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HISTORY OF NEUROLOGY

The discovery of Nipah virus: A personal account

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THE INITIAL PREOCCUPATION WITH JAPANESE ENCEPHALITIS

The outbreak of Nipah virus was a tragic event for the people of Malaysia especially for those in the pig-farming industries.[1] Though, officially the outbreak was recorded as starting in September 1998 in the northern part of peninsular Malaysia, the virus had most likely spilled into the swine population as early as 1997.[2] This was substantiated with a finding of 6 encephalitis patients who were admitted to Ipoh General Hospital in 1997 and whose stored sera were found to have anti-Nipah virus IgG.[3] The outbreak of febrile encephalitis in humans was preceded by the occurrence of respiratory illness and encephalitis in pigs in the same region. Unusual deaths in pig population were reported by pig farmers to veterinary officers in Veterinary Research Institute, but the cause of swine mortality was initially assumed to be due to classical swine fever (Personal communication, Dr Jasbir Singh, Veterinary Research Institute, Ipoh, Malaysia). Unfortunately, when human beings came down with encephalitis, a label of Japanese encephalitis was put on it.

In the early period, acting on the opinion of experts and the country's senior consultant virologists, drastic control measures based on the control of Japanese encephalitis epidemic were proactively and intensively taken by the government to control the outbreak. Very intensive fogging of insecticides was carried out at the outbreak areas and other pig-farms. Thousand of doses of Japanese encephalitis vaccines were rushed in from Japan to vaccinate the farmers and those who stayed near the pig-farms.

With the failure to control the outbreak, a conference was initiated by the Ministry of Health, Malaysia and the Veterinary Research Institute to brainstorm issues regarding the outbreak. The meeting was held in Veterinary Research Institute on the 24th of February 1999. As I learned later from some physicians who attended the meeting, the unusual epidemiological features of the outbreak with involvement mainly adults, which was not the usual scenario of Japanese encephalitis outbreak in Malaysia in the past, was brought up by some physicians. Unfortunately, this was interpreted as a different trend in Japanese encephalitis, as reported by the newsprint quoting Professor SK Lam, Head of Medical Microbiology Department, University of Malaya.[4] At that time, there were some pig farmers who had already been given 2 and even 3 doses of mouse brain derived Biken Japanese encephalitis vaccines. They were told to be safe to go back to pig farms to work. Some later became infected with encephalitis and succumbed to the illness. This was interpreted by the Head as the possibility that the Biken mouse brain derived Japanese encephalitis vaccine was not effective. He initiated and liaised with Dr Hyunsoo Kim in Republic of South Korea to seek the import of live attenuated Japanese encephalitis (SA14-14-2) vaccine for field trial.

At that time, in our Department, the concern of the Head was just to confirm Japanese encephalitis. Thus, only measurement of Japanese encephalitis-specific IgM was carried out in the laboratory and subsequently a post-doctorate scientist (Dr Brindha RN) was recruited to perform the Japanese encephalitis RT-PCR test on the same clinical samples. Probably because of asymptomatic endemic Japanese encephalitis infection[5], low cut-off threshold positive value or cross-reactivity with other flavivirus group such as dengue which was hyperendemic in this country, a high rate of positive Japanese encephalitis-IgM serology was reported, although quite a proportion of patients' sera that were tested positive in the department were subsequently found to be of false positive by Division of Arbovirus Diseases, CDC, Fort Collins headed by Dr Duane Gubler (Personal communication). I also had reservations of the many positive RT-PCR, which I believed were false positive. This was communicated to the

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Head of Medical Microbiology Department. To my disappointment, at the early March, the Head gave the feedback to the Ministry of Health that 40% of the cases was confirmed Japanese encephalitis. It was also mentioned that there was no necessity to confirm 100% during the Ministry of Health outbreak meeting.

THE DISCOVERY OF NIPAH VIRUS

I was officially involved in the outbreak investigation on 27th of February 1999 when a patient (PKL) from one of the outbreak farms in Bukit Pelandok was admitted to University Malaya Medical Centre with acute encephalitis. I isolated Nipah virus from his cerebrospinal fluid on the 9th day of inoculation into tissue cell culture, although his serum was also tested positive for Japanese encephalitis-IgM and Japanese encephalitis specific nucleic acids was apparently detected by RT-PCR on the third day of his admission. With the possibility that the outbreak was due to an unusual or even novel agent, I believed viral culture was necessary. I was wondering how to convince my Head of Department to give permission for virus isolation, as he believed then that the outbreak was due to Japanese encephalitis virus. I finally gave the reason that if it was a Japanese encephalitis outbreak, it could be from a different genotype of Japanese encephalitis virus, because of the unusual epidemiological features of the outbreak. With the isolated virus, we could sequence the complete virus genome. However, as soon as permission was granted, instead of inoculating the patient's cerebrospinal fluid and serum samples into C6/36 mosquito cell-line, which was the cells specifically used for isolation of Japanese encephalitis virus, I quietly inoculated the samples into a number of other continuous cell-lines seeded in 24-well cell culture plates at 10^5 cells per well. They were the MDCK cells from Madin-Darby canine kidney, Vero CCL-81 cells from African green monkey kidney, PS cells from pig spleen, Hep2 cells from human laryngeal carcinoma and MRC5 cells from human fibroblast. The culture plates were then carefully sealed and incubated at 37^0C.

On 1st of March 1999, our Department received additional cerebrospinal fluid and serum samples of 5 patients admitted to Seremban General Hospital with similar illness. One hundred microlitres of each clinical sample was similarly inoculated into the respective cell-lines for virus isolation. By 5th of March, one of the patients' (EKK) cerebrospinal fluid sample gave a syncytial type of cytopathic effect in Vero cells that was quite similar to the type of cytopathic effect seen in Hep2 cells due to respiratory syncytial virus. I was so excited and anxious to see such an unusual cytopathic effect and showed it to the Head immediately. He took a look at the culture under the inverted microscope, turned to me and told me it was mostly some form of contamination. He told me to ignore it and discard it. At that point of time, I was stunned and could not believe my ears. I just could not describe my disappointment but would not dare to challenge him.

On 6th of March, 2 more of the original 5 cerebrospinal fluid samples from the Seremban General Hospital patients (TCC, G) produced similar cytopathic effect in Vero cells. I immediately rushed to tell the Head about the findings. To my great disappointment, he did not even want to look at the cell culture and signaled to me to ignore it. On the 8th of March, the cerebrospinal fluid sample of PKL gave the same pattern of cytopathic effect. By then, his serum sample was also tested positive for Japanese encephalitis-specific IgM, and Japanese encephalitis nucleic acid was apparently detected by RT-PCR by the Arbovirus Unit of our Department. At that time, I could sense it was something new and deadly. Thus, I did not follow the instruction to ignore and throw away the virus cultures. During that period, it was also a great challenge to convince other staff in the department the possibility of something new. The only person who believed me, was my faithful medical laboratory technologist, Ms PS Hooi. I was so desperate to convince other colleagues that I went around asking other academic staff to look at the cytopathic effect in the culture flask. I also caught hold of Professor CT Tan who was the physician in charge of the encephalitis patients in our Medical Centre. He did not think the outbreak was due to Japanese encephalitis, and favored an unusual or novel organism based on epidemiological reasons. He agreed with my finding but he was not an expert in laboratory virology. I also showed the findings to Dr Winnie Yeap, research assistant, and Dr Sazaly Abu Bakar, another virologist in the Department.

On the 7th of March 1999 (Sunday), the remaining cells in the first cell culture flask with cytopathic effect (EKK) had shown complete cell death. I carefully harvested the infected cells, washed with sterile phosphate buffered saline, transferred them into wells of 24-well Teflon coated slides and subsequently inactivated them

to be used as antigens for identification. At the same time, I carefully passed 0.5 ml of the culture supernatant into a 25 cm^2 flask of Vero cells to scale up the production of infected Vero cells for electron microscopic study. I knew if the virus was new, existing typing reagents would not be able to identify the virus. But the morphology of the virus under electron microscope would at least be able to tell me which family the virus belonged to. That would be able to influence the control measures of the outbreak. There was a great temptation to perform negative staining of the virus for electron microscopy. I refrained from doing so as I sensed that the virus was highly deadly judging at the ways it replicated in culture flask, and patients were dying. In addition, the facility for such technique was not available in the department and the functioning electron microscope in campus was about a kilometer away.

I then managed to prepare 3 10-well Teflon coated antigen slides. I used one of the slides and began to carry out serological identification of the virus using whatever commercial typing monoclonal antibodies against viruses that were known to cause syncytial cytopathic effect in tissue cultured cells. They were herpes simplex virus, respiratory syncytial virus, measles virus, mumps virus, and parainfluenza viruses. As expected, all turned out to be negative. Subsequently, I reasoned that if the patients were infected with the virus that I had isolated, they should have antibodies against the virus. Using another prepared Teflon slide, I carried out the detection of human antibodies against the isolated virus by indirect immunofluorescent assay using sera and cerebrospinal fluid derived from patients of Bukit Pelandok area and a patient from Ipoh (LKK) who was transferred to Tawakal Hospital in Kuala Lumpur. By 6.30 p.m. the stained slide was ready and when I looked at the finding, I could feel a chill going down my spine. All the patients' sera and cerebrospinal fluid included in the test had strong positive antibodies against the infected Vero cells. The infected syncytial cells lighted up as "bags of green fluorescence lanterns". I immediately called up the Head of Department in his house and asked him to come to the laboratory. I can still remember vividly, he arrived at the laboratory at about 7.00 p.m. After looking at my finding in the UV-microscope, he looked up with his facial expression changed, turned to me and said, "Chua, why can't nature be more straightforward". At that time, the faithful medical laboratory technologist, Hooi was by the side and we just looked at each other. I felt like answering him, "Prof, nature was actually straightforward, it was you who thought otherwise". However, I did not have the courage and remained silent. Even then, he was still doubtful of the finding and told us that it could be cross-reactive antibodies. By then, it was almost 8 pm, and both myself and Hooi had not even taken our lunch. I suggested to him since I still had one more antigen slide, Hooi would pull out some patients' sera from the outbreak area and at the same time include some sera of patients known to be infected with measles virus, mumps virus and human herpes simplex virus. We will pass these to him the following morning. He would re-code the serum samples and then pass to me for testing.

We did exactly as agreed on the following day. At noon of 8th March 1999, we met again to review my findings. The result was a 100% correct match. I was able to identify correctly the persons infected with the isolated virus. None of the serum samples from patients known to be infected with measles virus, mumps virus and human herpes simplex virus reacted with the isolated virus. I knew there was going to be a meeting with representatives from Ministry of Health and Veterinary Research Institute on the 9th of March 1999 in Institute of Medical Research down town. I persuaded him to release the finding in the meeting. I also managed to persuade him to include Professor CT Tan to the meeting despite earlier tension due to differences in opinion on the cause of encephalitis.

On the 9th of March, 2.00 pm, we met in the main meeting room of Institute of Medical Research and there was still a lot discussion about the outbreak as Japanese encephalitis. This was because the laboratory findings from the Arbovirus Unit, our Department had already confirmed 40% of the cases to be due to Japanese encephalitis. I was quite upset and worried that my Head of Department would back off and not release the finding. At that juncture, I decided that if he withheld the finding, I would disclose the result but prayed that I would not have to do so. I was relieved as towards the later part of the meeting, he finally mentioned the possibility of a new virus besides the Japanese encephalitis.

On our way home, it rained heavily and we were caught in a traffic jam. With Professor CT Tan sitting beside him, our neuropathologist Dr KT Wong and myself at the back, I told the Head that it was most likely a new virus and we needed international assistance urgently to identify the

virus as quickly as possible as the outbreak was getting out of hand. At that time, it was not easy to get courier company to ship consignment packed in dry ice overseas. I suggested to him to hand carry it out of the country after careful packing. To avoid being misunderstood that I liked to go overseas often as I just returned from Perth prior to this outbreak on work related to a chikungunya outbreak, I suggested to him to send a medical technologist. However, on reaching home at almost 8.00 pm, I told my wife to pack my clothing because I knew I would probably have to do the job personally. It was likely to be a very deadly virus.

True enough, on meeting the Head in the morning of 10th March, the first thing he told me was that should any one be needed to ship out the material for identification, it would be me. At that point, I did not have a USA visa and suggested to him to call up the Minister of Health for help. I was very impressed and touched by the Minister, the Honorable Dato' Mr. JM Chua, as he must have called the US embassy. I was given a 10-year US visa within 24 hours.

THE IDENTIFICATION OF NIPAH VIRUS

On 11th of March, after 3 days of fixation and embedding in epon, the inactivated Vero cells infected with the virus were ready for viewing under electron microscope. No functioning electron microscope was available in the Faculty of Medicine of the University. I took the ultra-thin sections of electron microscopy slides prepared by Ms Elsie Wong of Department of Pathology to the Institute of Higher Learning of the University. To my disappointment, the electron microscope in the Institute was not well maintained and only some blurred images of envelope viral particles ranging in size of 100+ to 200+ nanometer with thickening of infected cell membrane were visible. No conclusive information could be obtained. However, as the virus caused infected cells to fuse and lead to formation of syncytial giant cells in tissue culture Vero cells, with the finding of thickening of certain parts of cell membrane under electron microscope suggesting viral morphogenesis at the membrane, a paramyxovirus seemed most likely.

On 12th of March 1999, I boarded the airplane and headed for the Division of Arbovirus-borne Diseases, Center for Disease Control and Prevention (CDC), Fort Collins, USA. I was sent to CDC in Fort Collins instead of CDC in Atlanta because the Head still held on to the idea that the outbreak was due to an arbovirus. I arrived at Denver Airport around 11.30 pm 13th of March, and took the Sambrock shuttle van service to Fort Collins. At 8.00 am 14th of March, Dr Nick Karabatsos picked me up from the hotel and took me to his laboratory. On arriving at the laboratory, he asked me whether I was familiar with immunofluorescence assay technique. I told him I had no problem and he immediately took out a whole panel of typing antibodies against known arboviruses and handed it to me. Using the antigens slides I brought along, 2 hours later, we found that none of the antibodies in the panel reacted with the virus infected cell. By then, Dr Bruce Cropp came in and led me to his electron microscope laboratory. On seeing his electron microscope (Philip EM410), which was an older version of the Institute of Higher Learning (University of Malaya)'s electron microscope (Philip CM12), my heart sank. The old scope still needed liquid nitrogen system for cooling. However, to my amazement, the old electron microscope was so well maintained that the moment he put the section that I brought along into the scope and turned on the screen, I could recognize the "concrete ring-like" structures of the paramyxovirus nucleocapsids cut in transverse section immediately. At that moment, a sense of great fear overwhelmed me. My God!!! it was truly a paramyxovirus. To my limited knowledge then, all paramyxoviruses known to me spread by close contact and droplet. No wonder all the Japanese encephalitis control measures intensively carried out by the Ministry of Health failed. I really felt very sorry for the poor pig farmers and workers who were given the Japanese encephalitis vaccines, told they were protected and sent back to the farms and became infected.

I was so overcome by sadness, that as soon as we completed capturing the required electron micrograph images, I requested Dr Cropp to use the phone in CDC and called up my Head in Malaysia. I managed to get him at home. I still remember vividly what I told him over the phone; "Prof. Lam, Chua here, calling from CDC Fort Collins. Prof., listen! listen carefully! under the electron microscope, the virus has the morphology of a paramyxovirus. For God's sake, please do not talk about Japanese encephalitis anymore. I am quite sure now it's a paramyxovirus. Most likely it is a new paramyxovirus. The control measures for paramyxovirus are totally different from Japanese encephalitis virus. Please! I want you to urgently pass this message to the Ministry

of Health to stop all the Japanese encephalitis control measures and switch over to the following control measures …Prof, listen carefully, you must pass the information of what I have just said to the Ministry of Health as soon as possible…. (I knew there will be a meeting in the Ministry of Health regarding the outbreak on the 17th of March)…I'll get Dr Bruce Cropp to process the electron microscopy photograph and fax it to you as soon as it is ready." There was a fairly long silence and he did not reply to my words.

On the following day, which was 15th March for USA, and 16th of March for Malaysia, the electron micrograph of Nipah virus was ready and I faxed it to him. On the same day, there was a teleconference between CDC at Fort Collins and CDC at Atlanta. A decision was made to transfer all the materials I brought along from Fort Collins to Atlanta. These were the virus isolates, patients' serum samples, antigen slides and fixed electron microscopy sections of infected Vero cells. Soon after the teleconference, I requested Dr Duane Gubler to help me confirm the earliest flight to return to Malaysia. All I could think of, at that time was the unfortunate pig farm workers, and wanted to rush back to assist the control of the outbreak. As Duane's secretary was arranging the flight schedule, there was a telephone call from Atlanta requesting me to go over. In brief, at CDC, Atlanta the virus was rapidly identified as a novel paramyxovirus by the wonderful team of experts working together. The moment the virus was identified, I made a special request to Dr Brain Mahy, who was the head of the Viral and Rickettsial Disease Division, to dispatch a team of experts to Malaysia to assist the control of this tragic outbreak, which I felt had already became too extensive for the local experts. He responded that CDC needed an invitation from the Ministry of Health of Malaysia. I phoned the Head about my request to Dr Brain Mahy, and the need for an official invitation. He subsequently called up the Minister of Health to send the required letter to Dr Brian Mahy.

In conclusion, epidemiology and clinical knowledge opened the mind to the possibility of other virus than Japanese encephalitis during the 1998/99 Malaysian viral encephalitis outbreak. Viral culture led to discovery of the novel infective agent which was not possible with serology and RT-PCR.

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