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Phylogenetic Analysis of Human Respiratory Syncytial Virus from Children with Acute Respiratory Infection Admitted to Yangon Children's Hospital

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Human respiratory syncytial virus (RSV) is one of the most important pathogens responsible for acute respiratory tract infection (ARI) outbreaks in children worldwide. RSV is a member of the family *Paramyxoviridae* in which differentiated into two groups (A and B) based on antigenic and genetic variability. To date, 11 genotypes for RSV group A and 23 for RSV group B have been described based on changes in the G gene coding for the attachment glycoprotein. In this study, nasopharyngeal swab samples were collected from hospitalized pediatric ARI cases at Yangon Children Hospital from January to September, 2014. Of 160 cases, non-structural protein 1 (NS1) gene of RSV was detected in 16.3% (26/160), comprising RSV-A strains 52% (11/21) and RSV- B strains 48% (10/21). Furthermore, 21 NS1 gene-positive nasopharyngeal swab samples were processed for genotyping by sequencing of C terminal of the G gene, second variable region. G gene of the RSV was successfully sequenced in 61.9% (13/21) of samples. RSV-A strain was the larger group, accounting for 53.8% (7/13), followed by RSV-B, 38.5% (5/13) and one case 7.7% (1/13) was a mixed infection. The phylogenetic analysis revealed that all group-A strains clustered as the ON1 genotype. Additionally, 72 nucleotide duplication in the second highly variable region of attachment G gene was observed in all RSV ON1 genotype in subgroup-A isolates. Moreover, one isolate of ON1 genotype showed G284S substitution as a novel mutation. Molecular surveillance of RSV infection should be conducted in Myanmar.

Key words: Phylogenetic analysis, Human respiratory syncytial virus, Children with acute respiratory infection

INTRODUCTION

Acute respiratory infection (ARI) is responsible for high morbidity and mortality among under five children, especially in developing countries.[1] Viruses are considered the most important agents in ARI of the lower respiratory tract (LRT) that require hospitalization. Respiratory Syncytial Virus (RSV) is one of those viruses that tops the list among respiratory viruses responsible for annual epidemic ARI outbreaks in infants and pre-school children worldwide, frequently causing bronchiolitis and pneumonia mostly in infants less than six months old.[2] RSV is classified in the genus *Pneumovirus* belonging to the *Paramyxoviridae* family and has an envelope, non-segmented, single-stranded, negative sense RNA genome of approximately 15.2 kb and contains 10 genes encoding at least 11 proteins.[3]

RSV is differentiated into two groups (A and B) based on antigenic and genetic variability. Further studies of genetic variability among RSV strains belonging to

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A and B groups revealed the existence of different genotypes, each with a distinct genetic pattern. The G protein of RSV is a type II surface glycoprotein of about 300 amino acids in length, consisting of a cytoplasmic domain, a transmembrane domain and an ectodomain. It is associated with attachment of the virus and shows the largest antigenic and genetic differences between the two HRSV groups and is one of the targets for neutralization and protective antibody responses.[4, 5]

The G protein gene contains two hypervariable regions (HVR2); the second variable region, which corresponds to the C- terminal region of the G protein (HVR2), reflects overall G protein gene variability and has been analyzed in molecular epidemiological studies.[6-9] The G protein is heavily glycosylated with N-linked and O-linked sugars. Sequencing of the second hypervariable region at the C-terminal end of the G gene, which encodes the G protein, has been widely used to further subdivide RSV-A and RSV-B into genotypes and facilitated differentiation between RSV isolates. To date, 11 RSV-A genotypes, GA1-GA7, SAA1, NA1-NA2, and ON1,[9-11] and 23 RSV-B genotypes, GB1-GB4, SAB1-SAB3, SAB4, URU1, URU2, BAI-BAXII, and THB[9, 12-20] have been described based on nucleotide sequence analysis.

The genetic variability of RSV circulating in Ontario, Canada during 2010-2011 winter seasons was investigated by sequencing and phylogenetic analysis of the G glycoprotein gene. RSV-A (55.7%) was more commonly observed than RSV-B (42.3%). Furthermore, a 72 nucleotide duplication in HVR2 nucleotide sequence was first detected in 10% (11/110) of the subgroup A strains and designated the ON1 gene.[13]

In China, 557 HRSV antigen-positive nasopharyngeal aspirates were selected during 2012/2013 to 2013/2014 seasons in Beijing for group typing. Among them, 37.2% (207/557) were group A and 62.8% (350/557) were group B. Phylogenetic analysis revealed that they belonged to either genotype ON1 66.2% (49/74) or genotype NA1 33.8% (25/74).[21] In Myanmar, a study on RSV was conducted at YCH during 2014 (January to September). A total of 160 nasopharyngeal swabs were collected from under five children attending YCH with acute respiratory infection (ARI). RT-PCR and sequencing was done for identification of Non-Structural protein 1 (NS1) gene. It was found that NS1 gene was detected in 16.25% (26/160) of ARI cases; RSV-A comprised of 52% (11/21) and RSV-B 48% (10/21).[22] Study on molecular characterization of the RSV has not yet been done in Myanmar. Therefore, there is lack of information on the prevalent genotype and subtype.

The aim of this study was to continue further genotyping of previously NS1 gene positive samples by sequencing of G gene. In this study, prevalent genotypes of RSV in Myanmar were explored and this baseline data was useful for development of vaccine against RSV, and might predict future clinical and epidemiological threat among children in Myanmar.

MATERIALS AND METHODS

Twenty-one NS1 gene-positive nasopharyngeal swab samples were used. RNA was extracted by using the QIAamp viral RNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

RT-PCR

RT- PCR was done for amplification of the C terminal of the G gene. The second hyper variable region at the carboxy terminal of the G gene was amplified using primers (Table 1) for A and B groups. The cycling protocol included 30 minutes of reverse transcription at 50°C, a 15-minute activation step of the enzyme at 95°C followed by 40 cycles of 30 seconds of denaturation at 94°C and 30 seconds of primer annealing at 50°C and 45 seconds of extension at 72°C, followed by a final extension step of 5 minutes at 72°C in Veriti 96-well thermal cycler (Applied Biosystems).

Nested PCR for RSV-A and RSV-B

These positive samples were subjected to nested PCR. Primers pairs used for A and B subgroups (Table 1). Thermo cycling conditions included a 5 minutes activation step of the enzyme at 95°C, followed by 25 cycles of 30 seconds of denaturation at 94°C, 30 seconds of primer annealing at 50°C, and 45 seconds of primer extension at 72°C followed by a final extension step of 5 minutes at 72°C.

Table 1. Genotyping primers

RSV A group	
GPA (511-530)	5'-GAAGTGTTCAACTTTGTACC-3'[10]
RSAG (539-558)	5'-ATATGCAGCAACAATCCAAC-3'[23]
F1A (3-22)	5'-CAACTCCATTGTTATTTGCC-3'[10]
RSV B group	
GPB (494-515)	5'AAGATGATTACCATTTTGAAGT-3'[10]
RSBG (512-531)	5'-GTGGCAACAATCAACTCTGC-3'[23]
F1B (3-22)	5'-CAACTCCATGGTTATTTGCC-3'[10]

10=Ref: 10, 23=Ref: 23

Gel documentation

The PCR products were verified by agarose gel electrophoresis and visualized under UV light Transilluminator, (BioRad). The size of the cDNA for each group of RSV: RT-PCR were 492 bp for RSV-A, 509 bp for RSV-B and 572 bp for RSV-BA and for Nested-PCR were 465 bp for RSV-A, 461 bp for RSV-B and 524 bp for RSV-BA.

DNA sequencing methodology

(i) The PCR products were purified by using Montage's Purification Method.

(ii) Cycle sequencing was done by using Big-Dye V.3.1, Applied Biosystems. Amplification was done under the following conditions: 1 minute of activation at 96°C followed by 25 cycles of 10 seconds denaturation at 96°C, 5 seconds of annealing at 50°C and 4 minutes of extension at 60°C.

(iii) Purification of Cycle sequencing product was done by ethanol purification method to remove excess Big-dye.

(iv) DNA sequencing was done by using Applied Biosystems 3500 XL Genetic Analyzer, Hitachi at Department of Medical Research.

Phylogenetic analysis

Sequence quality was checked by Bio Edit software v.7.0.5 and manual correction was done. A 462 nucleotide segment of HVR2 were aligned with that of other sequences available from GenBank by using Clustal W 1.6 method of MEGA software version 6.06. Phylogenetic tree was generated by the neighbor-joining method with 1,000 replicates of bootstrap values. RSV-A stains G gene sequences were submitted to GenBank and the accession numbers are KY320500 to KY320507.

Table 2. RSV group A G protein gene GenBank sequences used in the study

Strain	GenBank accession no.	Country of isolation	Years of isolation
A2	M11487	Australia	1961
ON67-1210A	JN257693	Canada	2010
CU2011/216	KC342446	Thailand	2011
CU2011/211	KC342444	Thailand	2011
RSVA/GN435/11	JX627336	Korea	2011
MY-2444006-11	JX256871	Malaysia	2011
CU2011/192	KC342434	Thailand	2011
AS12-047	AB808774	Japan	2011
CN-A011-12	KU681162	Korea	2012
BJ/39979	KC461212	China	2012
RXH/ON1/001	JX885730	South Africa	2012
Chiba-C/24226	AB808757	Japan	2012
NIV1212316/12/A	KF246640	India	2012
NIV1212334/12/A	KF246641	India	2012
HR3445-12	KF057865	Croatia	2012
WUE/16397/12	JX912364	Germany	2012
WUE/14576/12	JX912357	Germany	2012
KEN/Kilifi/116160/25	KF587959	Kenya	2012
BJ/43849	KM434001	China	2013
HD12114	KJ710386	Germany	2013
JPN/21413	AB918735	Japan	2013
LA2-85/2013	KJ672471	USA	2013
1308-509AN	KC858245	Italy	2013
BJ/51238	KM434024	China	2014
HN-5787	KT781390	China	2014
BJ/55543	KM434062	China	2014
BJ/52897	KM434039	China	2014
BJ/52137	KM434034	China	2014

Amino acid analysis

G protein of Myanmar RSV-A strains were compared to references strains from GenBank to identify amino acid substitutions by MEGA v6.06.

RESULTS

Detection of RSV

Among twenty-one NS1 gene-positive RSV isolates, RSV-A was identified in 53.8% (7/13), RSV-B in 38.5% (5/13), and one case (7.7%) was mixed infection of these two groups (A+B).

Sequence alignments and phylogenetic analysis

Sequences of the second hyper variable region of the G gene from 8 RSV-A (RSVA-7 and Mixed-1) and 6 RSV-B (RSVB-5 and Mixed-1) samples were aligned with sequences of reference strains and prototype strain (M11486) from GenBank. After the phylogenetic analysis (Fig. 1. A) all Myanmar RSV-A groups were clustered as the ON1 genotype and they were found to be closely related to ON67-1210A/2010/Canada, JPN/ 214.13/ 2013/ Japan, HN_5787/2014/China, CN-A002-14/2014/Korea and CU2011/216/ 2011/ Thailand, respectively.

Regarding RSV-B, only one sequence RSV-B139 Myanmar strain belonged to BA9 genotype and closely related to one strain from Japan 2006 (NG-022-06). The remaining 5 sequences belonged to RSV-B group (Fig. 1. B).

Deduced amino acid sequence analysis

Amino acid alignment of all the genotypes of RSV-A is shown (Fig. 2). The Myanmar RSV ON1 genotype has a characteristic of a 72- nucleotide duplication in the second highly variable region of attachment G gene and it was first detected in Canada in 2010.[13] The ON1 genotype could be defined by E232G or T253K substitutions. The eight Myanmar ON1 strains were compared with those strains from other countries with reference to the original strains from Canada (JN257693) and showed that there are four characteristic substitutions as given below. Thorough amino acid analysis was done except RSV-A083 because of the shortness of sequence length.

- One ON1 strain RSV-A100 Myanmar showed S250F substitution unlike the strains from Canada and all other countries. The remaining 7 Myanmar ON1 genotypes did not have such substitution like the strains compared in this study.
- There were amino acid substitutions at position (L274P, L298P, and Y304H) seen in ON1 strains from China, Japan, India, Germany, Kenya and Italy (KM434034; AB808757; KF246640; KF246641; JX912364; KF587959; KC858245). However, this type of substitution was not seen in Canada strain and all Myanmar ON1 strains.
- (G284S, Y280H) substitutions were seen only in RSV-A102 ON1 strain which was different from other Myanmar ON1 strains and other countries. This is unique for Myanmar strain. In addition, there was also E295K substitution in RSV-A102, which is similar to Germany ON1 strain (KJ210386). However, all other ON1 strains showed no such amino acid substitution.
- Seven Myanmar ON1 strains (except RSV-A083) did not show L310P substitution which was different from Japan, India and Kenya (AB808757, KF246640, KF246641 and KF587959) ON1 strains, but similar to Canada ON1 strains.

Clinical and epidemiological features of RSV infection at YCH

Among RSV-positive patients, the ratio of boys to girls was 1.2:1; the age range was 1 month to 20 months, the majority of patients (92.3%, 12/13) were under one year old. Diagnosis in the majority of patients was bronchiolitis (55%) followed by pneumonia (15.0%), severe pneumonia (15.0%) and AVI with no pneumonia (7.5%).

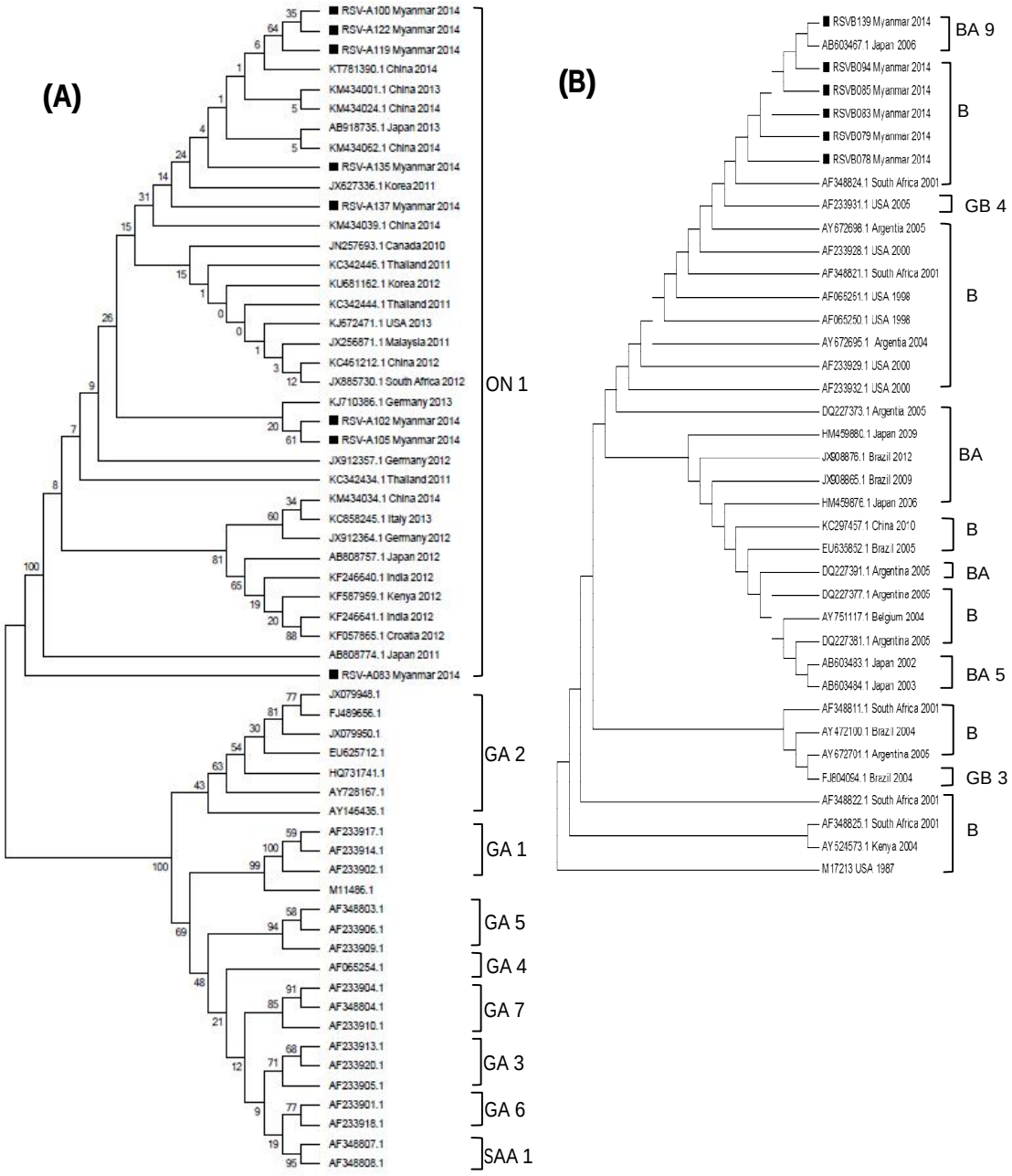

Fig. 1. Phylogenetic tree of RSV-A/RSV-B strains and reference sequences of identified genotypes. Phylogenetic trees for RSV-A (A) and RSV-B (B) strains were constructed with Neighbor-joining tree method with 1,000 bootstrap replicates using MEGA 6.06 software. RSV strains from Myanmar are indicated by "RSV-A and RSV-B" followed by their strain identification number. Number of identical strains is indicated in brackets after the strain identifier. Reference strains representing known genotypes were retrieved from GenBank and included in the tree (labels include accession number) (Table 2). The genotype assignment is shown on the right by brackets. Prototype strains (M11486 for subgroup A and M17213 for subgroup B) were used.

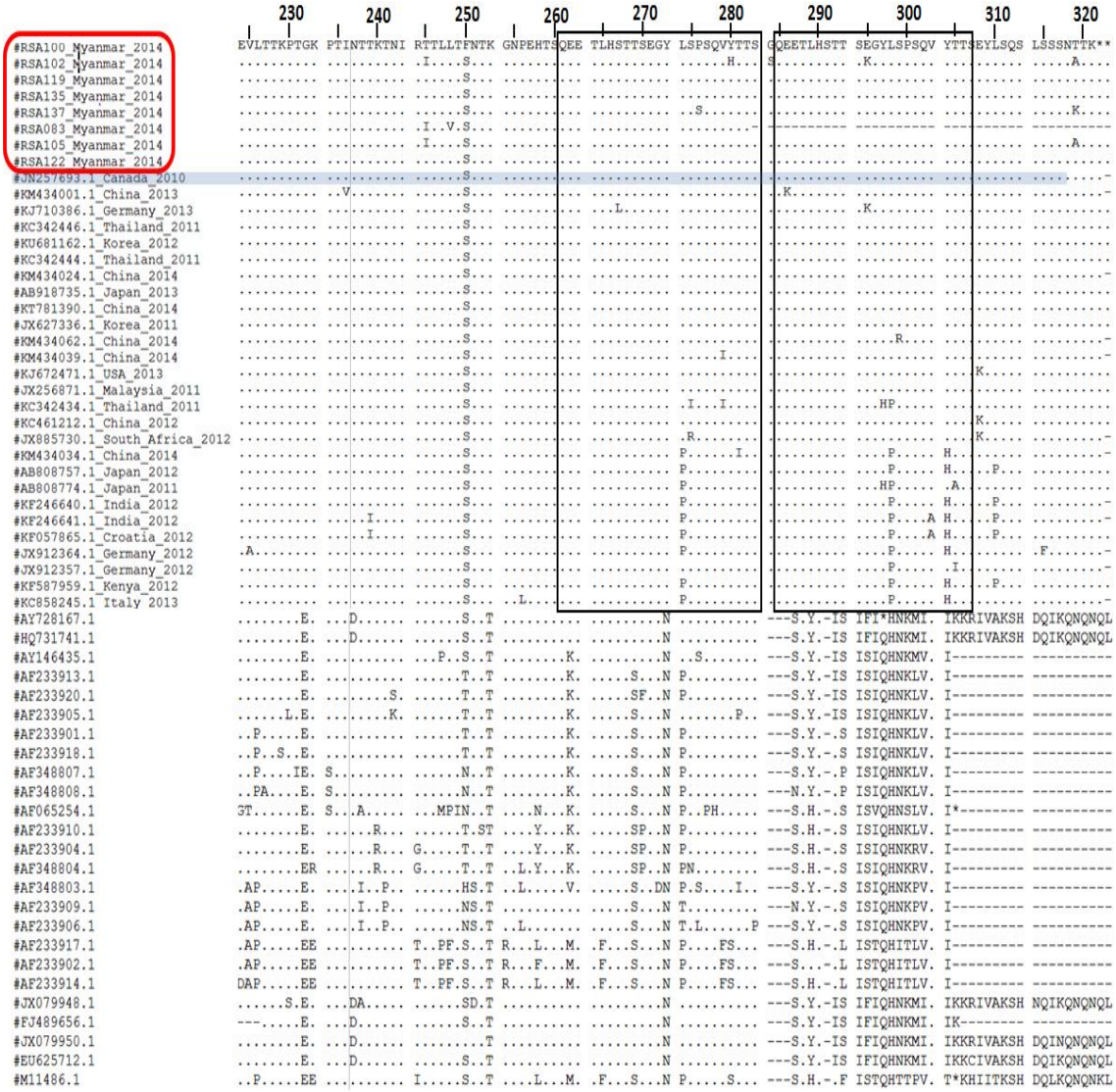

Fig. 2. Alignments are shown relative to the sequence of ON1 strain first described in Canada, JN257693. Alignment of sequences was performed using the ClustalW 1.6 method in MEGA 6.06 software. The amino acid positions correspond to positions 224 to 323 of the G protein of the prototype strain A2. Identical residues are indicated by dots, asterisks indicate the position of stop codons. Boxes frame the 23 amino acid duplicated region of the 24 amino acid insertion. Myanmar RSVA-ON1 strains were highlighted in red box.

DISCUSSION

RSV is the major pathogen of lower respiratory tract infections in infants and young children worldwide.[24] The prevalence of RSV among under five children of ARI cases in YCH, confirmed by IFA, in 2012 is 39%.[25] In the study done during January to September 2014, RSV infection was detected in 16.25% (26/160) of under five children with ARI at YCH. It was found that 52% (11/21) of RSV infection belonged to Group RSV-A and 48% (10/21) to Group RSV-B as done with NS1 gene.[22] In the present study, after sequencing of G gene of 21 RSV-positive samples, RSV-A was detected in 53.8% (7/13), RSV-B in 38.5% (5/13) and one case (7.7%) was mixed infection.

After nucleotide alignment and phylogenetic analysis, all Myanmar strains (61.5%, 8/13) of the group A clustered under the ON1 genotype which is characterized by a 72 nucleotide duplicated genetic region. Similar finding has also been reported in some countries in Asia such as China (KM434024), Korea (KU724061), Japan (AB918735), Thailand, (KC342444) and India (KF246641), in Europe such as Germany (JX912364), Italy (KC858245) and Spain (KF915266).

Most of the RSV-infected patients were younger than 6 months. This finding is similar to China report.[26] The diagnosis in the majority patients was bronchiolitis followed by pneumonia.[24] One study reported that ON 1 genotype was associated with less severe cases such as bronchiolitis. However, some countries, but not all, reported that the ON1 may be associated with greater clinical severity of ARI infection. It is unable to report any such clinical association in Myanmar because of the limited number and time period of the study done so far. More studies are needed.

Detection of ON1 strains in the present study is a first time report in Myanmar. Worldwide, the new ON1 strains has replaced all other strains in RSV-A group and is significant because this may indicate a greater resistance to host immune mechanism as reported from Germany, etc.[24] Although it cannot be definitely said that there is replacement of ON1 strains in RSV-A groups in Myanmar because DNA sequencing of RSV has never been done, the finding that ON1 strains are the prevalent strains in Myanmar is in accordance with the current prevalent strains globally.

The finding of G284S substitution in ON1 genotype of RSV-A102 in Myanmar is unique, it has not been reported from any other country so far. However, Heidelberg strain from Germany is also shown to have a unique substitution at E287K, which has not been reported elsewhere. These unique substitutions are of scientific interest and may be scientifically significant. Regarding RSV-B strains, one strain of RSV-B in Myanmar has already changed to RSV-B/BA9 strain. In other countries, this has led to replacement of all RSV-B strains by RSV-B/BA9 and it need to be confirmed whether this will also happen in Myanmar.

The important finding in the present study is that a new novel strain of RSV-A/ON1 has been detected in RSV isolated from ARI cases in under 5 children at YCH and probable replacement of all RSV-A by ON1, and the existence of the second hypervariable regions in the G protein genes of ON1 suggests the likelihood that the RSV strains in Myanmar are likely to evolve and change rapidly from time to time. It may be difficult to develop a vaccine against these rapidly varying strains. The detection of unique amino acid replacement in one strain of RSV-A/ON1 is of scientific interest and it may need further in-depth study.

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