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# Rapid replacement of prevailing genotype of human respiratory syncytial virus by genotype ON1 in Beijing, 2012–2014

Guanglin Cui [1], Runan Zhu, Jie Deng, Linqing Zhao, Yu Sun, Fang Wang, Yuan Qian *

*Laboratory of Virology, Beijing Key Laboratory of Etiology of Viral Diseases in Children, Capital Institute of Pediatrics, 2 Yabao Road, Beijing 100020, China*

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ABSTRACT

Human respiratory syncytial virus (HRSV) is the most common viral pathogen causing lower respiratory infections in infants and young children worldwide. HRSV ON1 genotype in subgroup A with a characteristic of a 72 nucleotide duplication in the second highly variable region of attachment glycoprotein gene, has been reported in some countries since it was first detected in clinical samples collected in Canada in 2010. In this study, 557 HRSV antigen-positive nasopharyngeal aspirates were randomly selected during 2012/2013 to 2013/2014 HRSV seasons in Beijing for subgroup typing and for ON1 genotype screening by using a PCR based method developed for easily identifying genotype ON1 out of strains of subtype A. It was found that subgroup B was dominant in the 2012/2013 season and sudden shift of subgroup dominance from B to A and rapid replacement of previously prevailing NA1 genotype by ON1 genotype occurred in the 2013/2014 season. Reversible amino acid replacement in the G protein gene was found in a new branch of ON1 genotype. The evolutionary rate of the 351 global ON1 sequences was estimated to $7.34 \times 10^{-3}$ nucleotide substitutions per site per year (95% highest probability density intervals, HPD, $5.71 \times 10^{-3}$ to $9.04 \times 10^{-3}$), with the time of most recent common ancestor dating back to June 2009.



## 1. Introduction

Human respiratory syncytial virus (HRSV) is the leading viral cause of severe pediatric respiratory tract infections worldwide, causing 1.9 million deaths per year (Williams et al., 2002). Repeated infections with HRSV are common throughout life (Henderson et al., 1979). The virus is composed of a non-segmented, single-stranded negative sense RNA genome packaged in a lipid envelope. It belongs to the genus *Pneumovirus* in family *Paramyxoviridae* of order *Mononegavirales*. Two major subgroups, HRSV-A and -B, have been identified by both antigenic and genetic analyses. The genome of HRSV is approximately 15.2 kb and contains 10 genes encoding at least 11 proteins (Collins and Crowe, 2007).

The attachment glycoprotein (G), one of the virus encoded transmembrane surface protein, is a type II integral protein, containing cytoplasmic domain, transmembrane domain and ectodomain (Roberts et al., 1994). The ectodomain is comprised of two mucin-like highly variable regions (HVR1 and HVR2) separated by a putative receptor-binding domain that is highly conserved within and between the two HRSV subgroups (Johnson et al., 1987). The G protein is involved in virus binding to cell membranes and is capable of stimulating neutralizing antibodies (Melero et al., 1997). Being the most variable protein, the G protein varies in its amino acid composition, length and distribution pattern of potential N- and O-linked glycosylation sites. These characteristics of the G protein may give HRSV the ability to evading the preexisted host immune response, facilitating recurrent infections and even large outbreaks. Thus, most of the studies on HRSV variability and evolution have been focused on G proteins, especially the HVR2.

By phylogenetic analyses for nucleotide sequences of HVR2, HRSV-A have been classified into 11 genotypes (GA1–GA5, GA6–GA7, SAA1, NA1–NA2 and ON1) and HRSV-B into 22 genotypes (GB1–GB4, BA1–BA6, BA7–BA10, BA-C, SAB1–SAB3, SAB4, URU1, URU2 and CB1) (Cui et al., 2013b). In 2003, Trento et al. (2003) reported three new HRSV-B strains isolated in 1999, named BA genotype, with the characteristic of a major change in the HVR2 introduced by a duplication of 60 nucleotides. Since then, BA genotype has gradually replaced other genotypes of HRSV-B and

*Abbreviations:* HRSV, human respiratory syncytial virus; HPD, highest probability density; HVR, highly variable region; TMRCA, the time of most recent common ancestor; CIP, Capital Institute of Pediatrics; NPA, nasopharyngeal aspirate; PCR, polymerase chain reaction; RT, reverse transcription; ESS, effective sample size; BEAST, Bayesian evolutionary analysis sampling trees.

* Corresponding author.
*E-mail address:* yqianbjc@263.net (Y. Qian).
[1] Present address: Clinical Laboratory, The First Affiliated Hospital of Zhengzhou University, 1 Jianshedong Road, Zhengzhou, Henan 450002, China.

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has become the predominant one worldwide (Trento et al., 2010). The duplicating segment was then used as a natural tag to track the global dissemination and evolution of HRSV (Trento et al., 2010). Coincidentally, a 72 nucleotide duplication in HVR2 nucleotide sequence was found in 10% of the subgroup A strains isolated in Ontario, Canada during 2010–2011 winter season (Eshaghi et al., 2012). HRSV strains with this 72 nucleotide duplication were designated genotype ON1. Up to date, HRSV genotype ON1 has been found in China (Cui et al., 2013b; Liu et al., 2014; Ren et al., 2014), Japan (Hirano et al., 2014; Tsukagoshi et al., 2013), South Korea (Lee et al., 2012), Germany (Prifert et al., 2013), South Africa (Valley-omar et al., 2013), Malaysia (Khor et al., 2013), Italy (Pierangeli et al., 2014), India (Choudhary et al., 2013), Thailand (Auksornkitti et al., 2013), Kenya (Agoti et al., 2014), Latvia (Balmaks et al., 2013), Spain (GenBank accession number: KF915266), Philippines (AB846656), Peru (KJ627264), Panama (KF301013), Croatia (KF057868) and USA (KJ672471).

The increasing frequency for detection of HRSV strains with ON1 genotype worldwide indicates that this genotype may have great advantages for escaping current host immunity. To easily screen and identify the strains with ON1 genotype, a PCR method was developed in this laboratory to find out the existence of this 72 nucleotide duplication. Rate of nucleotide substitution and the time of most recent common ancestor (TMRCA) of the global ON1 sequences were also estimated.

## 2. Materials and methods

### 2.1. Ethics statement

This study was approved by the ethics committee of the Capital Institute of Pediatrics (CIP).

### 2.2. Sample collection

From July 2012 to May 2014, NPAs were collected and transported immediately to the Laboratory of Virology in CIP for the antigen detecting for respiratory viruses by using direct fluorescent assay (Diagnostic Hybrids, Athens, OH, USA). During this period, 1601 NPAs were HRSV antigen-positive, among which 612 (38.2%) were from girls and 989 (61.8%) from boys. Some of the samples were inoculated into HEp-2 cells for virus isolation before storing at −80 °C.

### 2.3. PCR for subgroup typing and full length of G genes

The methods of RNA extraction, reverse transcription (RT), multiplex PCR and real time RT-PCR for subgroup-typing and PCR for amplifying full length attachment (G) gene were the same as those used in previous study by this group (Cui et al., 2013b).

### 2.4. PCR for ON1 genotype screening

Genotype ON1 was screened by using a PCR method to determine the presence of the 72 nucleotide duplication. The forward primer S5405 (5′-TCCAGAACACACAAGT-3′) corresponds to bases 5405–5420 of strain A2 (GenBank accession number: M11486) and the reverse primer A5534 (5′-TCATTTTGTTRTGTTG-3′) was complementary to bases 5519–5534 of strain A2. Briefly, the reaction was performed in a 25 μl final volume mixture containing 0.4 μM of each of forward and reverse primers, 4 μl of RT products from isolated HRSV strains or clinical specimens determined as subgroup A, 0.2 mM dNTP, 1 U of DNA polymerase (TransGen Biotech, Beijing, China) and 2.5 μl 10× PCR buffer (TransGen Biotech, Beijing, China) under the following thermocycling conditions: 94 °C for 5 min, 40 cycles of denaturation at 94 °C for 30 s, annealing at 49 °C for 30 s, and extension at 72 °C for 20 s, followed by a final extension at 72 °C for 7 min. The amplified products of 129 bp for non-ON1 genotype and 201 bp for ON1 genotype were visualized by electrophoresis in 2% agarose gels stained with ethidium bromide. Samples negative by this PCR screen were subjected to G gene sequencing. The insertion of the 72 nucleotide sequence in ON1 genotype can be easily identified after alignment with non-ON1 sequences.

### 2.5. G gene sequencing

PCR products from G genes were subjected to forward and reverse cycle sequencing with primers as those used in PCR for amplifying the full length G gene, as described previously (Cui et al., 2013b). Sequencing was performed by Invitrogen company using ABI 3730xl DNA analyzers. Chromatogram files were inspected using Chromas Lite 2.1 (Thehnelysium, South Brisbane, Australia). Sequence assembly was performed with DNAStar 5.01 (DNASTAR, Wisconsin, USA).

### 2.6. Phylogenetic analyses

Reference sequences of HRSV G genes with identified genotypes were downloaded from the GenBank (Table S1). They were aligned with the sequences from this study by using the ClustalW program embedded in Mega 5.05 software (Tamura et al., 2011). Nucleotide sequence alignment was rectified on the basis of their aligned predicted protein sequences. Identical sequences were identified with DAMBE software, version 5.3.10. Because the lengths of some reference sequences were much shorter than the full length G gene, only the HVR2 sequences were reserved in the alignment. Phylogenetic tree for the HVR2 sequences alignment was constructed by the Maximum Likelihood method within the Mega 5.05, using the General Time Reversible method for substitution model and complete deletion for gaps data treatment. The reliability of phylogenetic grouping was tested by 1000 replicates of bootstrap probabilities.

### 2.7. Analysis for evolutionary rate

A total of 301 nucleotide sequences (partial or complete G gene) for genotype ON1 HRSV strains isolated worldwide were retrieved from GenBank (date due: July 31, 2014) (Table S2). The information on sampling date of these isolates could be obtained. The HVR2 nucleotide sequences of these 301 sequences and 50 ON1 sequences from Beijing identified in this study were used for evolutionary analysis. Rate of nucleotide substitution per site and the time of the most recent common ancestor (TMRCA) were determined by two methods: an exploratory root-to-tip linear regression analysis (Path-O-Gen software) and the Bayesian Markov chain Monte Carlo approach (BEAST 1.8.0 software) as described by Agoti et al. (2014). Unlike previous studies on the evolutionary analyses for ON1 genotype (Agoti et al., 2014; Kim et al., 2014; Ren et al., 2014), identical sequences were not excluded in our study for the population genetic reason. The Path-O-Gen software (available at http://tree.bio.ed.ac.uk/software/pathogen/) takes the maximum likelihood tree as an input and analyzes the relationship between the genetic distance from the root and sampling date for each sequence. The Bayesian evolutionary analyses (BEAST, software available at http://beast.bio.ed.ac.uk/) were run through enough steps to make the effective sample size (ESS) not less than 200, under the HKY model of evolution.