Genotyping of Respiratory Syncytial Virus among influenza-like illness and severe acute respiratory infection cases of children in the Philippines from 2006-2016

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Abstract

Respiratory syncytial virus (RSV) is the leading cause of severe lower respiratory infection and therefore, a major threat to global health. In the Philippines, RSV is the second most common respiratory viral pathogen next to rhinovirus among children with severe pneumonia. Since 2006, national influenza-like illness (ILI) and severe acute respiratory infection (SARI) surveillances have been mainly focused only on influenza viruses. The prevalence and genetic diversity of RSV in the last decades were not completely elucidated. This study determined the epidemiological and molecular characteristics of RSV among (ILI) and (SARI) cases of children in the Philippines. The Philippine National Influenza Centre (PNIC) collected oropharyngeal swab and nasopharyngeal swab samples from patients under the age of five who are presented with ILI and SARI for the period of 2006-2016. These swabs have been examined for RSV subgroup by multiplex real-time qRT-PCR. Sequencing and phylogenetic analyses were used to determine the genotype of RSV samples. A total of 1,036 samples were systematically selected and tested. Of these samples, 122 were RSV-positive at 11.8 % prevalence rate, and 58.2% (71/122) were classified as RSV-A. Six genotypes were identified, which included NA1 (27/122, 22.1%), ON1 (5/122, 4.1%), GA2 (1/122, 0.8%) and GA5 (1/123, 0.8%) for RSV-A; and BA2 (13/122, 10.7%) and BA9 (1/122, 0.8%) for RSV-B. Most RSV-related cases were significantly associated with pneumonia and bronchitis. The pattern of RSV activity in the Philippines resembles the transmission of RSV globally.

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Abstract

Background: Respiratory syncytial virus (RSV) is the leading cause of severe lower respiratory infection and therefore, a major threat to global health. In the Philippines, RSV is the second most common respiratory viral pathogen next to rhinovirus (HRV) among children with severe pneumonia. Since 2006, national ILI and SARI surveillances have been mainly focused only on influenza viruses. The prevalence and genetic diversity of RSV in the last decades were not completely elucidated.

Objectives: This study determined the epidemiological and molecular characteristics of RSV among influenza-like illness (ILI) and severe acute respiratory infection (SARI) cases of children in the Philippines.

Study Design: The Philippine National Influenza Centre (PNIC) collected oropharyngeal swab and nasopharyngeal swab samples from patients under the age of five who are presented with ILI and SARI for the period of 2006-2016. These swabs have been examined for RSV subgroup by multiplex real-time qRT-PCR. Sequencing and phylogenetic analyses were used to determine the genotype of RSV samples.

Results: A total of 1,036 samples were systematically selected and tested. Of these samples, 122 were RSV-positive at 11.8 % prevalence rate, and 58.2% (71/122) were classified as RSV-A. Six genotypes were identified, which includes NA1 (27/122, 22.1%), ON1 (5/122, 4.1%), GA2 (1/122, 0.8%) and GA5 (1/123, 0.8%) for RSV-A; and BA2 (13/122, 10.7%) and BA9 (1/122, 0.8%) for RSV-B. Most RSV-related cases were significantly associated with pneumonia and bronchitis.

Conclusion: The pattern of RSV activity in the Philippines resembles the transmission of RSV globally. Data from this study can be utilized to improve diagnosis and patient management of respiratory infections in the Philippines.

Keywords: RSV-A, RSV-B, genotype displacement, influenza-like illness, severe acute respiratory infection

Background

Respiratory syncytial virus (RSV) is the leading cause of severe lower respiratory infections with nearly all children experiencing at least one infection by the age of two [1]. It is a major health threat which causes about 2350/100,000 hospitalization among children less than one year of age in the United States [2].

Clinical symptoms of RSV infection among children include prominent wheezing, severe bronchiolitis, cough, and shortness of breath [3]. It has been known that prematurely born infants, children with congenital heart defects or bronchopulmonary dysplasia, elderly and immunosuppressed patients have the highest risk of developing severe RSV infections [4]. Meanwhile, a study showed that 73% of hospitalized patients are children with no underlying medical conditions [5].

The classification of RSV varies antigenically and genetically. Based on the reaction of the monoclonal antibody to the surface antigen, RSV can be divided into two major subgroups: RSV-A and RSV-B [6]. Globally, molecular analysis showed that RSV-A has 11 known genotypes (GA1-GA7, SAA1, CB-A, NA1-4 and ON1), whereas RSV-B has 24 known genotypes (GB1-GB4, SAB1-SAB4, URU1-2, CBB, CB1, GB5 and BA1-12) [7].

In the Philippines, RSV is the most common respiratory viral pathogen next to rhinovirus (HRV) among children with severe pneumonia [8]. However, few reports have been published that described the genetic variability of RSV among children. This study examined the epidemiological features of children with RSV infection and characterized the RSV samples by molecular techniques to identify the prevalence and genotypes that circulated in the Philippines from 2006 to 2016.

In this study, we investigated the epidemiological features of children with RSV infections and characterized the RSV strains by molecular techniques to identify the prevalence and genotypes that circulated in the Philippines.

Study Design

Ethical consideration

The study was approved by the Institutional Review Board (IRB) of the Research Institute for Tropical Medicine (RITM) in Muntinlupa City, Philippines. Individual consent forms were waived since archived samples were used in this study. Permission was sought from the Philippine National Influenza Centre (PNIC) to access the samples and data respectively. The samples were devoid of any identifiers that may lead to the patient.

Study samples

The study include archived nasopharyngeal swab (NPS) and oropharyngeal swab (OPS) samples of the RITM-PNIC in Muntinlupa City, Philippines. Samples were selected systematically with the number of samples from each site proportionate to the number of samples collected from children age (<5y) with influenza-like illness (ILI) or severe acute respiratory infection (SARI) between January 2006 to December 2016 per year per site. The sentinel sites were composed of different 17 hospitals and 34 health centers nationwide that were chosen based on their geographic locations and their capacity to qualify based on the criteria set by the DOH. Samples were placed in a viral transport medium (VTM) or universal transport medium (UTM) and stored at the institutional biobank (-80°C). According to the World Health Organization (WHO), ILI cases were defined as patients with acute respiratory illness with cough, runny nose, and/or sore throat with history of fever (38°C or above) with or without other system manifestations within the past five days. Meanwhile, SARI cases were defined by the Philippine Department of Health (DOH) as patients with an acute respiratory illness that fits the ILI case definition and requires hospitalization. Specifically, the integrated management of childhood illness (IMCI) guidelines also included in the SARI surveillance any child between two months to five years of age suspect case for pneumonia with cough or difficulty of breathing. For children with severe pneumonia, any child between two months and five years of age with danger signs including: inability to drink or breastfeed, vomits everything, convulsion, lethargic or unconscious, and with chest indrawing or stridor in calm child .

Nucleic acid extraction

Viral RNA was extracted from 200 uL of each specimen using Roche High Pure Viral RNA (Roche Applied Sciences, Manheim, Germany) kit based on manufacturer's instructions.. RNA were eluted in 50 μL of elution buffer and were stored at -20oC prior to RSV screening [9]. cDNA was synthesized using primed viral RNA, 5x first strand buffer, 10mM DTT, one unit of RNAseOUT and five units of M-MLV RT (Moloney Murine Leukemia Virus Reverse Transcriptase) (Invitrogen, Carlsbad, CA, USA) for genotype identification.

Virus detection

For RSV screening, real-time RT-PCR was performed with subgroup-specific primers and probes used to amplify the partial nucleoprotein or N gene [10]. For genotype identification of all the RSV positive specimens, the 2nd Hypervariable Region (HVR) of the glycoprotein (G) gene was amplified by heminested PCR [11].

Sequencing and phylogenetic analysis

All amplicons were purified using QIAquick Purification Kit (Qiagen, Germany) according to manufacturer's instructions. Only PCR products with a concentration of [?]20 ng/μl were sent to 1st BASE Laboratories in Singapore for Sanger dideoxy sequencing. Resulting sequences from forward and reverse primers were analyzed to generate consensus sequences using MEGA 6 [12]. Consensus sequences were aligned using MAFFT [13] utilizing default parameters. Resulting alignment was manually inspected and confirmed using Aliview [14]. Appropriate model of nucleotide substitution for the dataset was chosen using jModelTest (Posada, 2008). Phylogenetic analysis was performed using maximum likelihood method with 1000 bootstrap replicates carried out in IQ-TREE (Nguyen, Schmidt, von Haeseler, & Minh, 2015) and resulting phylogenetic tree was visualized and edited using FigTree v.1.4.0 (Rambaut, 2012) and Interactive Tree of Live v.5.

Statistical analysis

Data were extracted from the PNIC database. Data was processed and analyzed using STATA 15 (STATA Corporation, College Station, TX, USA). Qualitative data, such as sex, were presented as counts and pro-

portions while quantitative data, like age, were presented as means or medians. T-test or Mann-Whitney U test were used to detect differences in quantitative variables from two groups while Chi-square test or Fisher's exact test were used to assess association in qualitative variables. The results with p value of <0.05 were considered significant.

Results

Patient characteristics

Between January 2006 to December 2016, a total of 30,527 oropharyngeal swabs (OPS) and nasopharyngeal swab (NPS) samples were collected from children below five years old at the SARI or ILI surveillance sentinel sites of the DOH of the Philippines. Overall, 1,036 samples were systematically chosen and of these 98.9% (1043/1055) were ILI cases and 1.1% (12/1,055) were SARI cases. RSV infection was detected in 11.8% (122/1,036) of the sample population by real-time RT-PCR.

Demographic characteristics of RSV (122) and non-RSV related (914) cases are compared in Table 1. Among male and female RSV and non-RSV related cases, no significant differences were observed. RSV related cases were and were observed during the first two years of life (<24 months).

Clinical characteristics of RSV infection

Aside from cough and fever, runny nose (88.1% RSV vs 11.9% non-RSV;*p-value: 0.021*) was significantly more common among RSV-positive cases. Moreover, most RSV-positive cases were significantly associated with pneumonia (80.6% RSV vs 19.4% non-RSV; *p- value: 0.015*) and bronchitis (71.7% RSV vs 28.3% non-RSV; *p-value: <0.001*).

RSV distribution

Annual positivity rates of RSV infection varied across the study period. Positivity rates ranged from 1.2% (2006) to 22.9% (2010) with an overall prevalence rate of 11.8% (122/1036). The highest positivity rates were observed in 2010 and 2011, respectively. RSV infection was observed all throughout the year, however increased positivity rate among specimens collected during the second half of the year (June to December). The study showed that 58.2% (71/122) of the RSV positive specimens belonged to the subgroup RSV-A while 40.1% (49/122) to RSV-B. Co-infection between both RSV subgroups were observed in 1.6% (2/122) of the samples. Co-circulation between both subgroups were observed for the period of 2007-2015, with higher number of RSV-A related cases (50.0-100.0%).

Phylogenetic analysis of RSV

Among the confirmed RSV samples, only 48 were amplified during the heminested PCR targeting the 2nd HVR of the G protein. The remaining 74 samples (37 RSV-A, 35 RSV-B and 2 co-infection) that failed to amplify were not included for sequencing and phylogenetic analysis, and were classified as untypable. Failure in the amplification of viral genomic fragments during the conventional PCR may be due to the variation in primer binding sites.

Phylogenetic trees were constructed for 35 RSV-A and 14 RSV-B strains by including 45 (23 RSV-A and 20 RSV-B) Philippine strains from previous studies [11] and 97 (46 RSV-A and 51 RSV-B) from other countries by BLASTn search. Philippine RSV-A strains in this study were distributed into four clusters NA1, ON1, GA2 and GA5. Philippine NA1 and ON1 strains clustered with previously reported circulating strains in the Philippines and other countries. Philippine GA2 strains clustered with strains from Brazil and HongKong whereas,, the Philippine GA5 strain clustered with strains from Malaysia and USA. Majority of the Philippine RSV-A strains were identified as NA1 genotype (27/122, 22.1%). Respectively, the following RSV-A genotypes were identified: ON1 (5/122, 4.1%), GA2 (2/122, 1.6%) and GA5 (1/122, 0.8%).

All Philippine RSV-B strains in this study clustered under the BA strains with 60-nucleotide duplication. Most of the Philippine BA strains were identified as BA9 genotype (13/122, 10.6%) except for one (1/122,

0.8%) that was identified as BA2. Philippine BA2 strains clustered under strains from Japan, Thailand and Malaysia whereas Philippine BA9 strains under strains from Malaysia and Colombia.

RSV genotype displacement

Genotype displacement among the circulating RSV-A and RSV-B genotypes was observed within the 10-year study period. NA1 genotype was detected as early as February 2007 in the Metro Manila, Philippines and continued to circulate until 2015. BA9 genotype was detected in the in the province of Capiz located in the western region of the Visayas islands in the Philippines in 2009 until 2013. This study was able to detect ON1 genotype in the province of Pangasinan, which is in the western area of Luzon as early as June 2010. Simultaneous detection of genotypes BA2 in the province of Zamboanga del Sur in Mindanao and BA9 (Cagayan, Philippines) were reported within the same year.

Discussion

This 10-year retrospective study is the first to provide long-term RSV data in the Philippines. We reported an overall prevalence rate of 11.8% among RSV-related ILI and SARI cases of children (<5y) in the Philippines. This is in agreement with rates reported from other countries including Thailand of 8.4% [15] South Korea of 13.3% [16] and France of 14.0% [17]. Meanwhile, higher prevalence rates which ranged from 19.3 to 40.6% [11, 18-20] were reported from previous studies in the Philippines, but it should be noted that these studies were done with hospitalized cases wherein a higher proportion is expected. These studies were among hospitalized patients, thus proportion of RSV cases were higher as expected. In other instances, active surveillances tend to report higher prevalence rates compared to retrospective studies [21]. Thus, the retrospective aspect of this study could have contributed to the lower reported prevalence rate. Variability among the prevalence rates around the world could be attributed to the difference in case definition, study design, sample size, location and study period.

Genetic variability of RSV viruses in general is due to the diversity of the ectodomain protein. Shifts in the dominance of RSV group can occur at different time intervals and different patterns of subgroup. RSV-A viruses were more frequently detected in epidemic seasons for the period of 1999-2007 in several countries including Germany, Belgium, Argentina and India [22]. On the other hand, the present study reported co-circulation between both RSV subgroups was notable. Increased RSV-A related cases was observed all throughout the study period, except for 2010. Similar occurrence was observed in previous studies in the Philippines (Malasao et al., 2015; Ohno et al., 2013) and other Asian countries (Auksornkitti et al., 2014; Khor, Sam, Hooi, & Chan, 2013; Yoshihara et al., 2016).

Globally, different time intervals for the replacement of RSV genotypes have been observed. A previous study reported genotype replacement every year [23]. Another study in Kenya showed that RSV-A genotype GA2 replaced GA5 in a span of seven years. On the other hand, the newly emerged RSV-A genotype ON1 only took four years to replace NA1. Notably, large epidemics were also attributed to newly emerged genotypes. In Japan, the lack of immunity against the newly emerged RSV-A genotypes (NA1 and NA2) for the periods of 2005-2007 and (ON1) 2014-2015 led to record high reported RSV cases [24, 25].

In this study, we reported the circulation of NA1, GA5 and ON1 in the Philippines between 2006 and 2016. The first RSV-A genotype to be identified was GA2 in 1998, published reports described the genotype to be more geographically widespread and epidemically active [26, 27]. In addition, outbreaks between 1996 and 2006 in Brazil were attributed to both subtypes. Previous studies have reported the global predominance of GA2 which started in 2000 [20, 27-30]. Different variants of GA2 such as NA1-NA2 [24] and NA3-NA4 (Cui et al., 2013) were identified. The present study report the detection of NA1 in the Philippines in 2007 and continued circulation up to the 2015. This indicates that NA1 circulated in some areas in the Philippines as early as 2007 which is a year earlier than the previously reported genotype detection in 2008 [11]. On the other hand, the finding is also consistent with published data from Cambodia [31], South Korea [32] and China [33] which indicated that NA1 predominantly circulated in Asia as early as 2006.

The genotype NA1 continued to be the predominant RSV-A genotype worldwide, until it was subsequently

replaced by ON1 between 2009 and 2010. It has been calculated that ON1 branched out from NA1 between 2005 and 2009 [28, 29, 34]. This study reported circulation of ON1 in Pangasinan, Philippines as early as 2010, which is a year earlier than the previous report [19]. The differences between the time of emergence may be due to the variability between the location and period of the studies. Despite the lack of ON1 related cases in 2015, ON1 continued to circulate in 2016. This may be attributed to the limited number of samples tested for this period. It must be noted that similar genotype displacement events between the prevailing RSV-A genotype NA1 and ON1 have been observed and reported globally in a period of three years [35-38].

Unlike previously mentioned RSV-A genotypes (GA2, NA1 and ON1), circulating patterns of non-predominant genotypes such as GA1, GA4 and GA5 may differ temporally or geographically. In European countries including Germany and Sweden, GA5 was the predominant genotype circulating from 1999 to 2007. The prolonged circulation of GA5 in these countries could be attributed to positive selection pressure or modulation of glycosylation sites among genotypes [39]. On the contrary, other studies have described GA5 to be an endemic genotype and have limited epidemic activities [26, 27]. Occasional detection of GA5 were reported from Netherlands [40], Spain [26] Vietnam [41] and South Africa [42]. A long term study in Malaysia reported the co-circulation of GA5 with NA1, NA2 and GA2 from 2003 until 2010 [43]. In this study, a sporadic case of GA5 strain was detected in Cagayan, Philippines in 2011. The sequence of Philippine GA5 strain is highly similar (x%) with GA5 strains from neighboring Asian countries including Japan, China and Malaysia. One explanation for the high similarity GA5 strains could be due to the availability of transportation in between these Asian countries as well as influx of tourists from these countries. Interestingly, it was observed that the predominance of GA2 in 2005 inhibited the endemic genotypes (reference or data mo ba ito). It was speculated that antibodies against GA2 have broad reactivity against GA3, GA5 and GA7 [26]. Thus, possible immune cross-reaction among antibodies produced during the GA2 infection could have prevented infection of other rare genotypes [27].

A nucleotide insertion similar to ON1 was also identified in the BA genotype. The genotype BA was first identified in Buenos Aires, Argentina in 1999 and different genotypes (BA1-BA10) have diverged over a period of 20 years [37, 44-46]. This study only detected RSV-B genotypes BA2 and BA9 within the study period. Although few RSV-B sequences were included, it was apparent that most RSV-B genotype were identified as BA9. This study reported the circulation of BA9 in the Philippines in 2009. This finding is concordant with previous reports which described the emergence and predominance of BA9 during epidemics among children in the Philippines from 2009 to 2011[19, 37]. The global transmission of BA9 could be attributed to travelers or selective fitness advantage due to the 72-nucleotide duplication [47]. Taken together with other data from different countries worldwide, BA9 continued circulate globally.

Gender was not identified as a factor of among RSV-related cases in this study. Meanwhile, the study showed majority of RSV-related cases were identified among infants below two years of age. This is consistent with reports from Mexico [48] and Kenya [49]. However, some studies reported increased detection of RSV among infants below one year of age [49]. Factors such as differing airway anatomy, immaturity of the immune system and waning levels of maternal antibodies may have affected the vulnerability of younger children to severe infections [50]. Nucleolin, which is a nucleolar protein, has been reported as a receptor for RSV that binds with the F protein (Tayyari et al., 2011). Interestingly, increased quantities of nucleolin can be found in surfaces of actively dividing cells such as the alveoli which continues to grow until about two years of age. This may potentially play a role in the preferential infection of the lower respiratory tract in young children.

Although the prevalence and variability of the circulating RSV genotypes in the Philippines were reported in this study, several limitations have been identified. Primarily, due to the retrospective nature of the study which used archived ILI and SARI surveillance samples from different PNIC sentinel sites and some of the provinces were not consistently represented in the 10-year surveillance scope. Also the use of ILI and SARI case definitions may have underestimated the detection of RSV cases in this study. ILI and SARI case definition required suspect cases to present fever prior to inclusion in the influenza surveillance. Contrary to the WHO Global RSV Surveillance case definition which defined RSV cases with at least one of the following: cough, shortness of breath, sore throat, and coryza. Suspect RSV cases did not require fever

presentation to be admitted in the RSV surveillance. It should be noted that there is a considerable fraction (>50%) of RSV-infected young children and elderly patients present without fever in the RSV surveillance. Another limitation is the low RNA concentration of the some samples. Several factors may affect the quality of viral RNA including: specimen collection procedure, timing of collection and specimen transportation conditions. Since this study included archived specimens in the biobank, frequent freeze thawing cycle could have contributed to the low viral RNA concentration. The distribution of RSV genotypes could have been underestimated due to the large number of RSV samples that were not amplified during heminested PCR and classified as untypable.

In conclusion, this study showed that RSV is more prevalent among younger children (<2y) in the Philippines. The study identified co-circulation of both RSV-A (GA2, GA5, NA1 and ON1) and RSV-B (BA2 and BA9) subgroups. Runny nose, bronchitis and pneumonia are clinical manifestations related to RSV infections among Filipino children. This work is the first study to report on a nationwide scale, the prevalence of RSV and genotype displacement over the period of 2006-2016.

Footnote Page

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