Role of Electron Microscopy in Nipah Virus Outbreak Investigation and Control

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SUMMARY

In 1998, a novel paramyxovirus (order *Mononegavirales, family Paramyxoviridae*, subfamily *Paramyxovirinae*, genus *Henipavirus*) emerged in peninsular Malaysia causing fatal encephalitis in humans and severe respiratory illness with encephalitis in pigs. The virus was successfully isolated in cultured mammalian cells. Transmission electron microscopy of infected tissue culture cells played a crucial role in the early preliminary identification of the causative agent of the outbreak. This in turn was pivotal to determine the correct direction of control measures that subsequently brought the epidemic under control. In light of this investigation, and indeed identification of infectious agents associated with other disease episodes, electron microscopy will remain an important frontline method for rapid diagnostic virology and investigation of any future outbreak of new and unusual cases of illness suspected of an infectious aetiology.

KEY WORDS:
Electron microscopy, Nipah virus, Outbreak investigation

INTRODUCTION

In late September 1998, an outbreak of acute febrile encephalitis associated with high mortality occurred in the suburb of Ipoh city, located in the northwestern part of peninsular Malaysia[1-3]. The outbreak of febrile encephalitis in humans was preceded by the occurrence of respiratory illness and encephalitis in pigs in the same region[4]. Initially, control measures were implemented based on the putative diagnosis of Japanese encephalitis (JE) epidemic. Despite intensive proactive control measures, the outbreak spread to Sikamat, a town in Negeri Sembilan (southwestern part of peninsular Malaysia) by December 1998. By late February 1999, a similar disease in pigs and humans was recognized in Sungai Nipah village and Bukit Pelandok district (the biggest pig-farming region) in the same state[3,4].

The pattern of spread of the outbreak was associated with the movement of pigs from the initial outbreak farms in the suburb of Ipoh and between farms[4-6]. The outbreak was only successfully controlled following the institution of a control measure based on the successful isolation and identification of a new aetiological agent isolated from a patient in early March 1999[2,3].

This report demonstrates the crucial role of electron microscopic examination for an early preliminary identification of the causative agent of the outbreak which subsequently determined the appropriate effective control measures.

MATERIALS AND METHODS
Patients and samples

On 27th of February 1999, a patient (PKL) from one of the outbreak farms in Bukit Pelandok, was admitted to University Hospital Kuala Lumpur with febrile encephalitis. The usual serological (JE-IgM) and molecular (RT-PCR) tests on the clinical samples were carried out by the Arbovirus Unit of the Department of Medical Microbiology to confirm whether JE virus was the cause of the outbreak. As part of the investigation, the patient's cerebrospinal fluid (CSF) and serum samples were also inoculated into a number of cell-lines seeded in 24-well cell culture plates (10^5 cells per well); MDCK cells (Madin-Darby canine kidney, need ATCC CCL-34 code), Vero CCL-81 cells (African green monkey kidney, need ATCC CCL-81code), PK13 cells (pig kidney, need ATCC CRL-6489code), Hep2 cells (human laryngeal carcinoma, need ATCC CCL-23 code) and MRC5 cells (human fibroblast, need ATCC CCL-171 code). The culture plates were carefully sealed and incubated at 37^0C.

Subsequently, on the 1st of March 1999, the Department of Medical Microbiology, University of Malaya received additional CSF and serum samples of five 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) CSF sample gave a syncytial type of cytopathic effect (CPE) in Vero cells that was quite similar to the type of CPE seen in Hep2 cells due to respiratory syncytial virus[7]. On the 6th of March, two more of the original five CSF samples produced similar CPE in Vero cells. On the 8th of March, the CSF of PKL gave the same pattern of CPE though by then his serum sample was also tested positive for JE-specific IgM, and JE nucleic acid was detected by RT-PCR (Unpublished data). By 7th of March 1999, the Vero cells inoculated with EKK's CSF sample had reached advanced stage of CPE and the infected Vero cells

were carefully harvested, washed and transferred into wells of a Teflon coated slide. The slide containing the infected cells was allowed to be air-dried under UV irradiation in a biosafety class II cabinet, inactivated and fixed in cold acetone, and subsequently used as antigen for serological identification. On the same occasion, five hundred microlitres of the infected culture supernatant were carefully inoculated into a 25-cm^2 cell culture flask containing a confluent monolayer of Vero cells.

Electron microscopy

Within 24 hours of post-inoculation, the infected Vero cells in the 25-cm^2 culture flask showed almost complete CPE. The supernatant was carefully removed and aliquoted for storage at –80^{0}C. The infected cells adhered to the culture flask were lightly washed (x1) with sterile phosphate buffered saline (pH 7.2, osmotic pressure 300 mosmol/kg), five millilitres of 4% (v/v) cold glutaraldehyde was added (1 hour) followed by 8 hours post fixation in a 15-millitre screwed-cap Falcon tube with the same fixation. This unusual fixation step was used as the fatal infectious agent was at this point in time not definitively identified. Cells were subsequently pelleted by centrifugation at 1000X g for 10 minutes, (Appendorf, Germany), the pellet rinsed with cacodylate buffer (pH 7.2, 300 mosmol/kg) and post-fixed with 1% (w/v) cacodylate buffered osmium tetroxide (2 h) and stored in the same buffer overnight. The following day, the cells were washed with double distilled water and dehydrated with graded concentrations of ethanol (35%, 50%, 70%, 96% and 100%). The cells were then rinsed with propylene oxide and infiltrated with propylene oxide/epon mixture, followed by 100% epon before polymerization at 60^{0}C for 24 hours. Polymerised blocks cells were sectioned (60-70 nm) with an ultramicrotome (Leica Ultracut F, Vienna, Austria), stained with uranyl acetate followed by lead citrate, and examined with a Philip CM12 transmission electron microscopes at 80 kV.

RESULTS

All electron micrographs illustrated in this report were imaged from ultrathin sections from the same block. Figure 1 is transmission electron micrograph of Vero cells infected with Nipah virus taken on the 11th of March 1999 (Malaysian Standard Time) using the Philip CM12 transmission electron microscope (Institute of Higher Learning, University of Malaya, Malaysia). Figure 1a shows foci of thickened electron dense plasma membrane and underlying nucleocapsids suggestive of a *'paramyxovirus'* infection. Within the same section occasional enveloped particles approximating 150 nm in diameter were also noted, these particles possessed surface projections (Fig. 1a). The cells within which the above structures were observed were syncytial in nature, that is, they were multinucleated (Fig. 1b). Within some of these nuclei, intranuclear inclusions were noted but no associated ultrastructural detail could be discerned. Collectively, whilst the ultrastructural data was suggestive of a *'paramyxovirus'* infection, no definitive taxonomic morphological data could be obtained in this electron micrograph.

Figure 2 shows the electron micrograph of the same ultrathin section taken on the 14th March 1999 (Fort Collins, Colorado, USA Central Time zone) using a Philip EM410

transmission electron microscope. Illustrated within this micrograph are distinct enveloped viral particles containing nucleocapsids. Also shown is an area of plasma membrane which is electron dense and thickened suggestive of a 'paramyxovirus' infection. Associated with these regions, but internal to the cell membrane, are distinct nucleocapsid-like structures which in cross section appear as 18nm hollow tubes. Aggregates of similar nucleocapsid-like structures are also apparent in the cytoplasm of infected cell.

Figure 3 shows transmission electron micrographs imaged on the 10th of June 2003 using the newly acquired LEO 972 AB EFTEM transmission electron microscope (University Putra Malaysia). Figure 3a is an image of an unstained ultra-thin section. Figure 3b was imaged from a section lightly stained with uranyl acetate followed by lead nitrate. Enveloped viral particles are apparent in both sections. Similarly, the characteristic electron dense layer underlying the plasma membrane (representative of M protein) is apparent as are the nucleocapsids.

DISCUSSION

Despite the rapid advances in modern medicine and biotechnology, infectious diseases still pose a serious potential threat to global health. The burden of infectious diseases is especially crucial and obvious among the developing countries. Compounded to the existing infectious disease burden, in the past two decades, the world has seen the scourge of an increase in incidence of infectious diseases due to the emergence and reemergence of deadly pathogens, especially those of viruses, where the availability of effective anti-viral agents is very limited. More new threats of such nature are expected in the time to come. Thus, the most potent defenses for successful outbreak management remain rapid early identification of the events and agents, treatment of victims, and containment of infection. Transmission electron microscopic diagnosis is uniquely suited for rapid identification of such emerging pathogen as exemplified in this report.

Figure 1 and 2 represent two historical landmark electron micrographs of Nipah virus. The photograph in Figure 1 is the first electron micrograph of Nipah virus. The photograph was recorded using the Philips CM12 transmission electron microscope. Whilst the basic ultrastructure of a paramyxovirus could be identified (nucleocapsids, syncytial cells) by a skilled and well practiced electron microscopical diagnostician others could struggle to detect and identify the structure. The poor quality of the initial image most-likely was the result of a modern trend where electron microscopes were losing favour and falling into disrepair. Thus, the resultant picture was of low quality resulting in the generation of data that could not definitively identify the virus as a member of the order *Mononegavirales*, family *Paramyxoviridae,* and subfamily *Paramyxovirinae*. Consequently, the original ultrathin sections were first hand-carried to the Division of Vector Borne Diseases, Fort Collins (Colorado) before being transferred to the main Center for Disease Control and Prevention, Atlanta (Georgia), USA for final characterization. Subsequent to this, the veterinary samples were sent to the Australian Animal Health
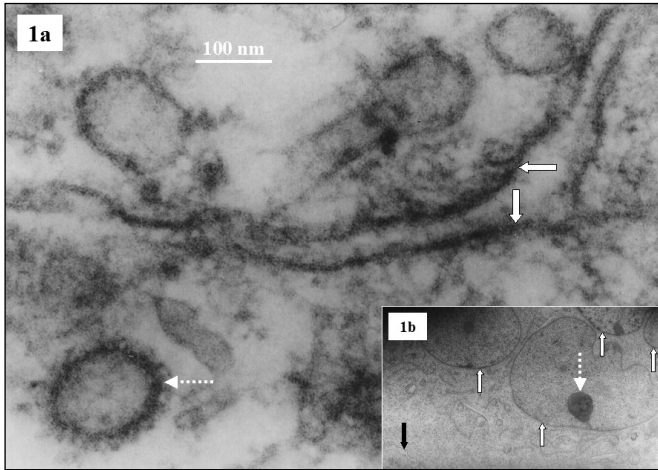
Fig. 1: Electron micrographs of Vero cells infected with Nipah virus. Figure 1b shows a region of syncytial cells with multiple rounded nuclei (solid white arrows). Broken white arrow shows an intranuclear inclusion. Figure 1a shows foci of cell membrane thickening suggestive of sites of viral morphogenesis (solid white arrows). Broken white arrow shows an envelope particle with surface projections suggestive of a viral particle. Bar represents 100 nm.
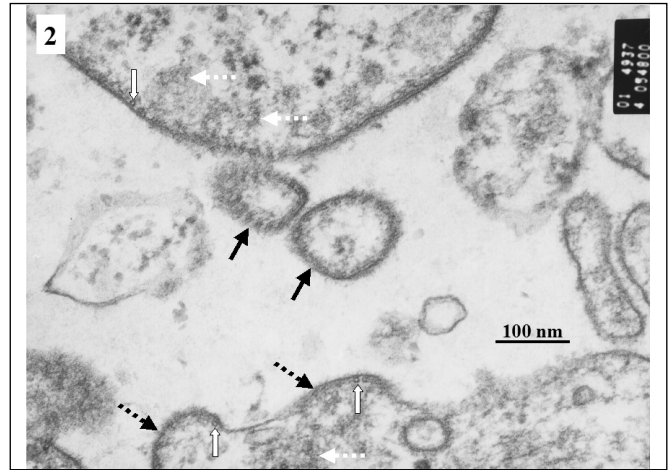

Fig. 2: Electron micrographs of Vero cells infected with Nipah virus. Distinct viral particles are indicated by solid black arrows. Broken black arrows indicate sites of cell membrane thickening suggestive of foci of viral morphogenesis at the plasma membrane. Just beneath the cell membrane, at the site of viral morphogenesis, ring-like structures (solid white arrows) indicative of cross-sections of paramyxovirus-like nucleocapsids are indicated. Aggregates of viral nucleocapsids were also visible in the cytoplasm of infected cells directly adjacent to the site of viral morphognesis (broken white arrow). Bar represents 100 nm.
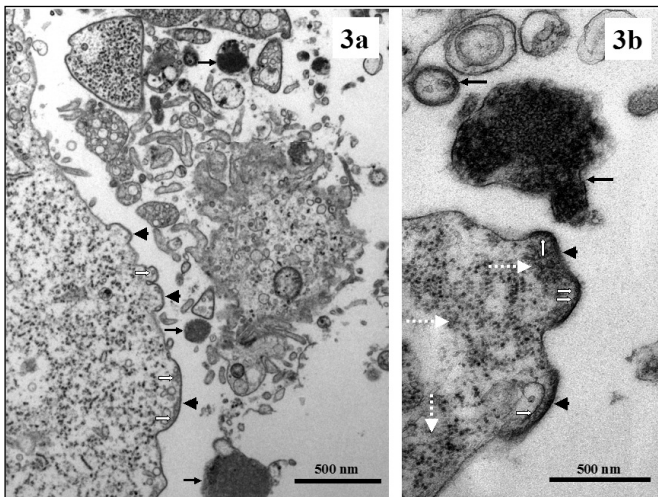

Fig. 3: Electron micrographs of Vero cells infected with Nipah virus. Figure 3a was captured from an unstained section and Figure 3b was taken from lightly stained section. Enveloped viral particles (solid black arrows) are obvious in both stained and unstained preparations. Sites of cell membrane thickening (arrow heads) indicative of foci of viral morphogenesis and cross sections of nucleocapsids (solid white arrows) are also depicted. Bar represents 500 nm.

Laboratory, Geelong, Australia where the virus was ultrastructurally and antigenically compared to the other virus within the genus *Henipavirus,* namely Hendra virus. This latter work is noteworthy as the investigation demonstrated electron microscopy to be a powerful diagnostic tool with the ultrastructural and antigen differentiation of HeV and NiV.[8]

The micrograph in Figure 2 was taken from the same tissue section as the first electron micrograph but using an older version of the Philips series of transmission electron microscope. Despite an older model, a much clearer picture was obtained to confirm the nature of the virus that was responsible for the outbreak. This was the landmark Nipah virus electron micrograph that was faxed back to Malaysia leading to a rapid and total change in outbreak control measures which culminated bringing the outbreak under control. These series of events demonstrate the importance of having a well maintained transmission electron microscope as a frontline instrument for diagnostic application in events of outbreak of infectious diseases.

In summary, for most disease outbreaks the morpho-diagnosis combined with clinical and epidemiological information is generally sufficient to permit a provisional diagnosis and to exclude the presence of more serious infections. When clinical data is inconclusive a range of assays can be used to identify associated infectious agents. However, if the agent is new there is a high probability that these assays will be ineffective and other multiplex assays such as electron microscopy must be used. The identification of NiV as described in this paper is compelling in its endorsement of electron microscopy as a critical diagnostic tool. That is because electron microscopy is not restricted by the existence of pre-defined probes (antibodies, antigens, nucleic acids), it will and should remain an important frontline method for rapid virus identification/exclusion investigation of any outbreak of new and unusual cases of illness suspected of infectious aetiology. Thus, to exploit the

potential of diagnostic electron microscopy fully, it should be quality controlled, co-ordinated and run in parallel with other diagnostic techniques. The results obtained should be interpreted with an open mind in relationship to the available relevant clinical and epidemiological features of diseases and outbreaks to arrive at the right meaningful conclusion.

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