dosages of both plasma- and yeast-derived vaccines in children and adults. The findings of these studies have been published in several peer-reviewed journals. However, this long-term follow-up study on the immunogenicity of low doses of yeast-derived vaccine in healthy children 1-12 years of age has not been published previously. It is now published here as part of the scientific events to commemorate the 25th anniversary of the national childhood immunisation programme against hepatitis B in Singapore.

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Review of the timing for gradual transition from containment to mitigation during the influenza A (H1N1-2009) pandemic in Singapore

The first outbreak of influenza A (H1N1-2009) virus was reported in Mexico in March and early April 2009[1]. The United States Centers for Disease Prevention and Control (CDC) confirmed 2 cases of influenza A (H1N1) on 17 April, and identified the virus to be swine influenza A (H1N1) based on preliminary genetic characterization of the influenza viruses[2]. A Public Health Emergency of International Concern was declared by the World Health Organization (WHO) on 25 April 2009[3]. WHO progressively elevated its influenza pandemic alert to phase 4 on 27 April and then to phase 5 on 29 April 2009, and declared the first influenza pandemic of the 21st century by moving to phase 6 on 11 June 2009[4].

The public health control measures against influenza A (H1N1-2009) adopted by Singapore can be broadly grouped into two phases[5]- containment and mitigation. During the containment phase when the cases were mostly imported and/or local clusters were linked to imported cases, the aim was to delay the spread of the disease in the community. During the mitigation phase when it had been ascertained that there was sustained community spread, the aim was to slow the spread of the disease, reduce morbidity and mortality from the disease, and minimise disruption to essential services. Most of the public health measures were common for both strategies of containment and mitigation[6]. The transition from containment to mitigation mainly involved stopping border screening and not actively carrying out contact tracing, isolation and quarantine. The focus was then switched to medical treatment and public education on hygiene and social responsibility not to spread influenza.

The determination of an appropriate time to initiate the change in strategy from containment to mitigation during an influenza pandemic is important to achieve optimal resource and case management. The timing of the decision by the Ministry of Health (MOH) to gradually switch the pandemic influenza control strategy from containment to mitigation was reviewed with respect to the indicators of influenza activity.

Materials and methods

We carried out a retrospective study of the quantitative indicators of influenza activity in Singapore based on epidemiological and virological data which MOH monitored on a daily basis between June and July 2009. The 2 indicators were the cumulative daily proportion of unlinked cases among local cases, and daily proportion of influenza A (H1N1-2009) among samples of patients with influenza-like illness (ILI) from polyclinics, private clinics and hospitals tested.

Influenza A (H1N1-2009) was made a legally notifiable disease in Singapore and medical practitioners are required to report all cases within 24 hours of diagnosis to MOH via fascimile or a dedicated website since 28 April 2009. Cases of influenza A (H1N1-2009) were laboratory-confirmed with H1N1-2009 specific polymerase chain reaction (PCR). Epidemiological information was obtained as part of active

contact tracing conducted via telephone interviews, which included demographic data, clinical symptoms, contact history, travel history during the period of 7 days prior to the onset of symptoms, and movement history from the onset of symptoms to the date when medical treatment was sought[7].

Daily biosurveillance of samples of patients with ILI (temperature>38°C with cough or sore throat) from polyclinics, private clinics and hospitals tested was also carried out.

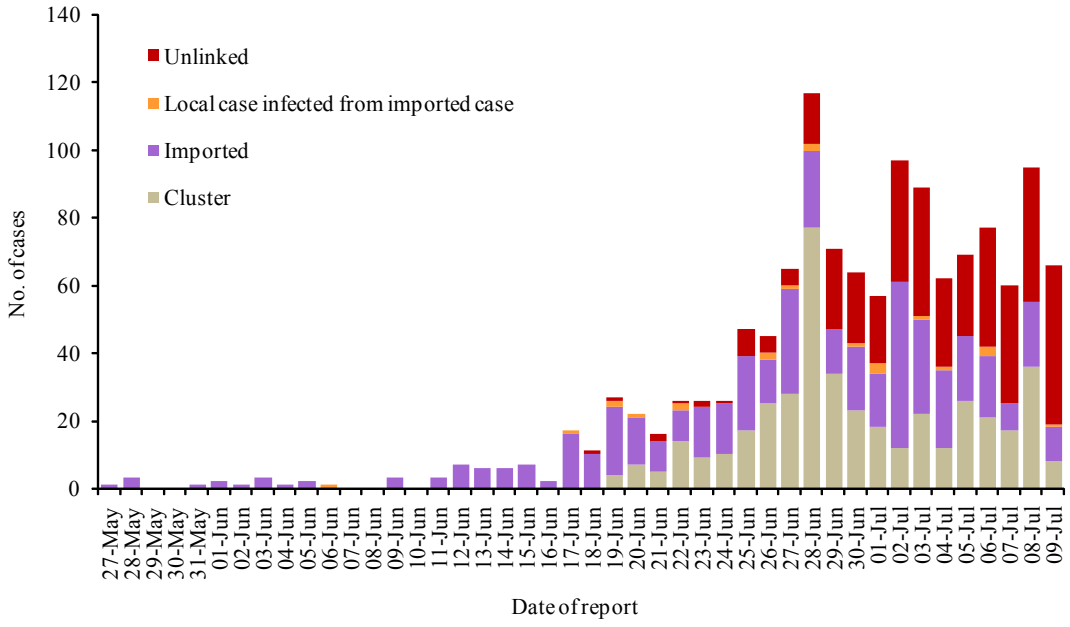
Results

The first case of influenza A (H1N1-2009) in Singapore was detected in a traveller returning from New York on 26 May – one month after the announcement by the WHO of the novel virus outbreak on 24 April 2009[8].

Three distinct waves of importation of cases were noted; the first wave comprised cases imported mainly from the United States during 27 May - 7 June, the second wave from Australia during 8 - 19 June, and the third wave from South-East Asia from 20 June onwards. The third wave of imported cases coincided with the beginning of local transmission (*Fig 3*).

The first case of influenza A (H1N1-2009) who was infected locally was reported on 18 June 2009. A total of 220 cases were reported between 27 May and 24 June 2009. Singapore began moving from the containment phase to early mitigation on 25 June. As of 30 June, medical practitioners were only required to notify clinically suspected cases who were seriously ill and who required referral to hospitals for treatment, and laboratory-confirmed cases of influenza A (H1N1-2009) within 24 hours of referral or diagnosis.

Figure 3
Influenza A (H1N1-2009) cases by classification, 27 May – 9 Jul 2009



Notifications of suspect cases were no longer required. Isolation of confirmed cases in hospitals was also no longer required from 1 July. Border screening was suspended from 8 – 9 July. Active contact tracing of laboratory-confirmed cases was no longer carried out after 9 July. The 993 ambulance service for transport of suspected cases to hospitals was terminated on 18 July. Since mid-July 2009, hospitals had to submit information of patients admitted due to clinical indications and positive for influenza A (H1N1-2009)[9].

The following 2 quantitative indicators were reviewed to determine the most appropriate date for implementation of the change in strategy from containment to mitigation.

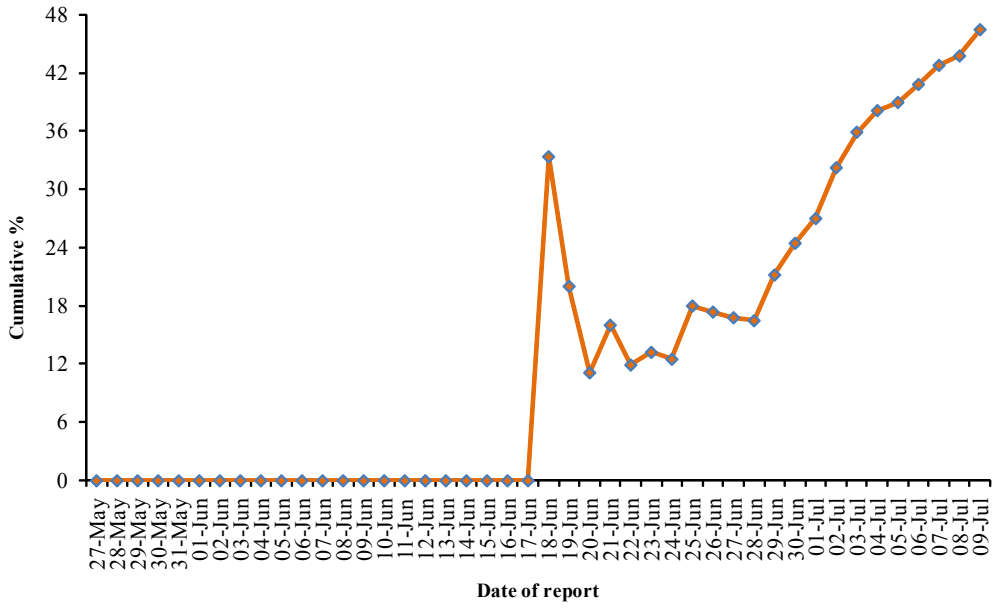
1. Cumulative daily proportion of unlinked cases among local cases

The cumulative daily proportion of unlinked cases of influenza A (H1N1-2009) among local cases first increased by 1.5 times from 11% on 20 June 2009 to 16% on 21 June, and increased by 1.4 times from 12.5% on 24 June to 18% on 25 June (*Fig 4*). The point of inflection of the cumulative unlinked cases was estimated to be around 25 June 2009, at which the trajectory of the pandemic started to increase when a lower increase or deceleration was replaced by a rapid increase or acceleration.

2. Daily proportion of influenza A (H1N1-2009) among ILI samples

The daily proportion of influenza A (H1N1-2009) among samples of patients with ILI from polyclinics, private clinics and hospitals tested was 1.1% with the first unlinked local case tested on 17 June 2009. It then increased to 5.4% on 20 June, dropped to 2.0% on 24 June and rose steeply to 8.5% on 25 June. The daily proportion of influenza A (H1N1-2009) remained above this level after 25 June (*Fig 5*).

Figure 4
Cumulative daily proportion (%) of unlinked local cases of influenza A (H1N1-2009)



Comments

We compared the development of the influenza A (H1N1-2009) pandemic and the public health response in selected countries with that of Singapore (*Table 1*).

Australia classified its response measures into 4 phases: delay, containment, sustain, and protect[10]. To respond to the international outbreaks, the "delay" phase was adopted on 28 April 2009, with a major focus on border control. The first imported case was confirmed on 9 May, and Australia adopted a containment strategy on 22 May in response to the first cases of influenza A (H1N1-2009), and subsequently changed its strategy initially in the most affected state of Victoria to a modified 'sustain' phase[11]. On 17 June, a new pandemic 'protect' phase was created to guide

Figure 5
Daily proportion (%) of influenza A (H1N1-2009) among all ILI samples tested

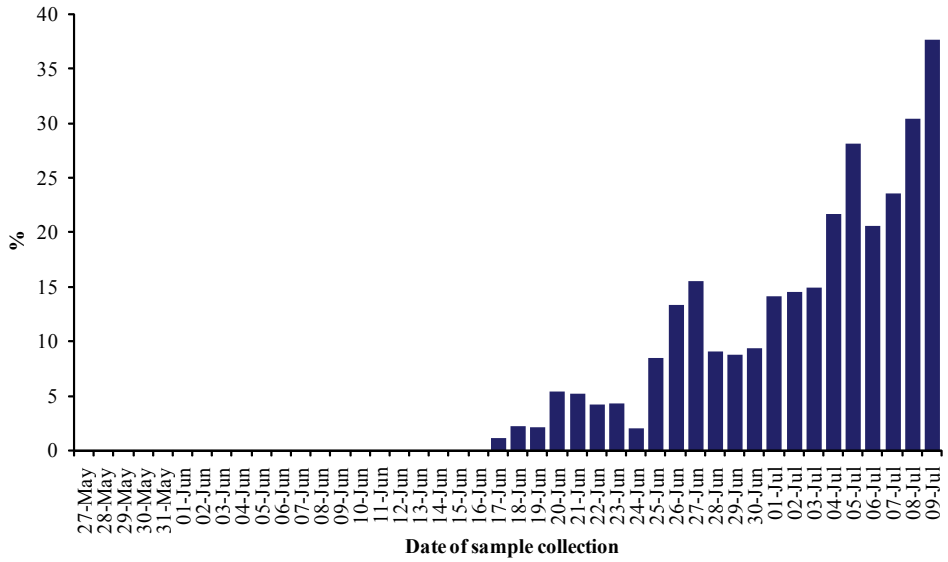


Table 1

Timelines of detection of first imported case(s), first local case(s) and change in strategy from containment to mitigation in selected countries

Country	Date first imported case(s) was detected	Date first local case(s) was detected	Change from containment to mitigation	No. of days from detection of first local case(s) to mitigation
Australia	9 May	22 May	22 May	0
Hong Kong	1 May	10 Jun	10 Jun	0
Japan	9 May	16 May	21 May	5
Singapore	26 May	18 June	25 Jun	7
United Kingdom	27 Apr	1 May	23 Jun	54
Taiwan	20 May	25 May	15 Jun	21

the ongoing Australian response to the disease, which took into account the less severe clinical characteristics of the current pandemic[12]. This change in strategy focused mainly on the early detection and adequate treatment of (potentially) severe cases.

Hong Kong confirmed its first reported imported case on 1 May 2009 and the first reported local case on 10 June[13]. Hong Kong switched from the containment phase in which all confirmed cases were isolated in hospital and their contacts were traced and quarantined to the mitigation phase when the first local case was confirmed after nearly 6 weeks since the first imported case was reported, and announced immediate closure of all educational institutions. Some containment measures including isolation of cases and prophylaxis of contacts were maintained until 27 June.

Japan reported its first 4 laboratory confirmed imported cases who returned from Canada on 9 May 2009. The first local cases were detected among 5 high school pupils in Ibaraki city, Osaka prefecture and 4 from Koke City in the neighbouring Hygo prefecture on 16 May[14]. With higher occurrence of school outbreaks, the focus of disease control was then shifted from "containment of disease outside of borders" to "strengthening medical systems" when large-scale community outbreaks occurred. ILI screening on board aircraft was terminated on 21 May.

On 15 April and 17 April 2009, novel swine-origin influenza A (H1N1) virus was identified in specimens obtained from two epidemiologically unlinked patients in the United States, which was of the same strain identified in Mexico, Canada, and elsewhere[15]. Between 15 Apr and 5 May, a total of 642 confirmed cases were identified in 41 states. By mid May, influenza A (H1N1-2009) had spread rapidly in the United States and confirmed cases had exceeded 3,000, and as a result, the country lifted its travel warning to Mexico on 15 May. On May 26, the focus of disease control was shifted from confirming infections case by case to monitoring the spread of disease and the development of severe cases.

The United Kingdom reported its first 2 confirmed cases in Scotland on 27 April 2009, which involved a couple returning from travel to Mexico. The first local cases were reported on 1 May[16]. On 23 June, UK aborted measures to contain the disease at affected areas through the use of antiviral prophylaxis and on 3 July, it focused mainly on treatment of cases.

Taiwan detected its first imported case on 20 May 2009, and the seventh confirmed case detected on 25 May was the first local case infected by imported cases. In mid-June, Taiwan shifted the focus of disease control to mitigation to be aligned with the direction of international disease prevention[17].

While Singapore moved to the early mitigation phase on 25 June 2009, about 1 week after the detection of its first case of local transmission, similar to Japan, some countries such as Australia and Hong Kong changed their strategy on the same day that their first local case(s) were detected.

The trends of the cumulative daily proportion of unlinked cases among local cases and daily proportion of influenza A (H1N1-2009) among ILI samples provided an early signal on 25 June for the gradual transition to mitigation in the week of 29 June 2009. This study suggests that these two quantitative indicators are useful to guide such a decision during an influenza pandemic.

(Reported by Ang LW, Tey SH, Cutter J, James L, Communicable Diseases Division, Ministry of Health)

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Molecular epidemiological investigation and risk assessment of *Plasmodium knowlesi* transmission in Singapore

Introduction

Plasmodium knowlesi, a simian malaria parasite incriminated by some as a potential fifth human malaria parasite[1], was first identified in India in 1931 from a long-tailed macaque imported from Singapore[2]. Its ability to infect human beings was first demonstrated in 1932 by Knowles and Das Gupta, who successfully transmitted the parasite from infected macaques to human volunteers via blood passages. However, the first natural transmission of simian malaria parasite in man was only reported in 1965, when an American surveyor acquired *P. knowlesi* infection after working in the jungle of Pahang, Peninsular Malaysia[3]. As natural infection by simian malaria parasites was thought to be rare, it was not recognized as a major public health problem and therefore not included in malaria eradication plans. However in 2004, a large focus of *P. knowlesi* infection, which had been misdiagnosed as *P. malariae* by microscopy, was reported in the Kapit district of Sarawak, East Malaysia[4]. Thereafter, reports of natural infection of *P. knowlesi* also surfaced in neighboring countries like Thailand[5] , Philippines[6], Peninsular Malaysia[7], Vietnam[8], Indonesian Borneo[9] and along the border of China and Myanmar[10].

Singapore reported the first locally acquired human *P. knowlesi* infection in 2007[11]. This index case involved a military personnel with no significant travel history, but had visited a restricted-access forest area in Singapore, where free roaming long-tailed macaques can be found. Comprehensive fever surveillance and monitoring among military personnel who accessed the affected forested area revealed an additional five human knowlesi malaria cases in 2007 and one in 2008.

Epidemiological investigation

As long-tailed macaques (*Macaca fascicularis)* are natural hosts to *P. knowlesi*, blood from 49 macaques caught in the affected forested area was sampled and sent to the Environmental Health Institute, National Environment Agency, for molecular screening of *Plasmodium* parasites. Out of the 49 macaques tested, 24 were found to harbour *P. knowlesi*. To determine if the human cases had acquired the knowlesi parasites from the macaques, molecular analysis of the human cases and *P. knowlesi* positive macaques was carried out.

Epidemiological investigation was conducted using phylogenetic analysis of the non-repeat region of circumsporozoite protein (csp*)* genes of the malaria parasites from four of the six human cases and the three monkey samples[12]. The non-repeat region of the *csp* genes of the malaria parasites formed a monophyletic clade with other *P. knowlesi csp* genes obtained from GenBank, reconfirming the identification of *P. knowlesi*. Each long-tailed macaque was found to harbor one to three genotypes of *P. knowlesi,* whereas only one genotype was observed from each human isolate.

In general, *P. knowlesi* sequences from human cases were not phylogenetically distinct from the

macaques' sequences. There were shared genotypes between three human cases detected in 2007 and two macaques caught in the same year. One of the genotypes was shared among the two macaques (SG/EHI/LT-001 and SG/EHI/LT-002) and a human case (SG/EHI/H-007). Another genotype was shared among the other two human cases (SG/EHI/H-001 and SG/EHI/H-002) and one of the macaques (SG/EHI/LT-002). The human case (SG/EHI/H-024) detected in December 2008 shared the same genotype as a macaque caught in June 2009 (SG/EHI/LT-013) *(Fig.6)*.

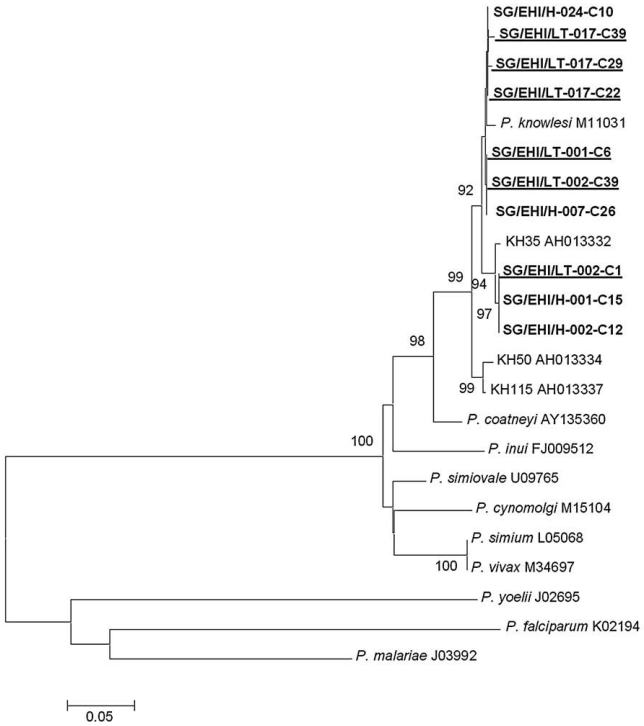
Evidence from molecular analysis suggested that the human cases reported in 2007 were epidemiologically linked to the two macaques caught about the same time in the affected forested area. Similarly, the most recent human case detected in December 2008 was linked to the macaque caught in 2009. The sharing of identical *P. knowlesi* csp sequence, in addition to the fact that none of the cases had significant travel history one month prior to onset of symptoms, strongly suggest that the human cases had acquired the infection locally.

Risk assessment of *P. knowlesi* transmission in Singapore

In addition to the screening of *P. knowlesi* in wild macaques caught from the restricted-access forest, malaria screening was also conducted on blood samples from peri-domestic macaques caught in the fringes of nature reserve and residential areas.

Figure 6

Phylogenetic tree based on the non-repeat region of the circumsporozoite protein genes of *Plasmodium* sp. produced by the neighbor-joining method. Clones highlighted in bold are obtained from Singapore isolates and clustered in the *Plasmodium knowlesi* clade. Clones highlighted are from human isolates, whereas those highlighted and underlined are from monkey isolates



A total of 33 peri-domestic macaques were screened and none were positive for malaria parasites. Similar results were also reported by Malaysian[7] and Thai[13] researchers; all long-tailed macaques caught in urban areas where competent vectors are most likely absent were free from malaria infections.

Conclusion

Long-tailed macaque is a natural host of *P. knowlesi* in Singapore, and human cases acquired their infections while working in areas where infected macaques are found. However, the risk of the general population of Singapore acquiring the infection may be low as macaques close to human dwellings were tested free from malaria parasites. Nevertheless, awareness of *P. knowlesi* being a potential 5th human malaria parasite should be made known to medical practitioners as imported cases of *P. knowlesi* from neighbouring endemic countries had been reported. During the early trophozoite stage, *P. knowlesi* resembles *P. falciparum* while at later stages it resembles *P. malariae*. Unlike these parasites, *P. knowlesi* exhibits a 24-hour life cycle. Therefore, when encountered with cases presented with daily fever peaks and blood smears suggestive of *P. falciparum* and *P. malariae*, diagnostic test for *P. knowlesi* should be requested. Accurate diagnosis and prompt treatment is critical as *P. knowlesi* infection is potentially fatal[14].

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Enforcement of treatment in recalcitrant TB treatment defaulters

Introduction

The control of tuberculosis (TB) is dependent on the effective treatment of infectious TB cases. Non-adherence to TB treatment prolongs infectiousness, sustains the spread of TB in the community, promotes the development of drug resistance, and contributes to the pool of patients in the community with poor outcomes, including relapse and even death. In Singapore, the National TB Registry, also known as the STEP (Singapore TB Elimination Programme) Registry, monitors the treatment progress of each notified TB case.

To address in part the issue of treatment non-adherence among the recalcitrant TB treatment defaulters, a public health legal framework using the provisions under the Infectious Diseases Act (IDA) to order such patients to submit for treatment was put into effect in 2004 (*Fig.7*).

Under the framework, cases found to be persistently defaulting on their anti-TB treatment despite efforts to support treatment adherence, will be referred to an independent review Committee appointed by the Ministry of Health (MOH) to deliberate on the merits of enforcing mandatory treatment in these cases. Cases identified for mandatory TB treatment would be surfaced to the MOH for legal actions to be taken under the IDA

Materials and methods

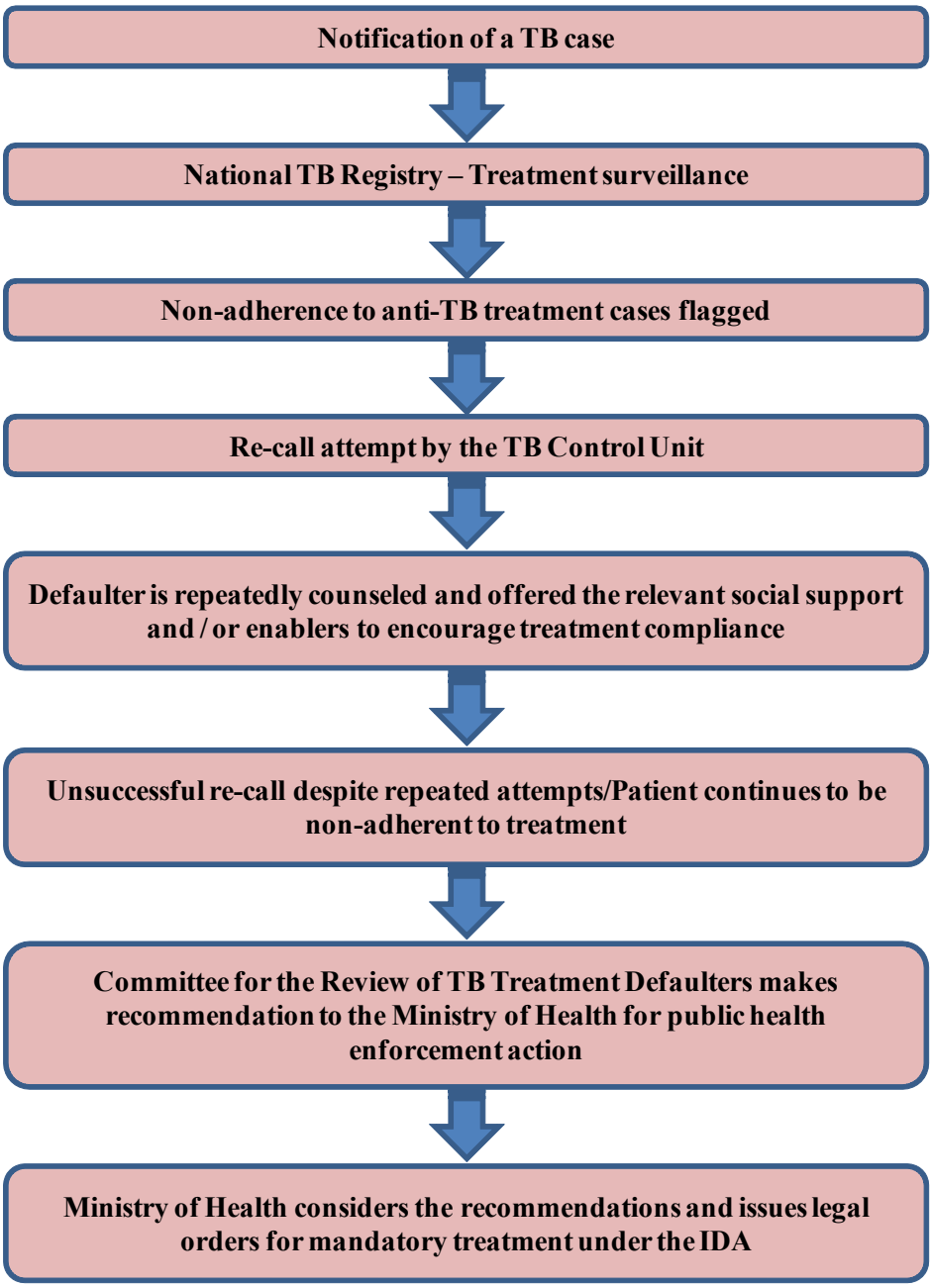
A retrospective study was conducted on the recalcitrant TB treatment defaulters who were issued with the legal orders by the Ministry of Health to undergo mandatory treatment for TB under the IDA between 2004 and 2008.

Results

Pulmonary TB accounted for the majority of notified TB cases in Singapore and between 2004 and 2008, accounted for 86.5%- 83.3% of new TB cases among Singapore residents.

From 2004 to 2008, 120 recalcitrant TB treatment defaulters were issued with a legal order under the IDA to undergo mandatory TB treatment. The annual number of legal orders served to TB treatment defaulters increased by 66.7% from 27 in 2007 to 45 in 2008. This coincided with the increase in the number of pulmonary TB diagnosed in 2007 (1074 cases) and 2008 (1208 cases) (*Fig. 8*)[1]. All patients who have been served with a legal order under this framework would be required to undergo either, (a) outpatient directly observed therapy (DOT) at their

Figure 7
Workflow for managing anti-TB treatment defaulters

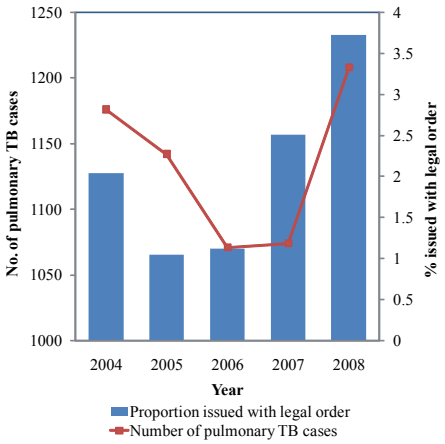


nearest polyclinic or at the TB Control Unit, or (b) inpatient treatment at a designated public sector hospital, depending on their treatment needs. Of the 120 patients issued with the legal order, 87.5% (105) were males and 12.5% (15) were females making a male to female ratio of 7:1. This is consistent with the gender distribution of TB cases notified to the STEP registry - the male: female ratio of all pulmonary TB cases notified between 2004 and 2008 was 2.7:1[1,2]. Most of the cases, i.e. 106 (88.3%) eventually completed treatment and 10 patients died.

Conclusion

Raising awareness of the TB disease and treatment compliance, and reducing barriers to treatment adherence continue to be important measures. However, when these measures fail, legal action can be employed as a last resort to compel treatment adherence among recalcitrant TB treatment defaulters.

Figure 8
Pulmonary TB cases and proportion issued with legal order



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