Incidence of viral infections and associated factors among children attending kindergarten in Taiwan

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Short title: Viruses among kindergarten attendees
Abstract

Background

Kindergarteners frequently encounter various infectious diseases, so we monitored viral infectious diseases and analyzed the incidences of various viral infections among kindergarten attendees to provide information for their health promotion.

Methods

We enrolled two to five year-old kindergarten attendees during the academic years of 2006 (Sep. 2006 to Jun. 2007) and 2007 (Sep. 2007 to Jun. 2008) in a Taipei City kindergarten. Daily monitoring of illness and regular biweekly physical examinations were undertaken. If kindergarten attendees had fever, respiratory symptoms, oral ulcer, skin rash or abnormal physical examinations, viral isolation and specific viral polymerase chain reaction followed by molecular typing were performed. Multiple infections were defined as one child having two or more laboratory-confirmed viral infections with different viruses or different serotypes during one academic year.

Results

The overall laboratory-confirmed incidence rate of viral infection was 239 per 100 person year in the 2006 academic year and 136 per 100 person year in the 2007
academic year. The attack rate for seasonal influenza (influenza B) was 17% (31/180) in the 2006 academic year and that for seasonal influenza (influenza A) was 27% (52/190) in the 2007 academic year. The enterovirus infection rate was 117 per 100 person year in the 2006 academic year but dropped to 18 per 100 person year in the 2007 academic year (P<0.001). The adenovirus infection rate was 103 per 100 person year in the 2006 academic year and 89 per 100 person year in the 2007 academic year (P=0.59). Boys and children with allergy had significantly higher risks to get multiple viral infections [OR 1.81 (95% CI 1.20-2.75), OR 1.56 (1.00-2.39), respectively]. Boys also tended to get enterovirus infections (OR 1.56, 95% CI 1.02-2.38) while children with allergy tended to acquire adenovirus infections (OR 1.71, 95% CI 1.12-2.66).

Conclusion

High incidence of adenovirus, enterovirus and seasonal influenza was found among the kindergarteners. Boys and children with allergy were more susceptible to multiple viral infections.

Key words: preschool children, kindergarten attendees, enterovirus, adenovirus, influenza, attack rate
Introduction

According to World Health Organization report, infectious diseases are one of the most important causes of death in children under 5 years [1], and most of them are preventable or treatable. Viral infection is extremely common among young children [2-5]. In the United States, approximately 25 million patients with upper respiratory tract infection are treated in the outpatient medical care setting annually [2]. In a survey among Canadian toddlers, during fall and winter, the average proportion of time with colds, diarrhea, or vomiting was 23.4% [3]. In a study by questioning about upper respiratory tract infection during the preceding 2 weeks, the reported incidence of upper respiratory tract infection was 24% for children less than 5 years of age and attending day care increased the risk [4]. A questionnaire-based cross-sectional study showed that 47.7% of the 3853 four-year-old preschool children contracted more than 2 common colds during the last 12 months [5]. Day care attendance and the presence of siblings significantly increased the risk of upper respiratory tract infections [5].

Since the birth rate is decreasing in developed countries throughout the world, it is important to prevent childhood diseases and promote the health of children. Taiwan also has a decreasing birth rate and there is usually a single child in the family. In Taiwan, the birth rate was 8.55 births per thousand population in 2013, one of the lowest birth rate in the world (215th among the 223 countries) [6]. In general, young
children tend to experience many more infections after they begin to attend kindergartens or day care centers than when they are taken care of at home, particularly when there is only one child in the family. For children less than five years of age, day-care attendance was associated with a significantly increased risk of both upper respiratory tract infection and acute ear infection [3]. We thus investigated and monitored viral infectious diseases among kindergarten attendees and hope that our results will help understand the incidence rates and risk factors associated with viral infections among children attending kindergartens. The results will provide information for the strategy on the prevention and management of viral infections of preschool children.
Methods and Materials

1. Study area and study population

In Taipei City, the population was about 2 million and 629 thousand people with 137,479 preschool children during our study period (September 2006 to June 2008).

There were 1184 kindergartens and 76 daycare centers. About 12% of children younger than 3 years of age were cared at daycare centers and 54% of 3 to 5 year-old preschool children attended kindergartens. The vaccine coverage rate of the preschool children was 98% for Bacillus Calmette–Guérin, hepatitis B, and Diphtheria, Tetanus,acellular Pertussis, Polio and Haemophilus influenzae type b (DTaP-IPV-Hib), 97% for measles, mumps and rubella, 95% for varicella and 63% for pneumococcal conjugate vaccine in this city.

We enrolled children from one public kindergarten in Taipei city. There were six classes: one class for 2 year-old children, two for 3 year-old children, two for 4 year-old children and one for 5 year-old children. Each class had 20 to 35 preschool children. Overall, there were 193 kindergarten attendees during the 2006 academic year (from September 2006 to June 2007) and 202 kindergarten attendees during the 2007 academic year (from September 2007 to June 2008).

After written informed consent was obtained from parents or guardians, the parents or guardians completed self-administered questionnaires on behalf of their children in
the beginning of the academic year. The questionnaires sought information about the
child’s age, gender, past medical history and vaccination history. A history of allergic
conditions, such as atopic dermatitis, asthma, allergic rhinitis, and allergic
conjunctivitis was also solicited. Study nurses would check the questionnaire later,
verified the contents and did telephone-interview to complete it if the data were not
all-inconclusive.

2. Ethical consideration

The institutional review board of National Taiwan University Hospital approved
this study and the education bureau of Taipei City agreed to this study. When the
academic year began, pediatricians and study nurses would meet with guardians or
parents of the kindergarten attendees and would explain the purpose, the methods, the
potential benefit of this study and the discomfort of sampling. Guardians or parents of
the kindergarten attendees gave their written informed consent after the meeting. We
gave the results of the viral workup to the guardians or parents of the kindergarten
attendees but did not provide any treatment for children found infected by viruses.

3. Data collection

In this public kindergarten, a full-time nurse was responsible for measurement of
participating children’s daily body temperature by infrared tympanic thermometers, examination for signs of oral ulcer and/or viral exanthema and aseptic care of their trauma. If any child had fever, respiratory symptoms, enterovirus-like illness such as herpangina or hand, foot, and mouth disease or viral exanthema, study nurses from National Taiwan University Hospital would take throat swabs from the ill children for viral isolation and specific polymerase chain reaction (PCR). Fever was defined as ear temperature over 38 °C.

Pediatricians from National Taiwan University Hospital did physical examinations for every participating child once every 2 weeks. If there were abnormal physical signs such as fever, injected throat, exudate of the tonsil, congested eardrum, oral ulcer, skin rash, or abnormal breathing sounds, the abnormal physical signs would be recorded and appropriate swabs would be taken for microbiological or viral tests including viral isolation and real time PCR for specific viruses such as enteroviruses, adenovirus, influenza virus, et al. If group A streptococcal tonsillitis was suspected, bacterial culture of the throat swabs would be performed. Because the winter vacation was in February and the summer vacation in July and August, we did not monitor their viral infections in February, July and August.

Overall, there were 35 instances of regular clinical examination by pediatricians and 81 study nurse visits which were for taking samples from ill children during the
two years of the study. A total of 2335 swabs including 2299 throat swabs, 11 rectal
swabs, 11 conjunctival swabs, 2 nasopharyngeal swabs for viral isolation and PCR
and 12 throat swabs for group A streptococcal screen were sampled during the two
years of the study. All the samples with appropriate transport medium were
transported to the labs of National Taiwan University, and viral isolation, PCR and
group A streptococcal screen were performed on the same day of collection.

4. Laboratory methods

(1). Virus isolation and identification

Throat swabs, rectal swabs, conjunctival swabs or nasopharyngeal swabs were
submitted for virus isolation to the virological laboratory of National Taiwan
University Hospital. Samples were inoculated into human embryonic fibroblast,
LLC-MK2, HEp-2 and rhabdomyosarcoma cell cultures. When cytopathic effect
involved more than 50% of the cell monolayer, cells were scraped and subjected to
indirect fluorescent antibody staining with specific antibodies or typed by specific
methods according to the suspected types of viruses.

(2). Molecular diagnosis for viruses

Molecular diagnosis and typing were performed at the molecular viral laboratory of
National Taiwan University Hospital. Viral RNA and DNA extraction: RNA and DNA
extraction from throat swabs, rectal swabs, conjunctival swabs or nasopharyngeal swabs were performed by using Isolation Kit (RNA and DNA extraction kit, Qiagen, Hilden, Germany), and reverse transcription was performed with 1st strand cDNA Synthesis Kit for RT-PCR (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s guide.

PCR for pan-enterovirus, pan-adenovirus, influenza A and influenza B were performed with the primers, probes and conditions listed in Table 1. In addition to the specimens, negative and positive controls were also used to assess the validity of each test.

(3) Molecular typing of the circulating enterovirus and adenovirus during the surveillance

If the samples had positive enteroviral and adenoviral viral isolation and/or real time PCR, further molecular typing of enterovirus and adenovirus would be done. For enterovirus serotyping, semi-nested RT-PCR was performed with primers according to a previous report [7], and the PCR product was purified. Then, auto-sequencing with the forward primer was performed. The serotypes of the enteroviruses were inferred by comparison of the partial VP1 sequence to those in the public gene database containing VP1 sequences for the strains of the human enterovirus serotypes including 3 polioviruses, 23 coxsackie A viruses, 6 coxsackie B viruses, 28
echoviruses, and enteroviruses 68-71. The detection sensitivity of this enteroviral
serotyping was about 1,000 copies of RNA.

For adenoviral molecular typing, primary PCR primer sets for amplification of
HAdV was 5’-TACAACATYGCTACCAGGG-3’ and
5’-GAGAASGGTBRCGSAAGTA-3’, the nested PCR was performed with primers
of 5’-AACTTCAGCCYATGAG-3’ and 5’-GRTTGACCTCRAARGTC-3’. The
HAdV positive PCR products were purified and the purified PCR products were
sequenced. The detection sensitivity of this adenoviral serotyping was about 100
copies of DNA.

5. Definitions and Data Analysis

Specific viral infection was defined as the presence of symptoms plus the positive
viral isolation and/or positive viral real time PCR. Age-specific incidence rate per 100
person year was estimated using the number of infections in the numerator and the
number of age-specific enrolled kindergarten children in the denominator during each
academic year. Multiple infections were defined as one child having two or more
laboratory-confirmed viral infections with different viruses or different serotypes of
enteroviruses or adenoviruses during one academic year. The number of specific viral
infections each month was divided by the total number of participating children to
calculate the virus-specific monthly infection rate. The overall monthly infection rate was the sum of the enteroviral monthly infection rate, influenza monthly infection rate and adenoviral monthly infection rate. Period of circulation for a specific virus was defined as the interval between the first date and the last date the specific virus was identified by either viral isolation or identified by PCR. For the attack rates of seasonal influenza, one child was only included once during one academic year. Upper respiratory tract infections included tonsillitis, pharyngitis, acute sinusitis or otitis media.

The data were analyzed with statistical package SAS system. The difference of incidence rates between different groups or different periods was measured with $\chi^2$ test. Risk factors associated with enterovirus infection, adenovirus infection, influenza infection and multiple infections were performed with univariate analysis followed by multivariate analysis; univariate analysis was done with Mantel-Haenszel $\chi^2$ test to screen statistically significant variables; if the variables were significant with $p$ values of less than 0.05 in univariate analysis, the variables would be included in multivariate analysis, which used multiple logistic regression analysis to adjust confounders simultaneously and to calculate the multivariate-adjusted odds ratios for risk factors. $P$ values were considered statistically significant if it was less than 0.05.
Results

Demography

After written informed consent was obtained from the parents or guardians, 180 (93%) of the 193 children enrolled at the kindergarten participated in this study in the 2006 academic year (from September 2006 to June 2007) and 190 (94%) of the 202 children enrolled at the kindergarten participated in the 2007 academic year (from September 2007 to June 2008). Their mean (standard deviation, SD) age and gender (%) for all the enrolled kindergarteners and different age groups are shown in Table 2.

Circulating viruses

Figure 1 (upper half) shows the types of circulating viruses and the periods of circulation in the 2006 academic year: the major circulating viruses were enteroviruses, adenoviruses and influenza B. Seven serotypes of enteroviruses circulated including coxsackievirus A4 (CA4, the most common serotype), CA2, echovirus 6, echovirus 4, CB2, CA6, and CA9 (in decreasing order) in the 2006 academic year. As for adenoviruses, five serotypes were identified: adenovirus type 2 was the predominant type, followed by adenovirus type 1, adenovirus type 3, type 5 and type 4. The major circulating influenza virus during the 2006-2007 influenza season was influenza B. Figure 1 shows that sometimes more than 5 respiratory
viruses co-circulated in the kindergarten simultaneously.

Figure 1 (lower half) also shows the types of circulating viruses and the periods of circulation in the 2007 academic year. In the study year, six serotypes of enteroviruses circulated in different periods of the year and CA2 was the most common, followed by enterovirus 68, CB4, CA10, CA4 and CA6. Four serotypes (type 3, type 2, type 1 and type 5 in decreasing order) of adenoviruses circulated in this kindergarten and the major serotype was type 3, which circulated almost all the year round. The seasonal influenza was influenza A which circulated from Nov 2007 to Feb 2008.

In comparison with the circulating viruses in the 2006 academic year, the major type of enteroviruses changed from coxsackievirus A4 in the 2006 academic year to coxsackievirus A2 in the 2007 academic year. The major type of adenovirus changed from type 2 in the 2006 academic year to type 3 in the 2007 academic year though adenovirus type 2 was still the second common in the 2007 academic year. Seasonal influenza changed from influenza B in the 2006-2007 season to influenza A in the 2007-2008 season. No *Streptococcus pyogenes* was ever cultured from throats swabs in this study and no chickenpox was found during the study period, either.

**Incidence rates**

The incidence rate of infection per 100 person year is shown in Table 2. The overall
laboratory-confirmed viral infection was 239 per 100 person year in the 2006 academic year and 136 per 100 person year in the 2007 academic year. The attack rate of seasonal influenza (influenza B) was 17% (31/180) in the 2006 academic year and that of seasonal influenza (influenza A) was 27% (52/190) in the 2007 academic year (p=0.02). Among the 31 cases infected with influenza B during the 2006-7 season, four (13%) received influenza vaccine; among the 52 cases infected with influenza A during the 2007-8 season, 7 (13%) received influenza vaccine. The influenza vaccination rates of uninfected children were 16% (24/149) during the 2006-7 season and 11% (15/138) during the 2007-8 season, not significantly higher than those of infected children. The overall vaccination rate for season influenza was 14%.

The enterovirus infection rate was 117 per 100 person year in the 2006 academic year but dropped to 18 per 100 person year in the 2007 academic year (p<0.001). The adenovirus infection rate was 103 per 100 person year in the 2006 academic year and remained comparably high, 89 per 100 person year, in the 2007 academic year (p=0.59).

Monthly infection rates of specific viruses and seasonality

Figure 2 (upper half) shows the virus-specific monthly infection rates during the
2006 academic year. The enterovirus monthly infection rate peaked (39%) in October and November 2007 and up to 39% of all the participating children had been infected with enterovirus each month then. Adenovirus monthly infection rate peaked in January 2007 when the highest monthly infection rate of adenoviruses reached 26%.

The seasonal influenza peaked in December 2006. The overall monthly infection rate (sum of all enteroviral, influenza and adenoviral monthly infection rate) range from 6% to 56% and was lowest in the first month (September) of the academic year and highest in the third month (November).

Figure 2 (lower half) shows the monthly infection rates of specific viral infections during the 2007 academic year. The peak (10%) of monthly enteroviral infectious rate occurred in September 2007, significantly lower than the peak rate (39%) in the 2006 academic year (p<0.001). The monthly infection rate of adenovirus peaked in January 2007, the same month as in the 2006 academic year. The peak of seasonal influenza infection was in January 2007, too.

Overall, seasonal influenza had obvious seasonality, usually in the winter (December to January); enterovirus circulated more in the summer and autumn and much less in the winter but adenovirus seemed to circulate all the year round, though somewhat more in the winter.
Clinical impacts

The most common diagnosis associated with enterovirus infection was upper respiratory tract infections (84%, 213/553) (including tonsillitis, pharyngitis, acute sinusitis or otitis media) followed by herpangina (15%, 39/253), the most common diagnosis associated with adenovirus and influenza infections was also upper respiratory tract infections (87% for adenovirus infections, 81% for influenza A, 90% for influenza B) followed by lower respiratory tract infections (including bronchitis, bronchopneumonia or pneumonia).

Six cases with enterovirus infections were hospitalized due to bronchopneumonia (3) or herpangina (3), 11 cases with adenovirus infections were hospitalized due to bronchopneumonia (7) or acute gastroenteritis (4) and 6 cases with influenza infections were hospitalized due to bronchopneumonia (5) or acute otitis media (1).

Factors associated with multiple, enterovirus and adenovirus infections

Table 3 shows the risk factors associated with multiple infections. With multivariate analysis, we found that males and children with allergy had significantly higher risk of acquiring multiple infections than girls and children without allergy. Age and influenza vaccination did not affect the risks of multiple viral infections.

We also found that boys had significantly higher risk of acquiring enterovirus
infection than girls (OR 1.56, 95% CI 1.02-2.38, p=0.04), and children with allergy had significantly higher risk of acquiring adenovirus infection than children without allergy (OR 1.71, 95% CI 1.12-2.66, p=0.01). No significant risk or protective factors were found for influenza infection.
Discussion

This study shows that a variety of viruses circulated among children at a kindergarten and the incidence rates were high. For example, the attack rate for seasonal influenza was 17-27% and the attack rates for enteroviruses and adenoviruses were even higher. Almost all the children would acquire enterovirus or adenovirus infection at least once a year. Moreover, three to six viruses circulated simultaneously in the same kindergarten during a certain period. This may explain frequent viral infections among children after joining a kindergarten.

Few studies have investigated the longitudinal dynamics of circulating respiratory viruses in kindergartens and this study provides a unique and long term observation of circulating viruses among the children at the kindergarten. Most of the other studies involved the sporadic outbreaks for a single pathogen or virus such as varicella, norovirus or other and calculated the attack rate of a specific pathogen [8-11]. This study carried out real time monitoring of the viral activity and provides a picture of various viruses co-circulating and causing repeated viral infections of the children attending that kindergarten. This is the reason why many parents frequently complain that their kindergarten children often get infections and sometimes one infection after another.

The attack rate of seasonal influenza among the kindergarteners was between 15
and 30% in this study, which was pretty high. The attack rate of seasonal influenza in this kindergarten was not lower than that rate (27%) of overall household transmission of pandemic (H1N1) 2009 virus [12]. Among the cases infected with influenza A or B, only 13% received influenza vaccine. Taiwan CDC provides free influenza vaccine for preschool children but most of the children in this study did not receive it. Infants and young children have been found to be at increased risk for hospitalization during influenza seasons [13, 14]. In this study, 6 (7%) children out of the 83 influenza cases were hospitalized due to bronchopneumonia (5 cases) or acute otitis media (1 case). Therefore, influenza immunization is highly recommended for pre-school children since the attack rate is high and hospitalization is sometimes needed [15, 16].

The attack rates of enteroviruses and adenovirus were strikingly high and almost all the children in this study acquired enterovirus and adenovirus about once a year. The reasons of such high attack rates may be related to the unavailability of a vaccine for non-polio enteroviruses and adenovirus and co-circulation of several serotypes. Because there is no vaccine available for non-polio enteroviruses and adenovirus, Taiwan CDC and kindergarten teachers advocate hand hygiene and aerosol precautions. It is generally difficult for kindergarten children to follow the strict rules of hand hygiene and aerosol precaution, thus we still observed very high attack rates. Our previous studies reported that kindergarten attendance was associated with
increased risk of enterovirus 71 infection and that EV71 household transmission was high (up to about 80%) among children [17,18]. If the transmission rate of enteroviruses is so high, other means of prevention, such as vaccine may be developed for certain important serotypes, such as EV71, which can cause severe brainstem encephalitis. This study also found boys to be at significantly higher risk (OR 1.56) of getting enterovirus infection. Previous studies also reported that male children were more susceptible to enteroviruses with the male to female ratio of 1.2 to 1.8 [19-22]. Therefore, more precautions, such as isolation of the enterovirus cases and avoidance of contact with ill children, should be taken by boys during an enterovirus season or outbreak.

Although most of the viral infections in this study were uncomplicated upper respiratory infections in this study, some lower respiratory tract infections or complicated upper respiratory tract infections such as otitis media or sinusitis did occur. Among them, 23 cases needed hospitalization mainly due to bronchopneumonia. The etiology of bronchopneumonia following viral infections may be due to the virus per se or secondary to subsequent bacterial infections. A limitation of this study is that we could not define whether bronchopneumonia was caused by the virus per se or by secondary bacterial infections. No matter what, we
consider that the role of respiratory viruses was important in upper and lower
respiratory tract infections and related hospitalizations.

Some studies have reported an increase in asthma exacerbations and admissions in
September in the pediatric age group and that the asthma-like symptoms commenced
after joining a kindergarten and many children started to experience the symptoms
within one month of beginning kindergarten [23, 24]. This might be explained by the
increased exposure to respiratory viruses after start of the new school year or after
beginning kindergarten. Our study provides evidence of exposure to many respiratory
viruses among children attending a kindergarten, and a possible explanation of why
asthma-like symptoms might increase when children attend kindergarten for the first
time. We also found that children with a history of allergy had significantly higher
risks of getting multiple viral infections (OR 1.56, 95% CI 1.00-2.3), especially
adenovirus infection (OR 1.71, 95% CI 1.12-2.66). The study’s findings suggest that
allergy may increase susceptibility to multiple viral infections including adenovirus.

Few other studies have reported the relationship between allergy and viral
susceptibility except one study which demonstrated asthma increased susceptibility to
influenza in mice model [25]. More studies are needed to further explore the
susceptibility to viral infection in children with allergy.
The early learning settings/child care in Taiwan somewhat differs from settings elsewhere in the world. Kindergartens can enroll 2 to 5 year-old children and the class size varies from 10 to 35 children. Younger age and bigger size of the class may partly explain the reasons why quite high attack rates of various viral infections occurred. In addition, illness exclusion policies in school/child care are only for enteroviral and chickenpox infection in Taiwan. For example, kindergarten children have to be cared for at home for at least one week if they get chickenpox, herpangina or hand, foot, and mouth disease. Otherwise, there is no clear illness exclusion policy for the other infections. In addition, sick children who did attend the kindergarten were not isolated from well children and not cared for separately in this kindergarten. Many other respiratory viruses such as adenoviruses, influenza viruses transmit via aerosol and spread easily among the kindergarten children. This also helps to explain high attack rates in this setting.

There are some other limitations in this study. First, we just monitored several viruses and many of viruses such as rhinovirus, coronavirus and herpes group viruses were not included. Second, the sample size in each subgroup was relatively small and that made the relatively wide confidence intervals in Table 3.

In conclusion, this study reveals that several respiratory viruses co-circulated in the kindergarten. The annual incidence rates of the various viruses were high, 15 to 30%
for seasonal influenza and even higher for enteroviruses and adenoviruses. Boys and
children with allergy had higher risks of acquiring viral infections than girls and
children without allergy, so more precautions may need to be taken for them.
Competing Interests

All the authors declared no potential conflict of interest.

Authors’ Contributions

LYC participated in the design and coordination of the whole study, the performance of this study, carried out the statistical analysis of the data and drafted the manuscript.

LMH participated in the design of the whole study, carried out the interpretation of the statistical data analysis and revised the draft of the manuscript. CYL, TYF, HC and ALC participated in the case enrolment, collection of the data and revised the draft of the manuscript. All authors read and approved the final manuscript.

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Figure legends

Figure 1. Circulating viruses and the periods of circulation during the 2006 academic year (upper half) and during the 2007 academic year (lower half). Adv1, Adv2, Adv3, Adv4 and Adv5 denote adenovirus serotype 1, 2, 3, 4, and 5; CA denotes coxsackievirus A, CB coxsackievirus B, ECHO echovirus, EV enterovirus and Flu influenza.

Figure 2. Monthly infection rates of specific viral infections including enteroviruses, adenoviruses and influenza B during the 2006 academic year (upper half) and during the 2007 academic year (lower half).
Table 1. Primers, probes and conditions for pan-enterovirus, pan-adenovirus, influenza A and influenza B real time PCR

<table>
<thead>
<tr>
<th>Primer or probe</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>Pan-enterovirus</td>
<td></td>
</tr>
<tr>
<td>Forward Primer</td>
<td>5’-TCCTCCGCCCCTGAATG-3’</td>
</tr>
<tr>
<td>Reverse Primer</td>
<td>5’-AATTGTCCACATAAGCAGCCA-3’</td>
</tr>
<tr>
<td>PanEV Probe (TaqMan)</td>
<td>6FAM-AACCGACTACTTTGGGTGTCCGCTTTTCXT-PH</td>
</tr>
<tr>
<td>Condition</td>
<td>Denature 95 °C 10 min</td>
</tr>
<tr>
<td></td>
<td>Amplification 95 °C 10s, 62 °C 10s, 72 °C 5s, 55 cycles</td>
</tr>
<tr>
<td></td>
<td>Cooling 40 °C 30s</td>
</tr>
<tr>
<td>Pan-adenovirus</td>
<td></td>
</tr>
<tr>
<td>Forward Primer</td>
<td>5’-GCCGCAGTGTCTTTACATGCACATC-3’</td>
</tr>
<tr>
<td>Reverse Primer</td>
<td>5’-GCACAGTGGGATTCACTAAACTT-3</td>
</tr>
<tr>
<td>Pan-adenovirus Probe (TaqMan)</td>
<td>6FAM-TGCACCAGACCCGGCTAGGTACTCCGA-TMR</td>
</tr>
<tr>
<td>Condition</td>
<td>Denature 95 °C 10 min</td>
</tr>
<tr>
<td></td>
<td>Amplification 95 °C 10s, 55 °C 10s, 65 °C 40s, 45 cycles</td>
</tr>
<tr>
<td></td>
<td>Cooling 40 °C 30s</td>
</tr>
</tbody>
</table>
Influenza A (SYBR Green I)

Forward Primer 5’-AGATGAGTCTTCTAAACCGAGGTCG-3’

Reverse Primer 5’-TGCAAAAAACATCTTCAAGTCTCTG-3’

Condition Reverse transcription 55 °C 10 min

Denature 95 °C 30s

Amplification 95 °C 0s, 62 °C 8s, 80 °C 13s, 55 cycles

Melting curve 95 °C 0s, 62 °C 10s, 95 °C 0s

Cooling 40 °C 30s

Influenza B

Forward Primer 5’-AAATACGTTGGATTAAAAATAAGCAA-3’

Reverse Primer 5’-CCAGCAAATAGCTCCGAAGAAA-3’

Influenza B Probe 6FAM-CACCCATATGGGGCAATTTCCTATGGC-TMR

(TaqMan)

Condition Reverse transcription 61 °C 20 min

Denature 95 °C 30s

Amplification 95 °C 1s, 56 °C 15s, 72 °C 15s, 50 cycles

Cooling 40 °C 30s

TaqMan denotes Taq polymerase plus PacMan principle.

The detection limitation (sensitivity) was ≤10 copies of in vitro transcribed RNA or DNA for pan-enterovirus, pan-adenovirus and influenza B real-time PCR and was
≤100 copies of in vitro transcribed RNA for influenza A. The linear regression coefficients of the standard dilution series for all the real time PCR were 0.99 to 1.
Table 2. Demography and Age-specific Viral Infection Incidence Rate per 100 Person Year of Enrolled Kindergarteners

<table>
<thead>
<tr>
<th>Period/Age Group</th>
<th>Mean (SD) Age in Year</th>
<th>Male Gender (%)</th>
<th>Overall Viral Infection Incidence Rate (95% CI)*</th>
<th>Enterovirus Infection Incidence Rate (95% CI)*</th>
<th>Adenovirus Infection Incidence Rate (95% CI)*</th>
<th>Influenza Infection Incidence Rate (95% CI)*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>2006 Academic Year</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall (N= 180)</td>
<td>4.04 (1.00)</td>
<td>103 (57%)</td>
<td>239 (208-271)</td>
<td>117 (99-136)</td>
<td>103 (82-124)</td>
<td>17 (11-22)</td>
</tr>
<tr>
<td>2 Years Old (N=25)</td>
<td>2.45 (0.26)</td>
<td>10 (40%)</td>
<td>348 (234-461)</td>
<td>174 (98-250)</td>
<td>139 (73-205)</td>
<td>26 (3-49)</td>
</tr>
<tr>
<td>3 Years Old (N=67)</td>
<td>3.54 (0.29)</td>
<td>34 (51%)</td>
<td>268 (210-326)</td>
<td>117 (88-147)</td>
<td>136 (95-178)</td>
<td>13 (5-21)</td>
</tr>
<tr>
<td>4 Years Old (N=55)</td>
<td>4.48 (0.26)</td>
<td>39 (71%)</td>
<td>207 (158-257)</td>
<td>109 (74-144)</td>
<td>74 (47-101)</td>
<td>24 (12-36)</td>
</tr>
<tr>
<td>5 Years Old (N=33)</td>
<td>5.51 (0.26)</td>
<td>20 (61%)</td>
<td>159 (112-205)</td>
<td>94 (66-123)</td>
<td>59 (24-93)</td>
<td>6 (0-14)</td>
</tr>
<tr>
<td><strong>2007 Academic Year</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall (N=190)</td>
<td>4.00 (1.04)</td>
<td>94 (49%)</td>
<td>136 (114-157)</td>
<td>18 (12-24)</td>
<td>89 (73-106)</td>
<td>27 (20-35)</td>
</tr>
<tr>
<td>2 Years Old</td>
<td>2.40 (0.41)</td>
<td>16 (55%)</td>
<td>86 (30-143)</td>
<td>14 (0-31)</td>
<td>55 (15-95)</td>
<td>17 (0-35)</td>
</tr>
<tr>
<td>Age Group</td>
<td>Frequency</td>
<td>Percentage</td>
<td>Mean Age</td>
<td>SD</td>
<td>CI 95% Lower</td>
<td>CI 95% Upper</td>
</tr>
<tr>
<td>-----------------</td>
<td>-----------</td>
<td>------------</td>
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<td>----</td>
<td>--------------</td>
<td>--------------</td>
</tr>
<tr>
<td>3 Years Old</td>
<td>3.48 (0.26)</td>
<td>33 (49%)</td>
<td>150 (113-187)</td>
<td>15 (6-23)</td>
<td>106 (78-134)</td>
<td>29 (16-43)</td>
</tr>
<tr>
<td>4 Years Old</td>
<td>4.45 (0.29)</td>
<td>18 (34%)</td>
<td>162 (115-209)</td>
<td>21 (9-32)</td>
<td>104 (68-140)</td>
<td>36 (19-53)</td>
</tr>
<tr>
<td>5 Years Old</td>
<td>5.45 (0.23)</td>
<td>27 (68%)</td>
<td>113 (78-147)</td>
<td>23 (9-36)</td>
<td>68 (40-95)</td>
<td>20 (5-35)</td>
</tr>
</tbody>
</table>

SD denotes standard deviation and CI confidence interval. * Age-specific incidence rate per 100 person year was estimated using the number of infections in the numerator and the number of age-specific enrolled kindergarten children in the denominator during each academic year.
Table 3. Factors Associated with Multiple Infections

<table>
<thead>
<tr>
<th>Factor</th>
<th>Positive Number (%) with Multiple infection among Enrolled Children</th>
<th>Unadjusted OR (95% CI)</th>
<th>Unadjusted P Value</th>
<th>Adjusted OR (95% CI)*</th>
<th>Adjusted P Value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age &lt;=4 years vs. Age&gt;4 years</td>
<td>93/189 (49%) vs. 82/181 (45%)</td>
<td>1.17 (0.78-1.76)</td>
<td>0.45</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male Gender vs. Female</td>
<td>107/197 (54%) vs. 68/173 (39%)</td>
<td>1.84 (1.21-2.78)</td>
<td>0.004</td>
<td>1.81 (1.20-2.75)</td>
<td>0.005</td>
</tr>
<tr>
<td>Allergic Conjunctivitis vs. No Allergic Conjunctivitis</td>
<td>13/20 (65%) vs. 162/350 (47%)</td>
<td>2.16 (0.84-5.53)</td>
<td>0.10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Allergic Dermatitis vs. No Allergic Dermatitis</td>
<td>21/43 (41%) vs. 154/327 (47%)</td>
<td>1.07 (0.57-2.03)</td>
<td>0.83</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Allergic Rhinitis vs. No Allergic Rhinitis</td>
<td>41/80 (51%) vs. 134/290 (46%)</td>
<td>1.22 (0.75-2.01)</td>
<td>0.42</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asthma vs. No Asthma</td>
<td>11/21 (53%) vs. 164/349 (47%)</td>
<td>1.24 (0.51-3.00)</td>
<td>0.63</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Allergy** vs. No Allergy</td>
<td>70/128 (55%) vs. 105/242 (43%)</td>
<td>1.57 (1.02-2.42)</td>
<td>0.04</td>
<td>1.56 (1.00-2.39)</td>
<td>0.05</td>
</tr>
<tr>
<td>Influenza Vaccination vs. No Influenza Vaccination</td>
<td>79/157 (50%) vs. 96/213 (45%)</td>
<td>1.23 (0.82-1.87)</td>
<td>0.32</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Multiple infections were defined as one child had equal to or over 2 viral infections with different virus or different serotypes during one academic year. CI denotes confidence interval

*Adjusted OR and adjusted p value were measured with multiple logistics regression analysis.

**Allergy was defined to have medical history of allergic conjunctivitis, atopic dermatitis, allergic rhinitis, or asthma.
Circulating Viruses and the Periods of Circulation During 2006 Academic Year

Circulating Viruses and the Periods of Circulation During 2007 Academic Year