

# Influenza Virus-Associated Fatal Acute Necrotizing Encephalopathy: Role of Nonpermissive Viral Infection?



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**ABSTRACT:** In 2014, two unusual peaks of H1N1 influenza outbreak occurred in Nakhon Ratchasima Province, in Thailand. Among 2,406 cases, one of the 22 deaths in the province included a 6-year-old boy, who initially presented with acute necrotizing encephalopathy. On the other hand, his sibling was mildly affected by the same influenza virus strain, confirmed by whole-genome sequencing, with one silent mutation. Absence of acute necrotizing encephalopathy and other neurological illnesses in the family and the whole province, with near identical whole viral genomic sequences from the two siblings, and an absence of concomitant severe lung infection (cytokine storm) at onset suggest nonpermissive infection as an alternative pathogenetic mechanism of influenza virus.

**KEYWORDS:** influenza, acute necrotizing encephalopathy

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## Introduction

Precise incidences of influenza-related neurological illnesses are not known in Thailand.<sup>1</sup> From January 2006 to December 2011, an annual mean of 399,853 deaths occurred in Thailand, with an average of 13,554 (3.4%) underlying pneumonia and influenza deaths.

Nakhon Ratchasima is a province in northeast of Thailand with a population of 2,620,517 in 2014.<sup>2</sup> In the same year, there were a total of 2,406 reported cases of influenza and influenza-like illnesses in Nakhon Ratchasima. Of these, 22 cases reported death, 21 from severe pneumonia, and one developed acute necrotizing encephalopathy (ANE). There has been an increasing concern about neurological complications due to several observations at hospitals in Thailand, and elsewhere, since the outbreak of the pandemic H1N1 in 2009. Influenza surveillance has intensified ever since.

In view of the fact that brain and cerebral blood vessels can be considered nonpermissive, this often results in an abortive infection. However, dysfunctions have been known to occur.<sup>3</sup> In mice or hamsters, human influenza virus inoculated by intranasal, intravenous, or direct muscle injection route produced a nonpermissive viral infection in cerebral endothelial cells causing cerebral edema.<sup>3,4</sup> The viral nucleic

acid and viral antigens can be found for one to three days, but infected cells ineffectively produce infectious virions and tissue inflammation.

## Case Report – Clinical Presentations

In April 2014, a boy aged 6 years and 8 months was admitted to intensive care unit at Maharat Hospital due to fever and intractable seizures. One day earlier, he developed fever with mild productive cough, vomiting, and loose stool. There was mild injected pharynx with no evidence of pneumonia. He then developed high fever and repeated episodes of generalized tonic seizures, lasting one minute each at 18 and 14 hours prior to admission, which resolved spontaneously. He became comatose after a third episode (at 6 hours), which lasted 10 minutes. He was hospitalized at a local community hospital, and then transferred to the provincial Maharat Hospital. On admission, he was in decerebrate posture with persistent eye deviation to the left and had Glasgow Coma Score of 4.

Blood test results showed white blood cell count of  $7.1 \times 10^9$  cells/mm<sup>3</sup> with 82.9% neutrophils and normal platelet count. Aspartate and alanine transaminase levels were 100 and 44 U/L, respectively. Rapid test on nasopharyngeal swab was positive for influenza A virus. Cerebrospinal fluid (CSF)

test showed traumatic tap with red blood cells of 500 cells/ $\mu$ L, 15 lymphocytes/ $\mu$ L, protein of 101.8 mg/L, and glucose of 5.8 mmol/L. Computerized tomography of the brain showed generalized brain edema with no uncus or tonsillar herniation. Noncontrast-enhanced hypodensity lesions were found in bilateral thalami (Fig. 1A) and deep white matter, in particular right frontal concomitant with left temporal regions. He was treated with oseltamivir and intravenous phenytoin and phenobarbital. Intravenous pulse methylprednisolone (30 mg/kg) was administered for three days in view of possible postinfectious encephalitis with brain edema. Seizures were controlled. During the whole admission course, he did not regain consciousness. He was on artificial ventilator support. Unfortunately, he had hospital-acquired bacterial pneumonia superimposed with subsequent development of septic shock and kidney failure. He died on eighth day of hospitalization.

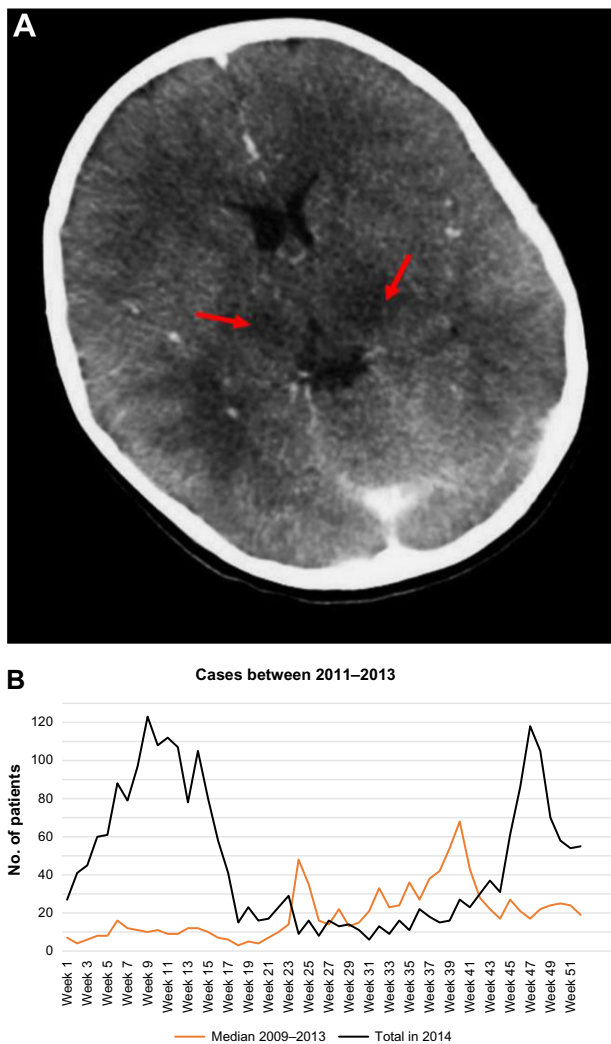
The patient's sister (4 years and 9 months old), who lived in the same house, developed fever and cough with rhinorrhea and vomiting on the same day as the patient. She was admitted to the same hospital two days later in fear of developing similar complications. Rapid test was also positive for influenza A virus. Oseltamivir was given on admission. She had an uneventful recovery in four days.

### Case Analysis and Interpretation

Simultaneous onset of influenza A virus infection (later revealed as H1N1 pandemic 2009) within the same household, but with only one sibling severely affected, was puzzling. The bilateral thalami lesions, elevated transaminase, and normal CSF glucose with elevated protein levels in this patient were consistent with ANE. Mutations of *RANBP2*, which are associated with familial and recurrent ANE, were not tested for due to lack of prior history of influenza-related encephalopathy within the family, and the family decided against genetic testing. Further, influenza-related encephalopathy had not been reported among those infected during this outbreak in the province.

The fatal case and the mild case were investigated in detail to see whether there were disparities between the viruses that affected the two siblings. Nasopharyngeal swab samples were collected from these two patients. The samples were positive for H1N1 influenza viruses identified by real-time reverse transcription polymerase chain reaction (RT-PCR). Deep sequencing technique was used to obtain complete genome sequence information from influenza virus isolates. RNA from influenza viruses isolated in Madin-Darby canine kidney (MDCK) cells was extracted, and the whole genomes were sequenced through the MiSeq Illumina platform. The sequences were evaluated using third-party analysis and visualization software such as MiSeq Reporter version 2.3.32, SAMtool, Integrative Genomics Viewer (IGV), and Molecular Evolutionary Genetics Analysis version 6.0 (MEGA6).

A total of 14 million reads with high-quality score ( $\geq$ Q30) were analyzed. Sequences were assembled via MiSeq Reporter. SAMtool was used to generate the consensus sequence. BLASTN revealed that these two sequences showed 99% similarity to influenza H1N1 2014 (Influenza A virus (A/NewYork/WC-LVD-14-044/2014(H1N1))). Different analytical processes were applied to confirm that the infection in the two siblings were from the same virus strain, which were identified using IGV and MEGA6. IGV is a visualization tool used to explore the two sequences before the consensus sequence was produced. On the other hand, MEGA6 aims to infer the molecular evolutionary patterns of genes, genomes, and species over time to accurately report strains. Deep comparison between the two consensus sequences found 100% similarity, although IGV showed one variation point, A756C on segment 8, which codes for nuclear export protein and nonstructural protein 1. The ratio of this variation, potentially resulting in a mutation, in the fatal versus the mild case was



**Figure 1.** (A) The brain imaging (computerized tomography scan) showing bilateral thalamic lesions (red arrows) in the patient, suggesting ANE; (B) the unusual pattern of influenza outbreak in Nakhon Ratchasima Province in 2014, compared to the usual peak of outbreak in previous years (2011–2013).



62:37. To our knowledge, this does not explain the difference in clinical severity as this variation results in a synonymous substitution (silent mutation). In addition, evolutionary divergence of nucleotide and amino acid sequences were evaluated using MEGA6, which revealed 0.01 and 0.00 substitutions per site for the fatal and mild cases, respectively.

## Discussion

Of 22 fatalities (19 H1N1 2009 and 3 influenza B) in 2014 at Nakhon Ratchasima Province, all except the subject of this paper died of severe pneumonia. Severe encephalopathy, as in this case, was unusual and uncommon. In 2014, none of the cases in outpatient clinics and the emergency departments, which led to hospitalization in the provincial hospital, presented with predominant cerebral symptoms as in the case reported in this paper. Studies on the classification of neurological manifestations of influenza, such as acute onset cytokine storm, where local inflammation spills over into the systemic circulation, producing systemic sepsis,<sup>5</sup> as proposed in the study by Goenka et al.,<sup>6</sup> has not yet been systematically conducted in Thailand. This includes acute hemorrhagic leukoencephalopathy, ANE, and other benign forms with compromised blood–brain barrier (BBB).

Massive perivascular edema without cellular infiltrates found in brain pathology supports the role of BBB leakage in the case of ANE.<sup>7</sup> Magnetic resonance imaging and angiography showed severe brain edema and cerebral vasculopathy.<sup>8</sup> Cytokine levels in the serum and/or CSF correlated with the severity of encephalopathy and outcome,<sup>3</sup> and possibly affected the integrity of BBB. Pneumonia complicating the pandemic 2009 H1N1 infection is a primary source of elevated cytokine production.<sup>9</sup> Although cytokine storm, as a response to concomitant pneumonia, is an attractive hypothesis, our patient initially manifested with seizures, which progressed to intractable state without any evidence of pneumonia associated with the influenza infection. None of the remaining fatal cases in the province had brain manifestations except at the preterminal stage.

*RANBP2*, an autosomal dominant missense mutation of a component of the nuclear pore, has been associated with ANE manifestations in two consanguineous patients<sup>10</sup> and was possibly responsible for a relapsing course.<sup>11</sup> However, the presence of a single ANE case in the entire province in 2014 argues against the role of this mutation.

It has been unclear whether the influenza virus itself plays a direct role in ANE. Evidence of influenza replications in organs outside the respiratory system has been inconsistent.<sup>3,12</sup> Reports on the presence of influenza viral antigen/RNA in human brain cells, in capillary vessel walls, or in the CSF has been conflicting,<sup>3</sup> as the virus could not be isolated in all patients.<sup>13</sup> Molecular and postmortem studies in at least one fatal adult case of H1N1-related ANE revealed no evidence of direct viral involvement in the brain.<sup>14</sup> On the other hand, human embryonic stem cell-derived neural progenitors have

been shown to support highly pathogenic avian H5N1 influenza virus infection.<sup>16</sup> Influenza virus can directly enter the CNS via the olfactory system and may have caused encephalitis in patients who died of H5N1 without exhibiting respiratory symptoms. Virus was isolated from the CSF in that case.<sup>16</sup>

Seasons of influenza and influenza-like illnesses at Nakhon Ratchasima Province in 2009–2013 were between weeks 24 and 50. However, in 2014, there were two peaks; first one between weeks 1 and 19 and another between weeks 35 and 52 (Fig. 1B). The unusual months of 2014 H1N1 influenza outbreaks (Fig. 1B), along with higher numbers as compared to previous years, raises the possibility of an unusual strain. However, whole genomic sequencing of H1N1 viruses from the two siblings did not confirm this. Although viral propagation using MDCK cells may reduce viral population diversity in the clinical specimens, only a slight genomic divergence, a synonymous mutation, was found.<sup>17</sup>

ANE in our case may suggest nonpermissive cell theory as the initial event, followed by cytokine storm ignited by superimposed pneumonia. Although the viral genome sequences from the siblings showed one variation point that resulted in a synonymous mutation, this does not rule out the possibility that this variation may have allowed for nonpermissive infection to occur. Several factors such as host genetic immune cell permissiveness, in response to virus infection, may have orchestrated the fatal outcome. Strict epidemiologic surveillance at the village/subdistrict levels should be encouraged, to keep a close eye on the situation, and health authorities should be alerted of any peculiar incidences like this.

## Genbank Accession Numbers

KU051428–KU051443 (influenza virus sequences reported in this manuscript).

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## Author Contributions

Conceived and designed the experiments: AM, JC. Analyzed the data: TH, SW, YJ, AJ, AR, SG. Wrote the first draft of the manuscript: TH, AS. Contributed to the writing of the manuscript: SG, AS, TH. Agreed with manuscript results and conclusions: AM, JC, SW, YJ, AJ, AR, SG, AS, TH. Jointly developed the structure and arguments for the paper: SG, AS, TH. Made critical revisions and approved the final version: SW, SG, AS, TH. All the authors reviewed and approved the final manuscript.



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