Title

Does respiratory virus co-infection increase the clinical severity of acute respiratory infection among children infected with respiratory syncytial virus?

Authors

Yoshitaka Harada MD,* Fumiko Kinoshita MD,† Lay Myint Yoshida MB BS, PhD,* Le Nhat Minh MSc,* Motoi Suzuki MD, MSc,* Konosuke Morimoto MD, PhD,* Yuichirou Toku MD,† Kunio Tomimasu MD,† Hiroyuki Moriuchi MD, PhD,‡ Koya Ariyoshi MD, PhD*

* Department of Clinical Medicine, Institute of Tropical Medicine, Nagasaki University, Sakamoto 1-12-4, Nagasaki, 852-8523, Japan

† Department of Pediatrics, Nagasaki Municipal Hospital, Shinchi 6-39, Nagasaki, 850-8555, Japan

‡ Department of Pediatrics, Nagasaki University School of Medicine, Sakamoto 1-7-1, Nagasaki, 852-8102, Japan

Address for correspondence

Koya Ariyoshi, MD, PhD

Department of Clinical Medicine

Institute of Tropical Medicine

Nagasaki University

Sakamoto 1-12-4

Nagasaki, 852-8523, Japan

kari@nagasaki-u.ac.jp

Telephone number: +81-(0)95-819-7842

Fax number: +81-(0)95-819-7843

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Abstract

Background: Respiratory syncytial virus (RSV) is a leading cause of acute lower respiratory infection in children less than 5 years of age. The impact of non-RSV respiratory virus co-infection on the severity of RSV disease is unknown.

Methods: This hospital-based prospective study was conducted in Nagasaki, Japan, on all children less than 5 years of age with acute respiratory infection (ARI) who had undergone a rapid RSV diagnostic test between April 2009 and March 2010. Thirteen respiratory viruses were identified from nasopharyngeal swab samples using a multiplex polymerase chain reaction (PCR); PCR positive samples were considered as confirmed respiratory virus infections. The cases were classified into three categories (pneumonia; moderate-to-severe non-pneumonic ARI; and mild ARI) according to the findings of the chest radiograph and the hospitalization records.

Results: Among 385 cases enrolled, 372 were eligible for analysis, of whom 86 (22%) were classified as pneumonia cases; 137 (36%), moderate-to-severe non-pneumonic ARI cases; and 162 (42%), mild ARI cases. RSV was detected in 172 cases (61.6%), and 31 cases (18.0%) had double or triple infections with other respiratory viruses. RSV infection was more frequently observed in pneumonia cases (OR, 2.2; 95% CI, 1.27–3.8) and moderate-to-severe non-pneumonic ARI cases (OR, 2.95; 95% CI,

1.82–4.78) than in mild ARI cases. The association with moderate-to-severe non-pneumonic ARI cases was stronger with RSV/non-RSV respiratory virus co-infection (adjusted OR, 4.91; 95% CI, 1.9–12.7) than with RSV single infection (adjusted OR, 2.77; 95% CI, 1.64–4.7).

Conclusions: Non-RSV respiratory virus co-infection is not uncommon in RSV-infected children and may increase the severity of RSV disease.

Background

Acute respiratory infection (ARI) is the leading cause of morbidity and mortality among infants and young children less than 5 years of age.[1] According to estimates from the World Health Organization and UNICEF, 2 million childhood deaths in the world are attributable to ARI annually.[2] Respiratory syncytial virus (RSV) is the most important viral pathogen, causing bronchiolitis, pneumonia, and severe forms of ARI in young children.[3] In addition to RSV, a wide range of respiratory viruses, including influenza virus, human metapneumovirus (HMPV), and human rhinovirus (HRV), are known to cause childhood ARI.[4]

Recently, multiplex polymerase chain reaction (PCR) has been used to detect concurrent infection with multiple respiratory viruses and has demonstrated that co-infection with more than one respiratory virus is common in children with and without ARI.[5-13] However, the clinical implications of multiple viral infections remain controversial. Several studies have shown that respiratory virus co-infection may increase the clinical severity of RSV disease,[5,7-9] while other studies have reported opposite findings.[10-12] These differences may be attributable to variations in clinical settings, study populations, and the seasonality of circulating viruses. No similar studies

have been formally conducted in East Asian countries, including Japan.

The aim of this study was to determine the frequency of respiratory virus co-infection with RSV and to investigate the impact of RSV/non-RSV respiratory virus co-infection on the severity of ARI among children less than 5 years of age.

Materials and Methods

Participant and clinical data collection

This hospital-based prospective study was conducted in Nagasaki, Japan. The total population in the city in 2010 was 443,000, with 16,700 (3.8%) individuals less than 5 years of age. Nagasaki City Municipal Hospital (NCMH) is one of two referral hospitals for sick children in Nagasaki. NCMH has 414 beds, including 38 beds in the pediatric ward. The hospital also cares for patients who walk in with mild symptoms. About a half of outpatients are cases without referral letters. We expect that the characteristics of these children do not differ from those patients in neighboring clinics.

We approached all children less than 5 years of age who showed signs of ARI and who were tested using the rapid RSV diagnostic kit in either the outpatient department (OPD) or the pediatric ward of NCMH. The children were evaluated by hospital pediatricians regardless of their participation in the study and the rapid diagnostic tests were ordered by them based on their clinical judgment; no standardized testing/admission criteria were used in the study. All children who had been administered the rapid RSV diagnostic tests were enrolled in the study regardless of their test findings." A commercial RSV diagnostic kit (a rapid

116 immunochromatographic assay; Check RSV, Alfresa Farmer Co., Ltd., Japan)[14] was
117 routinely used whenever RSV infection was clinically suspected. The sensitivity and
118 specificity of the RSV diagnostic kits which were commercially available in Japan were
119 32-79% and 100% when compared with RT-PCR.[15]
120
121 The study period was from April 1st 2009 through March 31st 2010 including
122 the four seasons: spring (March-May), summer (June-August), fall (Spring-November),
123 and winter (December-February). Information from clinical findings, chest radiographs,
124 and blood examinations were retrospectively obtained from the hospital records. The
125 information on some risk factors for severe diseases such as prematurity and
126 breastfeeding status were not always available in the hospital records, and the blood
127 examination was not usually done for the majority of outpatients. Thus these data were
128 not collected in our study.
129
130 **Sample collection and storage**
131 Residual nasopharyngeal swabs were temporarily stored at −20°C in the
132 laboratory department of NCMH after being used for the rapid RSV diagnostic test.
133 Within a week, the samples were transported to the Institute of Tropical Medicine at

Nagasaki University for storage in a deep freezer (−80 °C) until they were used for further molecular tests.

Multiplex PCR analysis

The viral nucleic acid was extracted using a QIA viral RNA minikit (QIAGEN Inc., Valencia, CA). The following four multiplex PCR assays were performed for each sample to detect 13 respiratory viruses in the RNA: (1) RSV, influenza A/B (FLUA, FLUB), and HMPV; (2) human parainfluenza virus (HPIV) types 1–4; (3) HRV and human coronavirus OC43/229E (HCoV); and (4) human adenovirus (HAdV) and human bocavirus (HBoV). The details of the multiplex PCR assays are published elsewhere.[13,16] Briefly, reverse transcription-PCR (RT-PCR) assays were performed using the one-step RT-PCR kit from QIAGEN. Confirmatory-PCR was performed using a hemi-nested PCR assay on samples that were positive in the initial PCR test. Samples positive for both PCR assays were defined as positive, and PCR positive samples were considered as confirmed respiratory virus infections. The results of rapid RSV diagnostic test kit were not used to define our cases.

Data analysis

The patients were classified into one of the following three case categories: (1) a pneumonia case, if the hospitalized child had consolidation on the chest radiograph; (2) a moderate-to-severe non-pneumonic ARI case, if the hospitalized child did not have consolidation on the chest radiograph; and (3) a mild ARI case, if the child was not hospitalized and did not have a chest radiograph. Most of the moderate-to-severe non-pneumonic ARI cases had bronchiolitis or severe bronchitis. Children who were hospitalized but did not have a chest radiograph were excluded from the analysis.

Patient characteristics were compared using the chi-square test for categorical variables and the Kruskal-Wallis test for continuous variables. Odds ratios were calculated to demonstrate the associations between the viral infection status and the disease severity (i.e. mild ARI vs. moderate-to-severe non-pneumonic ARI and mild ARI vs. pneumonia). The effect of RSV infection with or without other respiratory viral co-infection on the disease severity were investigated using logistic regression models. Sex and age group (<12 months, 12-23 months, and 24-59 months) were included in the final model as potential confounders. The Hosmer-Lemeshow test was used to determine the goodness-of-fit of the model. All data management was performed using Microsoft Excel 2007, and statistical analyses were performed using STATA 11.2

(STATA Corp., USA). The significant level was taken to be 5% for two-tailed tests.

Ethics approval

The study was approved by the Institutional Review Board (IRB) of NCMH and the IRB of the Institute of Tropical Medicine, Nagasaki University, Nagasaki, Japan.

Results

During the study period, a total of 385 children were tested for RSV, and all were recruited for this study. In all, 86 (22%) children were classified as pneumonia cases, 137 (36%) were classified as moderate-to-severe non-pneumonic ARI cases, and 149 (39%) were classified as mild ARI cases. Thirteen (3%) children were hospitalized but did not have a chest radiograph; these children were excluded from the analysis. Among the 372 children, 227 (61%) were male, and the median age (inter-quartile range) was 12 (5.5) months, ranging from 0 to 96 months. When the characteristics of the three groups were compared, there were no significant differences in sex and age. However, pneumonia cases had significantly higher white blood cell (WBC) counts and C-reactive protein (CRP) levels than the moderate-to-severe non-pneumonic ARI cases (Table 1).

At least one virus was detected in 279 (75%) of the cases by the multiplex PCR assay. Of these, two or more viruses were concurrently detected in 44 (15.8%) cases; 39 (14.0%) cases had dual virus infections, and 5 (1.8%) cases had triple infections. RSV was by far the most common pathogen and was identified in 172 (61.7%) children, followed by HRV (n = 63, 22.6%), HMPV (n = 23, 8.2%), HPIV-1 (n = 22, 7.8%), and

195 HAdV (n = 21, 7.5%) (Table 2). Among the 172 children with confirmed RSV infection,
196 31 (18.0%) of RSV positive children had double or triple infections with other
197 respiratory viruses; HRV was the most common co-infecting virus, followed by HAdV
198 (Table 2). Among the 200 RSV-negative children, 107 (53.5%) were positive for at least
199 one non-RSV respiratory virus, and 13 were double or triple infections. HRV/HAdV (n
200 = 3) was the leading combination followed by HRV/HBoV (n = 2) and HRV/PIV-1 (n =
201 2).
202
203 We then analyzed associations between infection with each virus and the
204 disease categories. RSV infection had significant associations with both pneumonia
205 cases and moderate-to-severe non-pneumonic ARI cases in comparison with mild ARI
206 cases; the level of association was stronger with moderate-to-severe non-pneumonic
207 ARI cases (OR, 2.95; 95% CI, 1.82–4.78; $P < 0.001$) than with pneumonia cases (OR,
208 2.2; 95% CI, 1.27–3.8; $P = 0.004$). In addition, HPIV-3 infection was strongly
209 associated with pneumonia cases (OR, 7.54; 95% CI, 1.56–36.4; $P = 0.012$) but was not
210 significantly associated with moderate-to-severe non-pneumonic ARI cases (OR, 2.21;
211 95% CI, 0.4–12.3; $P = 0.4$) (Table 3).
212

213 Because we found the most significant association between moderate-to-severe
214 non-pneumonic ARI cases and RSV infection, we further analyzed the possible risk
215 factors for moderate-to-severe non-pneumonic ARI. An age of older than 24 months
216 was protective against moderate-to-severe non-pneumonic ARI. Interestingly, when
217 RSV-infected children were stratified into those that were or were not co-infected with
218 other viruses, the association with moderate-to-severe non-pneumonic ARI was stronger
219 among RSV-infected children with non-RSV virus co-infection than in children with
220 RSV single infection. This association was even clearer in the multivariate analysis,
221 which adjusted for sex and age groups (Table 4). However, non-RSV respiratory virus
222 co-infection did not exhibit a significant effect on the degree of association with
223 pneumonia cases (data not shown). We also analyzed the association between
224 moderate-to-severe non-pneumonic ARI and non-RSV respiratory virus infection
225 among RSV-negative children. The association was stronger with multiple non-RSV
226 respiratory virus infections than single non-RSV respiratory virus infection, but the
227 confidence intervals were wide because of the small sample size (data not shown).
228
229 Results from the PCR and the RSV rapid test were almost compatible. The rate
230 of overall agreement between both assays was 86% (n = 332/385, Cohen's kappa

231 coefficient=0.72, p=0.0001); 30 samples were positive for PCR but negative for the

232 rapid test, and 23 samples were negative for PCR but positive for the rapid test.

233

Discussion

In our study population, we showed that respiratory virus co-infection with RSV infection had a prevalence of 18% and was associated with moderate-to-severe non-pneumonic ARI. The rates of respiratory virus co-infection in RSV-infected cases have varied substantially in past studies, ranging from 11% to 53%.[17,18] The co-infection rate may be affected by the composition of the study population; more hospitalized children may result in a higher rate of co-infection. In our case, OPD children constituted 40% of the total population, and their low co-infection rate (14.6%) may have reduced the overall rate. Seasonal variations in circulating respiratory viruses also determine the co-infection rate. In our one-year study, the co-infection rate during the cold season (from October to March, 19.7%) was higher than the rate during the hot season (from April to September, 12.5%). Thus, the comparison of co-infection rates between studies requires careful interpretation.

Our findings indicate that respiratory virus co-infections with RSV infection increases the risk of developing more severe ARI with respiratory distress, such as bronchiolitis, compared to RSV single infection. The effect of multiple viral infections on the clinical picture of ARI has been controversial. Previous studies have reported that

co-infection with non-RSV respiratory viruses, particularly HMPV, increased the risk of hospitalization, pneumonia, and the receipt of mechanical ventilation among RSV-infected children,[5,7-9] whereas other studies noted no association between respiratory virus co-infection and the severity of RSV disease.[10-12,19] Inconsistent findings have also been reported for associations between ARI severity and respiratory virus co-infections with non-RSV respiratory virus infections.[12,20] These variations may be explained by the different combinations of virus co-infections. Franz et al. reported that RSV/non-RSV co-infection was more strongly associated with pneumonia than RSV single infection, and the primary co-infecting virus in their study was HBoV (8.8% of all RSV infections, n = 14/160).[9] However, Martin et al. found that multiple viral infections did not increase the severity of RSV single infection; the leading combinations in their study were RSV/HAdV (25% of dual viral infections, n = 24/96) and RSV/HCoV (17.7% of dual infections, n = 17/96).[12] In our study, the most common virus that was present in an RSV co-infection was HRV (11%, n = 19/172). Therefore, our findings may not be directly comparable with those from previous reports.

Several hypotheses have been proposed to explain the association between

multiple respiratory virus co-infections and ARI severity; these hypotheses include modification of immune responses after the initial infection,[17,21] host susceptibility to multiple viruses,[8] and associations of specific respiratory viruses with colonized bacteria.[9,16] However, our study does not give a causal explanation for the observed phenomenon due to limited biological data. In our study, the evidence for an increased risk of developing pneumonia when co-infected with non-RSV respiratory viruses was not significant, which is probably due to the small sample size or because bacterial infection played a major role in the pathogenesis of pneumonia. Our results involving associations between clinical severity and higher WBC counts and CRP levels may reflect the latter explanation.

Despite the small sample size, we again demonstrate a strong and significant association of HPIV-3 infection with severe ARI in the Japanese population. We have previously shown a compatible association with children with ARI in Vietnam.[13] Both parainfluenza viruses and RSV are enveloped RNA viruses that are in the family Paramyxoviridae. Parainfluenza viruses have many epidemiologic and clinical similarities to RSV and thus require medical attention as the second most important viral cause of hospitalization for ARI in young children.[22]

288

289 On the other hand, the positivity of influenza was conspicuously low (1.3%) in
290 our children. This was because in our setting, a commercial rapid influenza diagnostic
291 test (RIDT) was routinely performed for any outpatient with influenza-like illnesses
292 prior to the RSV diagnostic test and children with positive RIDT results were usually
293 not tested for RSV. Consequently influenza cases were screened out from our study
294 population. Further investigations are needed to evaluate the association between
295 influenza infection and the disease severity.

296

297 In our study population, 25% of the children less than 5 years of age were
298 negative for any virus. This result was compatible with previous reports that found that
299 virus is not detected in a considerable proportion (10–30%) of patients with respiratory
300 diseases, despite intensive investigations.[6,7,9,12,20,23] Several reasons have been
301 proposed to explain this virus-negative population. First, conventional virus detection
302 tests, such as immunochromatography, are not sensitive. In our study, the sensitivity of
303 the RSV rapid test was 83.2%, referring to the PCR results. However, the sensitivity of
304 this rapid test is 100% if compared with other immunochromatography-based tests.[14]
305 Second, infection with other respiratory viruses, such as HCoV-NL63,[24]

306 HCoV-HKU1,[25] and human enteroviruses[26] that are not included as target viruses
307 in our current multiplex PCR system may have been missed. Third, targeted viruses
308 might have been cleared by the time of sampling because it was often several days or
309 over a week after the onset of disease when the sampling was performed. Such delayed
310 sampling may underestimate the true viral infection rate.[9,27,28] Fourth, some viral
311 RNA may have been degraded during the temporary storage at −20°C in the hospital.
312 However, the rate of viral detection was not low in our study as compared with previous
313 reports. Finally, there is always the possibility that an unknown virus might have caused
314 the disease.
315
316 In addition to the examples mentioned above, this study has additional
317 limitations. First is the nature of cross-sectional studies. We expect that sequential
318 sample collections will uncover the natural courses of respiratory virus co-infections
319 and their causal effects on the clinical outcomes. Second, our study lacked data
320 regarding the timing of infection and disease onset. Third, our specimens were not
321 tested for viral load by quantitative PCR. The viral load of RSV may have been
322 associated with the co-infection status and the disease severity.[12] Fourth, we did not
323 have information regarding bacterial infection because non-invasive collection of

appropriate sputum samples is not feasible among young children.

In summary, RSV is the leading viral cause of lower respiratory infections and causes substantial hospitalization burden during the winter season in Japan.[29] Our findings indicate that co-infection with RSV and other respiratory viruses is not uncommon and that co-infections may increase the severity of RSV disease in children. Further investigations are warranted to investigate the determinants of RSV disease severity.

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421
422
423

Table 1

Characteristics of the study patients by their case categories.

	Mild ARI N = 149	Moderate-to-severe non-pneumonic ARI N = 137	Pneumonia N = 86	Total N = 372	*P* value[a]
Sex, N (%)					
Male	94 (63.1)	83 (60.6)	50 (58.1)	227 (61)	n.s.
Female	55 (36.9)	54 (39.4)	36 (41.9)	145 (39)	
Age average (months), median (IQR)	12 (7)	12 (7)	12 (13)	12 (5.5)	n.s.
Age group (months), N (%)					
<12	62 (41.6)	63 (46)	26 (30.2)	151 (40.6)	
12–23	52 (34.9)	57 (41.6)	37 (43)	146 (39.2)	n.s
>24	35 (23.5)	17 (12.4)	23 (26.8)	75 (20.2)	
WBC (per mm^3), median (IQR)	n.a.	10,200 (5,900)	11,250 (7,200)	10,700 (6,400)[b]	0.04
CRP (mg/dl), median (IQR)	n.a.	0.9 (2.1)	3.1 (4.7)	1.6 (3.4)[b]	0.0001

NOTE. n.s., not significant; n.a., data not available; SD, standard deviation; IQR, inter-quartile range.

[a]P value calculated from univariate analysis that the chi-square test was used for categorical variables whereas the Kruskal-Wallis test was applied for continuous variables.

[b]Data was available for 223 patients.

Table 2

Virus distribution in the total samples and in the RSV-positive samples.

	No. of positives in total samples, N=372			No. of positives in RSV-positive samples, N=172	
	N	%		N	%
RSV	172	46.2	HRV	19	11.1
HRV	63	16.9	HAdV	6	3.5
HMPV	23	6.2	HBoV	3	1.7
HPIV-1	22	5.9	HMPV	2	1.2
HAdV	21	5.7	HPIV-1	2	1.2
HPIV-3	14	3.8	FluA	2	1.2
HBoV	7	1.9			
FluA	5	1.3			
HPIV-4	1	0.3			

NOTE. RSV, respiratory syncytial virus; HRV, human rhinovirus; HMPV, human metapneumovirus; HPIV, human parainfluenza virus; HAdV, human adenovirus; HBoV, human bocavirus; FluA, influenza A virus.

Table 3

Comparison of respiratory viral infections and clinical case categories.

		Mild ARI	Moderate-to-severe non-pneumonic ARI	Pneumonia	Mild vs. moderate-to-severe non-pneumonic ARI			Mild vs. pneumonia		
		N = 149 (%)	N = 137 (%)	N = 86 (%)	OR	95% CI	*P* value	OR	95% CI	*P* value
RSV	Pos	48 (32.2)	80 (58.4)	44 (51.2)	2.95	1.82–4.78	<0.001	2.2	1.27–3.80	0.004
	Neg	101 (67.8)	57 (41.6)	42 (48.8)	Reference			Reference		
HRV	Pos	25 (16.8)	30 (21.9)	8 (9.3)	1.39	0.77–2.51	0.3	0.5	0.22–1.18	0.1
	Neg	124 (83.2)	107 (78.1)	78 (90.7)	Reference			Reference		
HMPV	Pos	9 (6)	9 (6.6)	5 (5.8)	1.09	0.42–2.84	0.9	0.96	0.31–2.96	0.9
	Neg	140 (94)	128 (93.4)	81 (94.2)	Reference			Reference		
HAdV	Pos	10 (6.7)	6 (4.4)	5 (5.8)	0.64	0.23–1.80	0.4	0.86	0.28–2.60	0.8
	Neg	139 (93.3)	131 (95.6)	81 (94.2)	Refeference			Reference		
HPIV-1	Pos	11 (7.4)	9 (6.6)	2 (2.3)	0.88	0.35–2.20	0.8	0.3	0.06–1.38	0.1
	Neg	138 (92.6)	128 (93.4)	84 (97.7)	Reference			Reference		
HPIV-3	Pos	2 (1.3)	4 (2.9)	8 (9.3)	2.21	0.40–12.3	0.4	7.54	1.56–36.4	0.012
	Neg	147 (98.7)	133 (97.1)	78 (90.7)	Reference			Reference		

NOTE. OR, odds ratio; CI, confidence interval.

Table 4

Associations between RSV single/multiple infections and moderate-to-severe non-pneumonic ARI cases.

		Unadjusted OR	95% CI	*P* value	Adjusted OR	95% CI	*P* value
Sex	Male	Reference			Reference		
	Female	1.11	0.69–1.79	0.7	1.06	0.64–1.77	0.8
Age group	<12	Reference			Reference		
(months)	12–23	1.07	0.65–1.80	0.8	1.03	0.6–1.77	0.9
	>24	0.48	0.24–0.94	0.033	0.42	0.2–0.85	0.016
RSV	Negative	Reference			Reference		
	Single infection	2.68	1.61–4.47	<0.001	2.77	1.64–4.70	<0.001
	Multiple infection	4.56	1.80–11.57	0.001	4.91	1.90–12.7	0.001

NOTE. OR, odds ratio; CI, confidence interval.

Logistic regression analysis was done for mild ARI cases vs. moderate-to-severe non-pneumonic ARI cases. The multiple logistic regression model included the explanatory variables listed in the table. The result of the Hosmer-Lemeshow test indicated high goodness-of-fit for the final model (P=0.62).