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Genetic diversity of Nipah virus in Bangladesh

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ABSTRACT

Background: Nipah virus (NiV) infection, often fatal in humans, is primarily transmitted in Bangladesh through the consumption of date palm sap contaminated by *Pteropus* bats. Person-to-person transmission is also common and increases the concern of large outbreaks. This study aimed to characterize the molecular epidemiology, phylogenetic relationship, and the evolution of the nucleocapsid gene (N gene) of NiV.

Methods: We conducted molecular detection, genetic characterization, and Bayesian time-scale evolution analyses of NiV using pooled Pteropid bat roost urine samples from an outbreak area in 2012 and archived RNA samples from NiV case patients identified during 2012–2018 in Bangladesh.

Results: NiV-RNA was detected in 19% (38/456) of bat roost urine samples and among them; nine N gene sequences were recovered. We also retrieved sequences from 53% (21 out of 39) of archived RNA samples from patients. Phylogenetic analysis revealed that all Bangladeshi strains belonged to NiV-BD genotype and had an evolutionary rate of 4.64×10^{-4} substitutions/site/year. The analyses suggested that the strains of NiV-BD genotype diverged during 1995 and formed two sublineages.

Conclusion: This analysis provides further evidence that the NiV strains of the Malaysian and Bangladesh genotypes diverged recently and continue to evolve. More extensive surveillance of NiV in bats and human will be helpful to explore strain diversity and virulence potential to infect humans through direct or person-to-person virus transmission.

Introduction

Nipah virus (NiV) is a highly pathogenic zoonotic paramyxovirus causing severe respiratory disease and encephalitis in Southeast Asia with high mortality (Luby and Gurley, 2012; Satterfield et al., 2016). It is a negative-stranded RNA virus with a large genome of 18 kb and is classified in the *Henipaviruses* genus of *Paramyxoviridae* family along with its close relatives: Hendra virus, Cedar virus, and Mojiang virus (Rockx et al., 2012). NiV has an envelope with filamentous nucleocapsids containing a genome of six major structural proteins (N-P-M-F-G-L). NiV has been genetically categorized into two distinct genotypes: NiV-Malaysia (NiV-MY) identified in Malaysia and Cambodia and NiV-Bangladesh (NiV-BD) identified in Bangladesh and India (Harcourt et al., 2005; Wild, 2009). Differences in transmission patterns (direct and person-to-person) and mortality rates cause more concern about the virulence potential of NiV-BD than that of NiV-MY, which also correlates with its clinical manifestation, pathogenicity, route of transmission, and severity of infections (Mire et al., 2016).

NiV was first reported in Malaysia in 1998–1999, and subsequently, no further outbreaks have been reported from Malaysia. The initial outbreak was reported with 265 infected people and the case fatality rate (CFR) was ~ 40%, including 105 deaths (Chua et al., 2000). In 2001, 2007, 2018, and 2019, outbreaks

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were reported in India (Plowright et al., 2019; Yadav et al., 2019). Many of these outbreaks were propagated through person-to-person transmission, including the recent outbreak in Kerala, India, with a total of 23 cases, including 21 deaths (Arunkumar et al., 2018; WHO, 2018). Cases have been identified nearly every year in Bangladesh since 2001 (Arankalle et al., 2011; Chadha et al., 2006; Chua et al., 2000; Hsu et al., 2004). Most human cases of the Nipah infection have been reported from Bangladesh, where the outbreaks are more frequent but smaller than that of Malaysia and resulted in higher case fatality rate of up to 100% (average ~74%) with 260 cumulative confirmed cases from 2001 to 2015 (Satterfield et al., 2016). The consumption of fresh date palm sap is a cultural delicacy in Bangladesh and has been found to be the primary route of NiV transmission from bats to humans (Luby et al., 2009; Luby et al., 2006). During 2001–2014, a total of 248 NiV infections were reported in Bangladesh, and nearly one-third of cases (n = 84) occurred from person-to-person transmission (Hegde et al., 2016; Luby et al., 2009; Nikolay et al., 2019). Person-to-person transmission of NiV is common among close contacts and family members, particularly those with >12 h of exposure to patients' body secretions (Gurley et al., 2007; Hassan et al., 2018; Hossain et al., 2008; Sazzad et al., 2013).

Based on serological and virological evidence, large fruit bats (Flying foxes) from the genus *Pteropus* were identified as the natural reservoir for NiV (Chua et al., 2002; Enserink, 2000; Olson et al., 2002; Yob et al., 2001). *Pteropus* spp. bats occasionally shed NiV in their secretions and excretions (Chua et al., 2002; Islam et al., 2016; Wacharapluesadee et al., 2005). Epidemiological and experimental evidence show that NiV has the potential to infect a wide range of mammals, including pigs, dogs, cats, goats, horses, and rodents (DeBuysscher et al., 2013).

Our current understanding of the genetic and epidemiological dynamics of NiV in Bangladesh is mostly focused on human cases. There were only a few reports of NiV strains characterized from *Pteropus* bats, the most recent one was from P. medius in Bangladesh (Anderson et al., 2019). Besides, the available data are largely limited to serological detection of NiV and NiV like Henipaviruses in Pteropus spp., potential bat reservoirs, across different areas of the South Asian region (Kulkarni et al., 2013; Reynes et al., 2005). Molecular characterization of NiV and its genetic data will provide more knowledge on genotype diversity and potentially suggest links between genotypic differences and epidemiological characteristics, including the efficiency of human-to-human transmission.

The objective of this study was to compare genetic sequences of NiV strains from bats and humans in Bangladesh and investigate the maximum likelihood of phylogenetic relatedness of these and other publicly available NiV strains.

Material and methods

Sample collection

Six plastic tarps (each 4×6 m^2) were placed below flying fox roosts to collect urine excreted by bats in Joypurhat district from January to March in 2012 as part of an investigation into nearby human NiV infections. From the tarps, the urine was collected in 50 mL falcon tubes, which was then aliquoted (n = 456) in duplicate into VTM and lysis buffer (0.3 mL urine in 0.9 mL VTM/lysis buffer). Thirty-nine archived human throat swabs (collected during 2012–2018) with the evidence of NiV RNA using real-time reverse transcription- polymerase chain reaction (RT-PCR) were also available for attempted sequencing.

RNA extraction, reverse transcriptase PCR, and sequencing

Viral nucleic acid was extracted from 200 μl of pooled bat roost urine samples collected in lysis buffer using InviMag Virus DNA/RNA Mini Kit (Invitek, STRATEC Molecular, Berlin-Buch, Germany) on Kingfisher Flex 96 (Thermo Fisher Scientific Inc.) automated nucleic acid extraction system according to the manufacturer's instructions. The nucleic acids were eluted in 120 μl of elution buffer and stored at −80 °C. Initially, *Taq*Man PCR assay was used for the screening of NiV RNA using NiV N gene-specific primers and probe as described by Lo et al. (2012). One-step RT-PCR reactions were performed using the AgPath-ID one-step RT-PCR kit (Applied Biosystems, Foster City, CA, USA) as described previously (Lo et al., 2012). The real time RT-PCR positive samples were subjected to two-step conventional RT-PCR by using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) for reverse transcription to generate cDNA and Qiagen HotstarTaq Polymerase Kit (Qiagen, Hilden, Germany) for PCR amplification of NiV nucleocapsid gene (N gene) fragments. Briefly, 10 μl of extracted RNA was used in a final volume of 20 μl RT reaction with the kit-provided hexamer random primer for cDNA preparation. Subsequently, 2 μl of the cDNA was used as a template in 25 μl PCR reaction mixture to amplify overlapping N gene fragments of NiV with specific primers (Table 1) using the following thermal protocol: initial activation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s, 50 °C for 30 s, 72 °C for 45 s, and then one cycle of 72 °C for 10 min. All NiV N-gene fragment-positive PCR products were purified using the ExoSAP-IT (Affymetrix, California, USA). The cycle sequencing reaction was performed using the ABI BigDye Terminator v.3.1 cycle sequencing kit (Applied Biosystems, USA). The sequencing was carried out in an automated ABI 3500 XL genetic analyzer (Applied Biosystems, Foster City, USA) using the forward and reverse primers separately.

Table 1
Primers used for the NiV N gene amplification in this study.

Primers	Polarity	Sequence(5′-3′)	Region*	Amplicon size
NiV_N_F1	Plus	GGTCTTGGTATTGGATCCTCA	78–341	264
NiV_N_R1	Minus	TGAGTGTGAAAGCAGCTCCA		
NiV_N_F2	Plus	AGTGCTGCCGAGTCAATGAA	299–663	365
NiV_N_R2	Minus	TCGGGAGCTGTAACTGCTTT		
NiV_N_F3	Plus	GGAGATGGAAGGCTTGATGA	481–752	271
NiV_N_R3	Minus	GCCATTGCTGAGTTAGAGCA		
NiV_N_F4	Plus	ATGGGCTAAATACGTCCAACA	691–1117	427
NiV_N_R4	Minus	CATGGCAAAGCTCCACAATA		
NiV_N_F5	Plus	CAGAGAAATTGGCCCAAGAG	1015–1490	478
NiV_N_R5	Minus	GGCACACTGTTAGCAAGGGA		
NiV_N_F6	Plus	CCTTGCTAACAGTGTGCCGA	1474–1854	387
NiV_N_R6	Minus	TGGTTCTTCGGTTTGCCCAT		

* Positions are referred to the RefSeq NC 002728.

All sequences were deposited in GenBank under the accession numbers MT890702 to MT890707 and MT890709 to MT890731.

Molecular phylodynamics analyses

Molecular clock phylogenies were estimated using the Bayesian MCMC approach implemented in BEAST v 2.5.0 (Bouckaert et al., 2014; Drummond et al., 2012). We computed 3 independent runs of 100 million MCMC steps, sampling parameters and tree every 1000 steps. A maximum-likelihood phylogenetic tree was constructed using the general time-reversible model. A discrete gamma distribution was used to model the evolutionary rate differences among sites, and the rate variation model allowed for some sites to be evolutionarily invariable. A constant and exponential population size model with a strict clock was used, which assumes a uniform evolutionary rate among the branches of the tree that was utilized (Drummond and Rambaut, 2007). Hasegawa, Kishino, and Yano nucleotide substitution model and a codon position partition (positions 1 + 2 versus position 3) was employed. In each case, MCMC chains were run for a sufficient time to achieve convergence. Proper mixing of the MCMC was evaluated by calculating the effective sampling size for each parameter. Uncertainty in the data was measured by 95% highest-posterior density (HPD) intervals. The program Tracer v1.7 (Rambaut et al., 2018) was used to evaluate MCMC chain convergence and to compute marginal posterior distributions of parameters, after the removal of 10% of the chain as burn-in. The programs Tree Annotator v1.7.541 and FigTree v1.4.050 were used to summarize the posterior tree distribution and to visualize the annotated Maximum Clade Credibility tree, respectively.

Results

Detection of NiV RNA

A total of 456 bat roost urine samples were collected from Joypurhat in the north regions of Bangladesh in 2012 and subjected to RT-PCR for NiV screening. Thirty-eight (8%) samples were positive for NiV RNA by initial real-time PCR with cycle threshold (Ct) values ranging from 32.7 to 38.5. NiV-N gene fragments (N ORF 188–1599) were successfully sequenced from 10 roost urine specimens.

Out of 39 samples from humans collected during 2012–2018, where NiV RNA was previously detected, RNA was still detectable in 31 (80%) by real-time RT-PCR and nucleotide sequences were retrieved from 21 samples by Sanger sequencing.

The mean age of the human NiV patients (n = 21) was 21 years (median age, 15 years; range, 6–48 years), and 68% (n = 16) were male patients. Ninety percent of the patients died (19/21) and the duration between disease onset and death ranged from 3 to 9 days. The clinical presentation universally started with fever (100%) and included other common features of Nipah, including cough and coma. Most human patients (85%) had a history of fresh date palm sap consumption (Table 2). Many of the NiV cases were epidemiologically linked to each other. Two of those (RS90412 & RS90612) who drunk date palm sap together were from Joypurhat outbreak in 2012, where the bat roost urine samples were collected. Groups of the NiV cases of 2013 and 2015 were reported from the same or adjacent districts in a short time frame Table A1.

Sequence comparison and phylogenetic relationship

The phylogenetic analysis revealed that all sequences, regardless of their biological sources and geographic locations demonstrated a tight phylogenetic affiliation with each other and formed a cluster along with the sequencing data of previously reported human cases in Bangladesh. The sequence similarity revealed that the N gene sequences obtained from bat roost urine from Joypurhat in 2012 were 98.6%–99.6% identical to the NiV-BD strains reported from Bangladesh, India, and Thailand. These strains had 94.5%–95.4% similarity in the N gene fragments of NiV-MY strains (FN869553, AF212302). The genetic data of the NiV strains from bat and human samples in this study shared >99% similarity to each other. Pairwise sequence comparisons of bat roost urine derived NiV N gene fragments indicated up to 1.5% nucleotide variations among each other and up to 1.4% with that of human cases.

Table 2
Clinical characteristics of NiV-positive human cases in Bangladesh, 2012-2018 (n = 21).

Variables	
Age, years	
Mean ± SD	20.5 ± 13.403
Median (range)	15 (6–48)
Sex, n (%)	
Male	16 (80%)
Female	4 (20%)
Involvement in P2P transmission (Spreader)	4 (20%)
Exposure to DPS	17 (85%)
Fever (Highest 105 °F)	20 (100%)
Altered mental status	19 (95%)
Vomiting	11 (55%)
Headache	14 (70%)
Nausea	10 (50%)
Drowsiness	13 (65%)
Personality change	12 (60%)
Difficulty in breathing	9 (45%)
Joint pain	5 (25%)
Muscle pain	5 (25%)
Rash	1 (5%)

None of the study strains had identical nucleotide sequences for the N gene fragment. However, the predicted amino acid (aa) sequences of NiV N gene fragments isolated from bats and humans in Bangladesh were either identical or very similar. A particular aa substitution at a position 457 (N→D) was found frequently among the Bangladeshi N gene sequences regardless of its biological sources. Amino acid substitution at position 464 (V→M) was observed in most of the N gene sequences (5 out of 9) detected in bats as well as in 2 human cases reported in 2012 (Table 3). The NiV strain that had neither N457D nor V464 M aa substitutions was reported almost every year from 2012 to 2018 outbreaks. Some other aa substitutions L214 P; I341 V, I341 M, and L355 P were infrequently observed among the human cases in Bangladesh.

Phylodynamic and evolutionary relationships

We estimated the most recent common ancestor (tMRCA) of NiV to be ~75 years back from the most recent available date (last accessed in August 2020), corresponding to the year 1945 (95% HPD:1924–1966). The evolutionary tree revealed that NiV-N gene sequences from Bangladesh fell into a single monophyletic clade I (Figure 1, bootstrap score = 99%), with the other sequences from India and Thailand (Wacharapluesadee et al., 2016). NiV N-gene sequences from Malaysia, Cambodia, and Thailand fall within clade II (Figure 1, bootstrap score = 86%). Indeed, clade I corresponds to genotype NiV-BD and clade II to genotype NiV-MY. Our analysis also suggested that NiV had two different lineages originating in two different time frames: one in 1995 (95% HPD: 1991–2001), which corresponds to genotype NiV-BD and the other in 1981 (95% HPD: 1972–1992), which corresponds to genotype NiV-MY. Broadly equivalent rates of nucleotide substitution were observed

Table 3
Amino acid differences among the available complete Nipah virus N gene.

Sequence and accession no.	Genotype	Amino acid position																												
		30	139	188	211	214	318	341	345	355	367	380	381	387	396	414	429	432	436	457	464	502	505	506	508	511	512	518	521
NiV/MYS/HU/1999/AF212302	MY	T	S	E	Q	L	I	I	M	L	A	N	R	D	V	K	I	G	I	N	V	I	R	T	G	E	K	L	A
NiV/MYS/PI/1999/AJ564622	MY	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
NiV/MYS/PI/1999/AJ564621	MY	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
NiV/MYS/PI/1999/AJ627196	MY	.	R	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
NiV/MYS/HU/1999/AJ564623	MY	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
NiV/MYS/HU/1999/AY029768	MY	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
NiV/MYS/BA/2000/AF376747	MY	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
NiV/MYS/BA/2010/FN869553	MY	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	V	E	.	D	.	.	.	.	.	.	.	.	.	.
NiV/KHM/BA/2004/AY858110	MY	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	V	E	.	D	.	T	.	.	.	G	.	P	T	
NiV/BGD/HU/2004/AY988601	BD	.	.	D	.	.	.	.	.	.	.	.	.	N	.	.	V	E	.	D	.	.	K	D	R	.	.	.	T	
NiV/BGD/HU/2004/JN808861	BD	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	V	E	.	D	.	.	K	D	R	.	.	.	T	
NiV/BGD/HU/2004/JN808862	BD	.	.	.	.	.	.	.	.	.	.	I	.	N	.	.	V	E	M	D	.	.	K	D	R	.	.	.	T	
NiV/BGD/HU/2004/JN808858	BD	.	.	.	.	.	.	.	.	.	.	.	.	N	.	N	V	E	M	D	.	.	K	D	R	.	.	.	T	
NiV/BGD/HU/2008/JN808857	BD	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	V	E	.	D	.	.	K	D	R	.	.	.	T	
NiV/BGD/HU/2008/JN808863	BD	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	V	E	.	D	.	.	K	D	R	.	.	.	T	
NiV/BGD/HU/2010/JN808859	BD	.	.	.	.	.	V	.	.	.	.	.	.	N	.	.	V	E	.	D	.	.	K	D	R	.	.	.	T	
NiV/BGD/HU/2010/JN808860	BD	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	V	E	.	D	.	.	K	D	R	.	.	.	T	
NiV/BGD/HU/2010/JN808864	BD	.	.	.	.	.	.	.	.	.	.	.	K	N	.	.	V	E	.	D	.	.	K	D	R	.	.	.	T	
NiV/IND/HU/2007/FJ513078	BD	.	.	.	R	.	.	.	.	.	.	.	.	N	.	.	V	E	.	D	.	.	K	D	R	.	.	.	T	
NiV/BGD/BA/2012/J 101	BD	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	V	E	.	D	.	.	K	D	R	.	.	.	T	
NiV/BGD/BA/2012/J 102	BD	–	.	.	.	.	.	.	.	.	.	.	.	N	.	.	V	E	.	D	M	–	–	–	–	–	–	–	–	
NiV/BGD/BA/2012/J 103	BD	–	.	.	.	.	.	.	.	.	.	.	.	N	.	.	V	E	.	D	M	.	K	D	R	.	.	.	T	
NiV/BGD/BA/2012/J 104	BD	–	.	.	.	.	.	.	.	.	.	.	.	N	.	.	V	E	.	.	M	.	K	D	R	.	.	.	T	
NiV/BGD/BA/2012/J 105	BD	–	.	.	.	.	.	.	.	.	.	.	.	N	.	.	V	E	.	D	M	.	K	D	R	.	.	.	T	
NiV/BGD/BA/2012/J 106	BD	–	.	.	.	.	.	.	.	.	.	.	.	N	.	.	V	E	.	.	M	.	K	D	R	.	.	.	T	
NiV/BGD/BA/2012/J 109	BD	–	.	.	.	.	.	.	.	.	.	.	.	N	.	.	V	E	.	.	–	–	–	–	–	–	–	–	–	
NiV/BGD/BA/2012/J 113	BD	–	.	.	.	.	.	.	.	.	.	.	.	N	.	.	V	E	.	D	.	.	K	D	R	.	.	.	T	
NiV/BGD/HU/2012/RS90412	BD	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	V	E	.	D	M	.	K	D	R	.	.	.	–	
NiV/BGD/HU/2012/RS90612	BD	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	V	E	.	D	M	.	K	D	R	.	.	.	–	
NiV/BGD/HU/2013/RPP176613	BD	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	V	E	.	D	–	–	–	–	–	–	–	–	–	
NiV/BGD/HU/2013/RPP175113	BD	–	.	.	.	.	.	V	.	.	.	.	.	N	.	.	V	E	.	D	.	.	K	D	R	.	.	.	T	
NiV/BGD/HU/2013/RajM115913	BD	–	.	.	.	.	.	.	.	.	.	.	.	N	.	.	V	E	.	.	.	.	K	D	R	.	.	.	T	
NiV/BGD/HU/2013/RajP115713	BD	–	–	–	–	–	.	.	.	.	.	.	.	N	.	.	V	E	.	.	.	–	–	–	–	–	–	–	–	
NiV/BGD/HU/2013/RPM175513	BD	–	.	.	.	.	.	.	.	.	.	.	.	N	.	.	V	E	.	.	.	.	K	D	R	.	R	.	T	
NiV/BGD/HU/2014/RP210214	BD	–	.	.	.	.	.	.	.	.	.	.	.	N	.	.	V	E	.	.	.	.	K	D	R	.	.	.	T	
NiV/BGD/HU/2014/FP54214	BD	–	.	.	.	.	.	.	.	.	.	.	.	N	.	.	V	E	.	.	.	.	K	D	R	.	.	.	T	
NiV/BGD/HU/2014/FP54114	BD	–	.	.	.	.	.	.	.	.	.	.	.	N	.	.	V	E	.	D	.	.	K	D	R	.	.	.	–	
NiV/BGD/HU/2014/FP51514	BD	–	.	.	.	.	.	.	.	.	.	.	.	N	.	.	V	E	.	.	.	.	K	D	R	.	.	.	–	
NiV/BGD/HU/2014/FP081915	BD	–	–	–	–	–	.	.	.	.	.	.	.	N	.	.	V	E	.	D	.	.	K	D	R	.	.	.	T	
NiV/BGD/HU/2015/FP086315	BD	–	.	.	.	.	.	.	.	.	.	.	.	N	.	.	V	E	.	.	.	.	K	D	R	.	.	.	T	
NiV/BGD/HU/2015/FP088315	BD	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	V	E	.	.	.	.	K	D	R	.	.	.	T	
NiV/BGD/HU/2015/RS202615	BD	–	.	.	.	P	.	.	.	.	.	.	.	N	.	.	V	E	.	D	.	.	K	D	R	.	.	.	T	
NiV/BGD/HU/2015/FP088215	BD	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	V	E	.	.	–	–	–	–	–	–	–	–	–	
NiV/BGD/HU/2015/RS195315	BD	–	.	.	.	.	.	M	.	.	P	.	.	N	.	.	V	E	.	.	–	–	–	–	–	–	–	–	–	
NiV/BGD/HU/2017/FP136517	BD	–	.	.	.	.	.	.	.	P	.	.	.	N	.	.	V	E	.	.	.	.	K	D	R	.	.	.	T	
NiV/BGD/HU/2017/RS276817	BD	–	.	.	.	.	.	.	.	.	.	.	.	N	.	.	V	E	.	.	.	.	K	D	R	.	.	.	T	
NiV/BGD/HU/2018/SQ00118	BD	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	V	E	.	.	.	.	K	D	R	.	.	.	T	
NiV/BGD/HU/2018/RS337518	BD	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	V	E	.	.	.	.	K	D	R	.	.	.	T	

in our data set of 61 NiV-N gene sequences and the estimated mean substitution rate was 4.64×10^{-4} substitution/site/year (95% HPD: 3.43×10^{-4} - 6.04×10^{-4}). Among the strains of genotype NiV-BD, Bangladeshi NiV strains demonstrated two major sublineages (NiV- BD 1 and NiV- BD 2) (Figure 1). These two sublineages of NiV-BD strains separated around the year of 2000 (95% HPD: 1996–2003) and both lineages consist of NiV strains obtained from bat roost urine and human cases. Most of the strains (7 out of 9) from bats in this study along with the strains from human cases, clustered together in the sublineage NiV- BD 2. The Thai NiV strains from *Pteropus lylei* in 2010–2011 also clustered in the sublineage NiV-BD 2. NiV strains from the first outbreak cases (2004) of Bangladesh phylogenetically clustered into the sublineage NiV-BD 1 with the strains from outbreaks between 2010 and 2018.

Discussion

Amongst the NiV strains that were successfully sequenced for N gene fragment, none were identical regardless of its host. Many of the NiV cases were epidemiologically linked to other Nipah case/s. Samples from two such patients from Joypurhat, RS90412 and RS90612, who drank date palm sap together were subjected to further analysis. The nucleotide sequences of the NiV N gene fragment were not identical for these patients. NiV is a single-stranded negative-sense RNA virus. RNA viruses exhibit a high mutation rate, undergo continuous genetic evolution, and often consist of nonidentical but very similar variants called quasispecies for better survival in the host environment (Duffy, 2018; Vignuzzi et al., 2006). Therefore, the same shedding event could generate

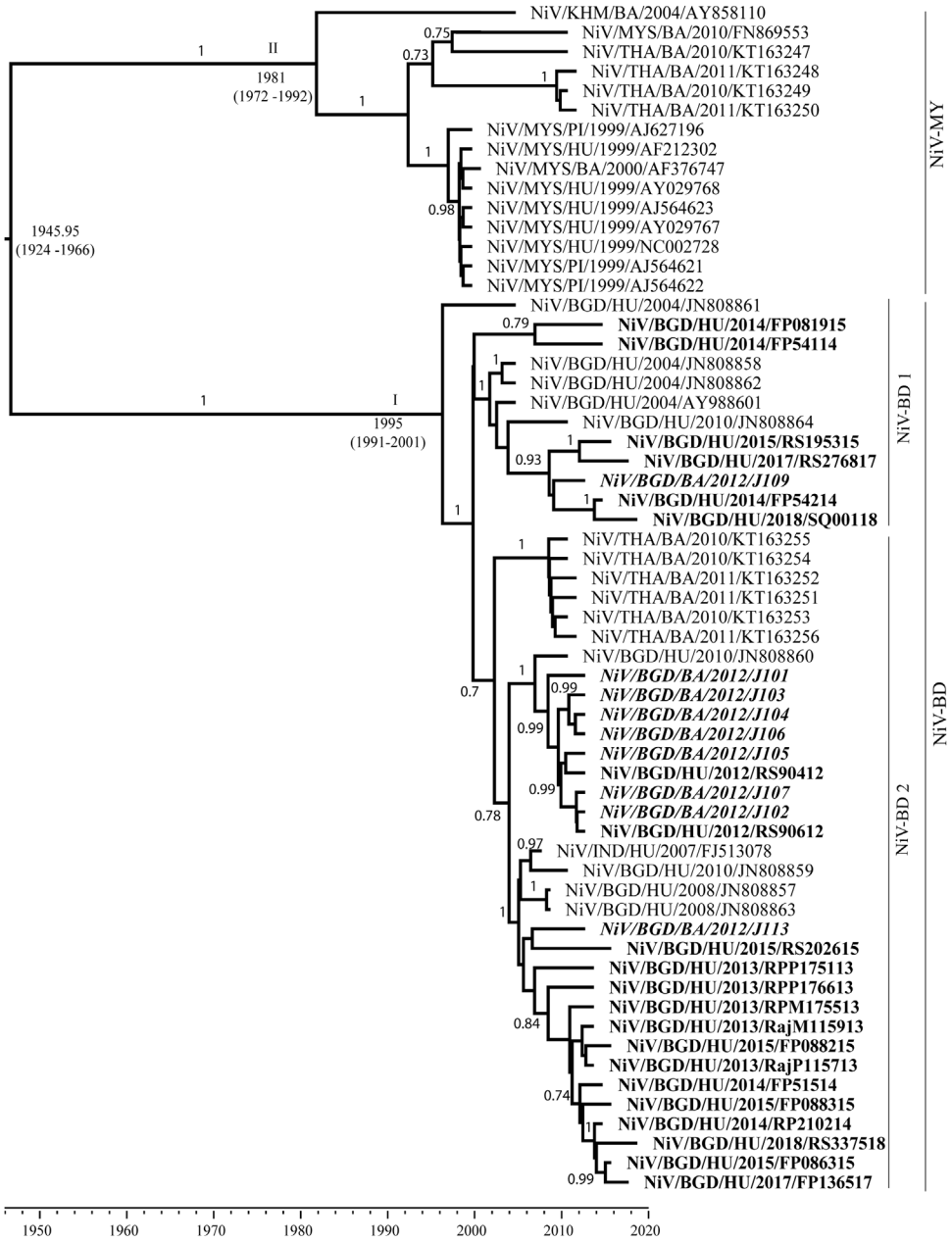

Figure 1. Bayesian maximum clade credibility phylogeny of NiV-N gene sequences from Bangladesh between 2012 and 2018. The number along the branches represents significant statistical support for the clade subtending that branch (posterior probability >0.70). The scale at the bottom of the tree represents time in years. Main clades and clusters are indicated. Two sublineages of NiV-BD were denoted as NiV- BD 1 and NiV- BD 2. Depending on the isolation host of the Bangladeshi study strains, strains from bat denoted as bold and italic and strains from human with only bold indicates. Nomenclature as follows: NiV/BGD, NiV-Bangladesh; NiV/MYS, NiV-Malaysia, HU, human; BA, bat; PI, pig; year, year of isolation; and GenBank accession number or isolate identification number.

different strains, possibly the quasispecies of NiVs and nonidentical NiV strains might be found in hosts that were linked epidemiologically. The finding was in line with the previously reported synchronous shedding of multiple paramyxoviruses that coincided with a spillover event from bats in the environment (Peel et al., 2019). However, some of the predicted amino acid sequences were identical to that of the strains from bat roast urine samples collected in 2012 from Joypurhat. Possibly, these strains were evolved from the same origin and over time, varied genetically but still possessed the similar phenotypic properties i.e., the similar amino acid sequences. The groups of cases reported from the 2013 outbreak from adjacent districts and from the 2015 outbreaks that occurred in the adjacent districts of the Nipah belt in Bangladesh in a very short time frame were possibly a part of the same shedding event. Despite variations in the nucleotide sequences, some of the predicted amino acid sequences for the NiV N gene from human cases were identical to that of NiV strains (n = 3) from bat roost urines. The amino acids in the five conserved positions characteristics to the NiV-BD and NiV-MY genotypes remained consistent among the NiV strains reported in this study (Lo et al., 2012). The predicted amino acid sequences of the minimum contagious region of NiV (aa 30–404) remained unchanged in the strains reported from 2004 to 2014, but there were some infrequent amino acid substitutions in that of the strains reported in 2015 and 2017. These molecular changes in the minimum contagious region might contribute to the NiV particle formation, copy number, virulence, and disease transmission (direct or person to person transmission) (Ong et al., 2009). The sequences in this study had more variations in the amino acid sequence at the carboxyl (C)-terminus of the N protein. This finding was in line with previously reported Bangladeshi NiV strains (Chan et al., 2004; Gill et al., 1988; Lo et al., 2012). Whereas, the most variable domain of the C-terminus of NiV N gene (aa 384–532) (Chan et al., 2004) and the highly conserved regions (aa 171–181, 267–277, 322–336) of NiV-N ORF (Gill et al., 1988) remained as reported earlier.

Time-scaled phylogenetic analysis suggested that more than 75 years ago, the common ancestor of NiV entered in the southeastern Asiatic regions. We estimated the date of tMRCA of NiV to be the 1945strain, which was earlier estimated to be 1947 (Lo Presti et al., 2016). Our study suggested that the NiV-BD was evolving during 1995, approximately 14 years (1981) after the evolution of NiV-MY strains. Considering all the 61 NiV sequences in this study, the mean substitution rate was 4.64×10^{-4} substitutions per site, per year (subs/site/year), which was somewhat lower than previously estimated (6.5×10^{-4} subs/site/year) (Lo Presti et al., 2016) but similar to that of other RNA viruses, such as the human respiratory syncytial virus and human parainfluenza viruses (Duffy and Holmes, 2008; Jenkins et al., 2002). The strains of the NiV-BD genotype diverged into two sublineages during 1995. The first NiV sequences from Bangladesh in 2004 were clustered in sublineage NiV-BD1, but later, there was a temporal shift, where strains from 2008 to 2010 belonged mostly to sublineages NiV-BD 2. After that, NiV strains from 2010 to 2018 were distributed in both the sublineages (NiV-BD 1 & 2). The occurrence of any particular sublineage of NiV strains was not limited to a specific outbreak region in Bangladesh. NiV strains from both the sublineages were reported from Rajshahi, Jaypurhat, and Faridpur districts. So far, only the NiV-BD 2 strains were reported from Rangpur, Gopalganj, and Manikganj districts.

The study suggested that NiVs diverged into NiV-MY and NiV-BD genotypes about 75 years ago. Because of the high mutation nature of RNA viruses, NiV may adopt enhanced virulence and evolvability that could bring further changes in viral characteristics, which further accentuates some of the differences between the Malaysian and Bangladesh strains. Both NiV-MY and NiV-BD strains were reported from Southern Thailand, a geographical location between Bangladesh and Malaysia (Wacharapluesadee et al., 2016). This finding indicated the overlapping geolocation for NiV strains where both NiV-MY and NiV-BD strains were cocirculating among its natural reservoir but in different host species. The presence of NiV-BD strains in *Pteropus* bats in Thailand suggested the attribution of different species of *Pteropus* genus for global dissemination and heterogeneity of NiV.

Our study had some limitations, including a limited number of samples from each year and incomplete coverage of the geographic area of Bangladesh, which limits our understanding on NiV transmission dynamics as well as the progression pattern. We focused on only the N gene, which might have restricted us from obtaining the complete picture of virus evolution as we would have if we could have used the whole genome approach. We also used bat roost urine rather than specimens from captured individual bats and thus could not differentiate the bat species in the roost. While collecting the roost urine samples, dilution with the negative ones minimized the detection of NiV RNA using the real-time PCR assay. Another major limitation of our study was that in the absence of Biosafety level 4 facilities for NiV growth in culture, we could retrieve only sequences of NiV-N gene. We used archived bat roost urine samples and previously extracted RNA samples stored at -80°C. These samples were reported as positive but contained trace amounts of NiV-RNA with borderline Ct values (>36). Therefore, because of the nature of RNA degradation and the low copy number of NiV-RNA in the samples tested, the recovery of NiV-RNA positive samples was lessened. Although the number of bat roost urine samples in this study may not represent the overall bat populations in Bangladesh, our results strengthen the molecular evidence for NiV transmission from bat to human.

Conclusion

The present study added information about the genetic diversity and molecular characterization of NiV strains, both from bats and humans in Bangladesh. This study provides molecular information to design laboratory methods for the early detection of NiV in the environmental samples to assess its epidemic potential in humans. The study supported that the strains of NiV continue to evolve, and therefore, further study on genetic variability between NiV strains from bats and humans may contribute to know its ability to cause infections and outbreaks and help in the selection of vaccine candidate strains.

Ethics approval and consent to participate

The study protocol was approved by icddr,b's institutional review board (IRB) (protocol no: PR-15,033).

Declaration of interests

The authors declare that they have no known financial interests or personal affairs to influence the work reported in this paper.

Authors' contributions

MZR, JHE, SPL, and ESG designed the study. MMI, MEH, MMR, and AS carried out the laboratory testing. AI, MSSH, SS, AI, and MR were involved in field data collection. MZR, SPL, ESG, MR, JDK, and MSF carried out data analysis. MZR, SPL, and ESG discussed the results. MZR drafted the first manuscript. MR, MR, JDK, MSF, JHE, SPL, and ESG reviewed and edited the manuscript. All authors critically read and gave their approval for publication.

Conflict of interests

The author declare that they have no competing interests.

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Appendix A.

Table A1
Short description of Nipah cases included in this study from 2012 to 2018.

Sequence ID	Sex	Age	Date of onset	Subdistrict	District	Primary or secondary	Spreader?	Isolated or clustered	connected to any others on this list?
NiV/BGD/HU/2012/RS90412	female	7	17-Jan-12	Joypurhat Sadar	Joypurhat	primary	yes	clustered	Clustered with RS90612 - drank date palm sap together. This case is linked with the bat sampling in Joypurhat in 2012
NiV/BGD/HU/2012/RS90612	male	6	17-Jan-12	Joypurhat Sadar	Joypurhat	primary	no	clustered	Clustered with RS90412 - drank date palm sap together. This case is linked with the bat sampling in Joypurhat in 2012
NiV/BGD/HU/2013/RPP176613	male	7	26-Jan-13	Fulbari	Kurigram	primary	no	isolated	No
NiV/BGD/HU/2013/RPP175113	male	9	14-Jan-13	Polashbari	Gaibandha	primary	no	isolated	No
NiV/BGD/HU/2013/RajM115913	male	43	16-Jan-13	Chatmohor	Pabna	primary	?	isolated	Onset just days apart from RajP115713, in a nearby district. Possibly part of same shedding event
NiV/BGD/HU/2013/RajP115713	male	8	19-Jan-13	Bodolgachi	Naogaon	primary	no	isolated	Onset just days apart from RajM115913, in a nearby district. Possibly part of same shedding event
NiV/BGD/HU/2013/RPM175513	male	48	23-Jan-13	Sayedpur	Nilphamari	primary	no	isolated	
NiV/BGD/HU/2014/RP210214	male	22	14-Jan-14	Sadar	Rangpur	primary	no	clustered	
NiV/BGD/HU/2014/FP54214	male	22	1-Feb-14	Saltha	Faridpur	primary	yes	clustered	Part of same cluster as next case
NiV/BGD/HU/2014/FP54114	female	32	31-Jan-14	Saltha	Faridpur	primary	yes	clustered	Part of same cluster as previous case
NiV/BGD/HU/2014/FP51514	male	14	14-Jan-14	Sadar	Magura	primary	yes	clustered	
NiV/BGD/HU/2014/FP081915	male	13	30-Dec-14	Mousha	Magura	–	–	–	
NiV/BGD/HU/2015/FP086315	male	7	18-Feb-15	Mokshedpur	Gopalganj	primary	no	isolated	There was another spillover around the same time in another place in the same subdistrict
NiV/BGD/HU/2015/FP088315	male	36	7-Mar-15	Shibchar	Madaripur	primary	no	clustered	Part of a cluster with FP088215
NiV/BGD/HU/2015/RS202615	male	42	23-Mar-15	Bagha	Rajshahi	primary	no	isolated	none
NiV/BGD/HU/2015/FP088215	female	15	10-Mar-15	Shibchar	Madaripur	primary	no	clustered	Part of a cluster with FP088315
NiV/BGD/HU/2015/RS195315	male	25	15-Jan-15	Manda	Naogaon	primary	no	clustered	none
NiV/BGD/HU/2017/FP136517	male	12	15-Feb-17	Joykalidangi	Faridpur	primary	no	?	none
NiV/BGD/HU/2017/RS276817	male	13	10-Feb-17	Uttar Raghobpur	Pabna	primary	no	?	none
NiV/BGD/HU/2018/SQ00118	male	26	8-Feb-18	–	Faridpur	primary	no	isolated	none
NiV/BGD/HU/2018/RS337518	male		3-Apr-18	–	Rajshahi	primary	no	isolated	none

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