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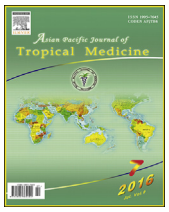

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Molecular epidemiology and phylogeny of Nipah virus infection: A mini review

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ABSTRACT

Nipah virus (NiV) is a member of the genus *Henipavirus* of the family Paramyxoviridae, characterized by high pathogenicity and endemic in South Asia. It is classified as a Biosafety Level-4 (BSL-4) agent. The case-fatality varies from 40% to 70% depending on the severity of the disease and on the availability of adequate healthcare facilities. At present no antiviral drugs are available for NiV disease and the treatment is just supportive. Phylogenetic and evolutionary analyses can be used to help in understanding the epidemiology and the temporal origin of this virus. This review provides an overview of evolutionary studies performed on Nipah viruses circulating in different countries. Thirty phylogenetic studies have been published from 2000 to 2015 years, searching on pubmed using the key words 'Nipah virus AND phylogeny' and twenty-eight molecular epidemiological studies from 2006 to 2015 have been performed, typing the key words 'Nipah virus AND molecular epidemiology'. Overall data from the published study demonstrated as phylogenetic and evolutionary analysis represent promising tools to evidence NiV epidemics, to study their origin and evolution and finally to act with effective preventive measure.

1. Introduction

Nipah virus (NiV) is member of the genus *Henipavirus* in the family Paramyxoviridae. Due to its highly pathogenicity and relative new finding, it is classified as a Biosafety Level-4 (BSL-4) agent. Moreover, the Centers for Disease Control and Prevention (CDC) and the National Institute of Allergy and Infectious Diseases (NIAID) have classified NiV as a Category C priority pathogen.

Nipah virus disease is a recently discovered zoonotic disease characterized by fever, constitutional symptoms, and encephalitis, sometimes accompanied by respiratory illness. NiV has an envelope with filamentous nucleocapsids [1], the genome consists of a single-stranded negative-sense RNA of approximately 18.2 kb. The genome encodes for six major structural proteins: nucleocapsid (N), phosphoprotein (P), ma-trix protein (M), fusion protein (F), glycoprotein (G), and large protein or RNA polymerase (L) [2].

The name 'Nipah virus' originated from Sungai Nipah (Nipah River Village), where the first isolates were obtained [3–5]. Bats of the genus *Pteropus* appear to be the natural reservoir of the virus. Nipah virus swept through numerous piggeries in Malaysia and killed 1 100 people during the period from 1998 through 1999.

NiV was identified as the etiological agent responsible of an outbreak, in pigs and humans, in Malaysia and Singapore. Transmission may be from consumption of contaminated food by bats secretion, or contact with infected pigs. Another way can be human-to-human spread. Since 1998 there have been several cases of infections in Bangladesh and India [6–18]. The case-fatality varies from 40% to 70% depending on whether encephalitic or severe manifestations are noted and whether adequate healthcare facilities are available. At present there is no antiviral drug available for Nipah virus disease and the treatment is supportive. Ribavirin has been used in few patients but its efficacy for Nipah virus disease has not yet been determined. Because of the lack of effective vaccines or therapies and the fact that NiV can infects animals such as pigs, NiV infection can be considered an emerging disease and a public health issue [12,14].

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Since NiV is considered an important pathogen, especially in South Eastern regions, phylogenetic, and evolutionary analyses can be used to help in understanding the epidemiology and the temporal origin of this virus. This review provides an overview of evolutionary studies performed on Nipah viruses circulating in different countries.

2. Phylogenetic analysis

Evolutionary analysis, over the last three decades, has increasingly been applied to the study of microbial pathogens. Phylogenetic and phylodinamic analysis are the fundamental tools to investigate how the genealogy of a pathogen population is influenced by the interaction between pathogen's demographic history and environmental, ecological and host immunological factors [19,20]. Phylogenetics and phylodinamics are a branch of molecular biology evaluating taxonomy and species evolution [21]. These methodologies are used as a complement to the 'classical epidemiology' [22] and represent powerful tools widely used to analyze epidemics especially, in particular settings such as in case of nosocomial outbreaks. By phylogenetic and evolutionary analysis, factors contributing to the evolution of novel and emerging microbial variants can be identified. In recent years, a number of methods that infer phylogenetic trees based on genetic distances, evolutionary parsimony, Maximum-likelihood and Bayesian theory, have been introduced [23–26]. Genetic distances and phylogenetic trees (coupled with a correct epidemiological design *i.e.*, cross sectional studies), inferred via different sequence evolutionary models and model selection criteria, are normally used to assign the genotype [27]. Coalescent theory and the molecular clock hypothesis are instead used to study the ancestral relationships of individuals sampled from a population (*i.e.*, longitudinal studies) which can be inferred from a gene genealogy (phylogenetic tree) [22,28–32].

A deductive and normally used cycle in phylogenetic analysis was started with microorganism isolation and sequencing, and an appropriate data set have to be built. The alignment with reference sequences, manual editing to delete 'indels' (insertions/deletions), and the determination of the phylogenetic signal is required. Phylogenetic and/or phylodinamic analyses represent the 'core' of the data analysis and hypothesis testing. To test for the best substitution model, to infer phylogeny using different algorithms (*e.g.*, genetic distance, Maximum-likelihood, Bayesian methods), to test the trees reliability (*e.g.*, by bootstrapping and posterior probability), are essential steps for evolutionary analyses [30].

The analytical power of the phylogenetic and Bayesian methods available today should prompt the researchers to use dataset as large as possible to monitor the epidemiological changes of the microorganism over the time. Performing phylogenetic analyses on the gene region and sometimes, when available, on the whole genome, may result in a better identification of novel subtypes or recombinants. Moreover, whenever it is possible, combined sequencing and phylogenetic analysis should always be used in order to gain information about the starting of the epidemic, its spread and the dynamics of viral strains.

Finally, phylogeographic methods can provide information about the spread of viral strains between different geographic regions. Phylogenetic analysis, can also be applied to define and characterize the possible viral vector, as it was identifying NiV in bats so as in other hosts [33].

Monitoring the genetic evolution of NiV represents an essential strategy to control the local as well as global epidemic and to develop efficient preventive and therapeutic strategies with a great impact in clinical practice.

3. Phylogenetic studies

About thirty phylogenetic studies on NiV have been published from 2000 to 2015 years, with weak peak in 2012, typing on pub-med the key words 'Nipah virus AND phylogeny', as reported in Figure 1. About twenty-eight molecular epidemiological studies from 2006 to 2015 have been performed, with weak peak in 2010, typing the key words 'Nipah virus AND molecular epidemiology', as in Figure 1.

The first phylogenetic studies were performed to investigate the similarity between NiV and Hendra virus, another member of the family Paramyxoviridae [12,30,34,35]. These studies

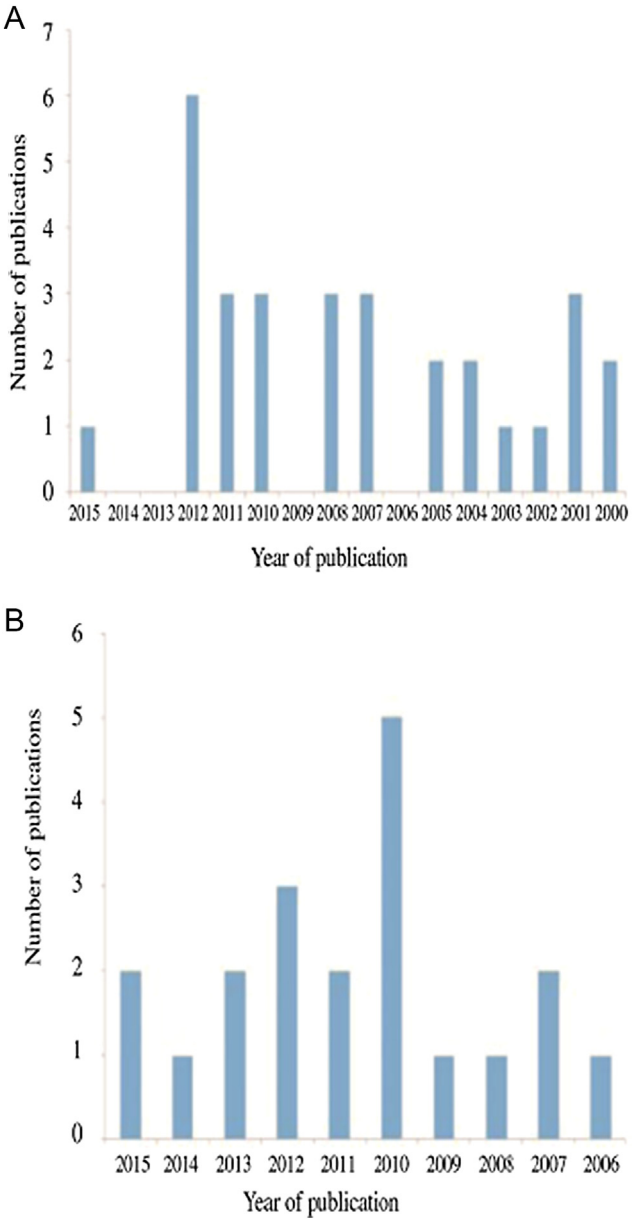

Figure 1. Number of publications on phylogeny of NiV by year of publication.

A, key words: 'Nipah virus AND phylogeny'; B, key words: 'Nipah virus AND molecular epidemiology'.

demonstrated the strong similarity between the two viruses, encouraging authors to include a new genus in the family Paramyxoviridae, named *Henipavirus*, consisting only of NiV and Hendra viruses. Phylogenetic studies were performed to follow and characterize the first important epidemics, the Malaysia in the 1999 and the Bangladesh one in 2004. Interestingly, some authors sequenced the whole NiV genome from strains isolated in the Malaysian outbreak of the year 1999. NiV genome, although 12 nucleotide longer than the Hendra virus genome, was identical within the regulatory genetic regions and the predicted aminoacid sequence of structural proteins and RNA polymerase [36].

Advances from these studies gave the opportunity to evidence that, during the Malaysian epidemic, at least two major strains of NiV were circulating in pigs, one strain from the initial outbreak in the north and the other strain from the subsequent outbreak, approximately 4 months later in the south. The isolates from the south had identical sequences to those detected from human infections, which confirmed that infections occurred in humans during the southern outbreak originated from infected pigs. This finding implied that the 1998 Malaysia outbreak was probably due at least to two different origins of Nipah virus infections [37]. The genome of NiV from the outbreak of Bangladesh was 6 nucleotide longer than the prototype Malaysian strain and the phylogenetic analysis demonstrated that this virus should represent a new strain of NiV, strictly correlated to the Malaysian strain, but exhibiting a higher interstrain nucleotide heterogeneity [38]. These findings could suggest differences in the way of transmission of the virus between the two countries: in Malaysia, the phylogenetic analysis suggested that at least two introductions of NiV into pigs have been occurred, whereas in Bangladesh the sequence heterogeneity observed should indicate multiple introductions of the virus in humans from different colonies of fruit bats. From this study emerged that NiV circulating in different areas have specific genetic characteristics and may have coevolved with the local natural reservoirs. This coevolution was further supported by Halpin *et al*. in 2007. These authors demonstrated the phylogenetic relationship between bats and their associated virus suggesting an important role of bats as the reservoir hosts of newly emergent viruses, such as Nipah virus, Hendra virus, and severe acute respiratory syndrome-like coronaviruses [39].

In the year 2010, Rahaman *et al.* [40] demonstrated that the putative reservoir for the 1998 NiV outbreak occurred in Malaysia was *Pteropus vampyrus (P. vampyrus)* bat. The virus isolated in bats resulted monophyletic with previous NiV and the phylogenetic analysis enforced the hypothesis that similar strains were co-circulating in sympatric reservoir species.

In 2011, an intra-familial NiV outbreak in West Bengal region of India, was described by Arankalle *et al.* [41]. The full-genome sequence of the virus showed 99.2% of nucleotide and 99.8% aminoacid similarity with the Bangladesh-2004 isolate, suggesting a probable common source of the virus. Phylogenetic analysis, interestingly, showed that viruses from Bangladesh and India clustered and diverged from the viruses of Malaysia.

Lo *et al.* [42] in the year 2012 reported the molecular phylogenetic analysis of available complete NiV gene sequences including those from the outbreaks in Bangladesh during 2008 and 2010. These authors proposed a genotyping scheme based on a 729-nt sequence window, localized in the N terminal region of the genome, but with a sequence variability comparable to that observed using the complete genome. This genotyping method produced a phylogenetic tree with high bootstrap values and proved to be a relatively accurate indicator of overall nucleotide variability useful for NiV sequences classification.

One of the most recent phylogenetic study on NiV infection, was performed by Lo Presti *et al.* [43] to investigate the genetic diversity of the virus, to estimate the date of origin and the spread of the infection. For the first time, these authors demonstrated, using the time-scaled phylogenetic analysis, with the root of the tree originated in 1947 when the virus entered in south eastern Asiatic regions. At the phylogenetic analysis the nucleocapsid gene sequences segregated in two main clades, indicating two different introductions: one in 1995 corresponding and the other in 1985. The phylogeographic reconstruction indicated that the epidemic followed two different routes spreading to the other locations facilitated by bats of the *Pteropus* genus that are able to travel to long distances. The molecular evolutionary approach was used by these authors to investigate also the presence of sites under positive and negative selection, using a selective pressure analysis method [43]. Only negatively selected sites were detected confirming the stability of the viral protein studied. Interestingly, some of these negatively selected sites were found in positions previously described as important interaction sites [44]. Even if a large proportion of amino acids are invariable, the occurrence of adaptive at certain sites of the genome, over the time, cannot be excluded, especially if infected pigs trade and bats migration are not adequately monitored. This situation is in analogy with Chikungunya virus where only one nutation determined a change of vector from *Aedes aegypti* to *Aedes albopictus* [45].

4. Nipah virus reservoirs

Paramyxoviruses are characterized by broad host range and for this reason they show an important zoonotic potential, like Hendra and Nipah viruses originating from bats. Bats represent the most successful mammals on earth including about 1 200 chiropteran species distributed worldwide. In the last decades Hendra virus, Nipah virus and other zoonotic viruses like Ebola, Marburg, and SARS virus, have been identified in various *Pteropus* spp. fruit bats [46–50].

The route of infection of NiV from bats to humans is by ingestion and consumption of NiV-contaminated or partially eaten fruits, or by contact with infected animals such as pigs, cattle and goats.

Rahman *et al.* in 2010 [40], reported the results of a prospective cohort study focused on a group of *P. vampyrus* flying foxes captured in two different locations in Malaysia. Authors showed that NiV detected in *P. vampyrus* differs from all known isolates from Malaysia for the amino acid changes at 44 positions. The phylogenetic analyses unequivocally showed that NiV *P. vampyrus* forms a monophyletic clade with other NiV isolates from Malaysia, but it differs from human, pig, and *Pteropus hypomelanus* bat isolates. When 56 NiV sequences from *Pteropus lylei* bats isolated in Thailand were included, NiV *P. vampyrus* phylogenetically grouped most closely with NiV *P. lylei*, and the monophyly of NiV sequences from Malaysia was lost. This close homology suggested that NiV is naturally

transmitted between these two species. From this study, the presence of NiV diversity in isolates from *P. lylei* bats has also emerged. This diversity demonstrated that multiple strains co-circulate within populations and that the ecology and sympatry of *Pteropus* spp., not coevolutionary patterns, are determinant for the NiV strain diversity observed in reservoir hosts.

In 2012, Yadav *et al.* [51] have surveyed the Indian states of Maharashtra and West Bengal to evaluate the presence of viral RNA and IgG against NiV in different bat populations belonging to the species *Pteropus giganteus*, *Cynopterus sphinx* and *Megaderma lyra*. Authors found NiV RNA in *Pteropus* bat thus suggesting it may be a reservoir for NiV in India. Furthermore, the phylogenetic analysis demonstrated that two phylogenetic lineages were formed for NiV sequences, one including Bangladesh and India sequences and the other Malaysia and Cambodia sequences. By phylogenetic analysis it was unmistakable confirmed that the same NiV strain circulates in India and Bangladesh and that it was different from that circulating in Malaysia and Cambodia. In the same period, a similar study was performed on free-ranging European insectivorous bats to assess the presence of paramyxovirus infection in these animals [52]. The study involved 120 deceased bats of 15 different European species. Bayesian reconstruction of phylogenetic trees was performed in concordance with the current proposals of Paramyxoviridae taxonomy. Interestingly, the phylogenetic analysis confirmed the presence of the first three paramyxoviruses in European insectivorous bats. The genetic distance between these three novel paramyxoviruses and the closest related member resulted higher than that observed in other members within the paramyxovirus genera. This data suggested that all three viruses might be considered as new paramyxoviruses. Since, infected bats were found in close proximity to heavily populated human areas, a potential risk for a zoonotic paramyxovirus infection in Europe cannot be excluded.

Recently, the occurrence of Henipaviruses in fruit bat populations in the north of Australia was explored [53]. In particular, these authors evaluated the possibility that NiV were restricted to the west of Wallace's Line. This line represents the biogeographic barrier existing between the Australo-Papuan and Wallacean region on the one hand, and Southeast Asia on the other, with different distribution of vertebrates and invertebrates. Data from this study demonstrated the presence of Nipah virus in both *P. vampyrus* and *Rousettus amplexicaudatus* the fruit bat populations localized on the eastern side of Wallace's Line.

5. Conclusions

Nipah virus causes a recently discovered zoonotic disease endemic in South Asia, where sporadic outbreaks have been reported in Malaysia, Singapore, India, and Bangladesh. The case-fatality varies from 40% to 70% depending on the severity of the clinical manifestations, such as encephalitis, and on the availability of adequate healthcare facilities. At present there is no antiviral drug available for Nipah virus disease and the treatment is just supportive. NiV infection can be considered an emerging disease and a public health problem [12,13] as a consequence of the lack of effective vaccines and therapies and of the evidence that NiV can infect pigs [12,13]. Phylogenetic and evolutionary analyses can represent very useful tools to elucidate the epidemiology and the temporal origin of this virus. Moreover, these analyses, especially the evolutionary analysis, could be advantageous to develop new therapy, vaccine and prevention strategies.

The circulation of NiV may be influenced by the presence of genetic polymorphisms along the virus genome. As a consequence, the antigenic variability is possible and may play an important role in the ability of the virus to escape the host immune response [42]. On this basis, monitoring will be important to implement possible intervention strategies. In Asiatic countries, there is a close contact between animals and humans, especially in rural settings. This aspect represents a vulnerability of Asia for outbreaks caused by zoonotic infections. This vulnerability is further increased by sociocultural beliefs and weak public health infrastructure [54].

Consequently, the need of a multidisciplinary approach to prevent and control zoonotic infections in this country is evident. Phylogenetic and evolutionary analysis represent promising tools to evidence epidemics, to study their origin and evolution and finally to act with effective preventive measure.

Conflict of interest statement

We declare that we have no conflict of interest.

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