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**Running title:** 25-OH vitamin D in RSV infection

本邦における生後 3 か月以下の RSV 感染乳児例における血清 25-hydroxy vitamin D の検討

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## Summary

Low levels of blood vitamin D have been reported in children who have frequent respiratory tract infections. We measured serum concentrations of 25-hydroxy (OH) vitamin D in Japanese infants less than 3-months old infected with respiratory syncytial virus (RSV). Serum levels of 25-OH vitamin D of the 10 infants, excluding those with underlying diseases, were between less than 4 to 29.8 ng/mL. In 8 out of 10 subjects (80.0%), serum 25-OH vitamin D levels were less than 20 ng/mL. There was no statistically significant association between levels of 25-OH vitamin D and age, duration of admission, respiratory severity score, white blood cell count, blood gas levels, and NT-proBNP levels. Levels of serum 25-OH vitamin D in children who required hospitalization owing to RSV infection were low, indicating deficiency. These results suggested that vitamin D deficiency affects the susceptibility to RSV infection, but not the severity of the RSV respiratory infection.

## Introduction

Respiratory syncytial virus (RSV) is a major cause of lower respiratory tract infections in Japan, and is the most common cause of the hospitalization of infants, resulting in clinical diseases, such as bronchiolitis and pneumonia (1). Vitamin D deficiency has been reported to correlate with RSV infection, as blood concentrations of 25-OH vitamin D have been reported to be associated with the incidence of RSV infection in the first year of life in foreign countries (2,3,4). Polymorphisms in *VDR* have also been reported to be associated with the genetic predisposition to RSV bronchiolitis (5,6). Vitamin D levels in infants differ according to their region of habitation, which affects the time and amount of sun exposure as well as their eating habits. To clarify the involvement of vitamin D deficiency in RSV infection in young infants in Japan, we measured serum 25-OH vitamin D levels in infants younger than 3-months old who were hospitalized owing to RSV infection.

## Material and Methods

Ten patients (6 boys and 4 girls) aged 0 to 3-months old, who were hospitalized in the Department of Pediatrics, Tokyo Medical University Hospital, from January 2017 to January 2019 were enrolled in this study. Patients with congenital heart disease or low birth weight were excluded. All other patients with no serious underlying diseases, who were diagnosed as bronchiolitis or pneumonia caused by RSV by the rapid antigen test (Check RSV; Alfresa, Japan), were included (Table 1). To evaluate the severity of respiratory symptoms, the respiratory severity score established by Nariai S. was used (7). Levels of serum 25-OH vitamin D were measured by the chemiluminescent immunoassay (CLIA) method and subjects were categorized into the following 3 groups: Normal ( $> 30$

ng/mL), Low (30–20 ng/mL), and Deficient (< 20 ng/mL). Serum 25-OH vitamin D levels of 10 age-matched hospitalized controls (4 with fever of unknown origin, 2 with urinary tract infections, 1 with influenza, 1 with asphyxia, 1 with bronchitis, and 1 with HIV infection) were also measured. N-terminal pro-natriuretic peptide (NT-proBNP) levels were also measured by the CLIA method to investigate possible involvement of the heart in RSV infection, and levels higher than 1,000 pg/mL were defined as extremely high. Renal function data of all subjects were in the normal range, and serum P and Ca levels were also normal in all subjects. Alkaline phosphatase (ALP) levels of all subjects were less than 1,000 IU/L. None of the subjects were diagnosed clinically as having rickets. Serum samples in admission were subjected for assay. Statistical analyses were performed using IBM® SPSS® Statistics version 25.0 software. A Pearson *r*-coefficient of greater than 0.8 was considered to indicate a statistically significant correlation (*p*-value < 0.05).

This study was approved by the Ethics Review Board of Tokyo Medical University (study approval no.: SH3841). Blood samples were collected from all patients after obtaining informed consent from the parents at the time of the patient's admission.

## Results

Serum levels of 25-OH vitamin D of the 10 subjects were between less than 4 to 29.8 ng/mL. None of the subjects showed levels higher than 30 ng/mL, which is considered normal. Eight out of 10 subjects (80.0%) had levels less than 20 ng/mL, which was defined as vitamin D deficiency. The serum levels of 25-OH vitamin D of the 10 subjects with RSV infection were tended to be lower than those of the age-matched controls. However, the difference between the 2 groups was not statistically significant (Figure 1).

Serum levels of NT-proBNP were significantly high in 2 out of the 10 patients (Figure 2). The levels of 25-OH vitamin D did not correlate with age (months), bronchiolitis vs pneumonia, duration of admission, requirement of respiratory support, respiratory severity scores, birth weight, respiratory rates, or WBC, SpO<sub>2</sub>, PCO<sub>2</sub>, HCO<sub>3</sub><sup>-</sup>, and NT-proBNP levels. On the other hand, respiratory severity scores of RSV infection correlated with duration of admission, PCO<sub>2</sub>, and HCO<sub>3</sub><sup>-</sup>. NT-proBNP levels correlated with duration of admission and PCO<sub>2</sub> levels. Levels of 25-OH vitamin D statistically correlated only with CRP levels. Data are shown in Table 2.

## Discussion

Vitamin D is associated with bone remodeling, and plays roles in the improvement of immune function and the reduction of inflammation. Recently, vitamin D has been shown to play a key role in the regulation of innate immunity (8). In this study, 8 out of 10 infants who were hospitalized owing to RSV infection had serum 25-OH vitamin D levels of less than 20 ng/mL. A level of 25-OH vitamin D of less than 20 ng/mL is classified as vitamin D deficiency. Vitamin D deficiency readily occurs upon low levels of its uptake and the lack of sun exposure, because the circulating half-life of vitamin D is only about 15 days (9). Preterm infants, inadequate intake, lack of sun exposure, fat malabsorption, obesity, and impaired absorption have been considered to be risk factors for vitamin D deficiency (10). In this study, none of the subjects had these conditions.

Belderbos et al. showed that vitamin D deficiency in healthy neonates was associated with increased risk of RSV-associated lower respiratory tract infection in the first year of life (3). Halasa et al. investigated the risk factors for severe respiratory symptoms, including vitamin D levels, in the Middle East and Arab countries. RSV-positive children

were more likely to be previously healthy without any underlying medical conditions, less likely to be born prematurely, and had lower median vitamin D levels compared with the RSV-negative group (2). In a rare report about vitamin D and respiratory infection in Japan, Inamo et al. investigated the association between vitamin D deficiency and the severity of respiratory infection by determining serum concentrations of 25-OH vitamin D in a group of hospitalized children with acute lower respiratory infections, including RSV infection. They found that there was a significant correlation between vitamin D deficiency (<15 ng/mL) and the need for supplementary oxygen and ventilator management (11). However, this study was not focused on RSV infection.

The pathophysiology of vitamin D deficiency has been studied by both immunological status and local defending status. Secretion of the peptides LL-37 and 13-defensin 2, which have anti-viral activity against RSV, is induced by vitamin D, and inhibits the production of new infectious particles and the spread of infection (12,13). Additionally, levels of 1,25-(OH)<sub>2</sub> vitamin D, which is the activated form of vitamin D, correlated with the suppression of inflammatory cytokines and the production of IgG. The production of NF-κB-linked proinflammatory cytokines following RSV infection was inhibited by vitamin D treatment, in experiments using human airway epithelial cells (14).

Although vitamin D is considered to be effective against severe RSV infections our results showed no statistically significant difference between the levels of 25-OH vitamin D and the duration of admission, respiratory severity scores, WBC counts, blood gas levels, and NT-proBNP levels. Beigelman reported that vitamin D status at the time of bronchiolitis was not associated with indicators of the severity of acute bronchiolitis, which is similar to our results. The following indicators of acute bronchiolitis severity did not differ between infants with and without vitamin D deficiency: duration of

hospitalization, lowest oxygen saturation, and bronchiolitis severity score, even after adjusting for age, and for infant formula consumption (15). On the contrary, according to the report by Hurwitz et al. the levels of retinol-binding protein and vitamin D were associated with severe outcomes in children hospitalized with lower respiratory tract infection and RSV infection. Low vitamin D levels were observed in 50% of the children and were associated with a significantly increased risk for the need of admission to an intensive care unit and invasive mechanical ventilation (16).

A systematic review by Eisenhut M. et al. showed that extrapulmonary manifestations of RSV infection included cardiovascular failure with hypotension, and inotrope requirement associated with myocardial damage, as evident from increased cardiac troponin levels (35%–54% of ventilated infants). NT-proBNP represents a marker of heart failure resulting from pressure overload, in RSV pulmonary infection. NT-proBNP levels in 2 out of the 10 patients were significantly high. However, there was no correlation with 25-OH vitamin D levels. RSV infection is usually complicated with myocarditis, and the patients seldomly have serious arrhythmia (17,18). Patients often have mild myocarditis with RSV infection. Further studies to identify potential confounding factors are necessary to conclude the effectiveness of vitamin D in the prevention of RSV infection in infants.

Recent studies have provided information regarding the association between cord blood 25-OH vitamin D levels and the risk of developing respiratory infections in infants. In a Korean birth cohort study, the authors investigated cord blood vitamin D levels and respiratory tract infection at a 6-month follow-up. The results showed that 34.3% of infants had 25-OH vitamin D concentrations of less than 25.0 nmol/L, and cord blood vitamin D insufficiency or deficiency in healthy neonates was associated with an

increased risk of acute nasopharyngitis by 6 months of age (4). Mohamed et al. also reported similar results of increased risk of acute lower respiratory tract infection in the first 2 years of life (19). Cord blood 25-OH vitamin D concentrations were strongly associated with maternal vitamin D3 supplementation during pregnancy. Therefore, enhancing routine vitamin D supplementation during pregnancy may be a useful strategy to prevent RSV-associated lower respiratory tract infections during infancy.

A limitation of this study is that the sample size was too small to demonstrate statistically significant data to support our conclusion.

#### **Author contributions**

HK designed the study; SM, SN, and YK performed the experiments, and collected and analyzed the data; HK wrote the manuscript; GY provided technical support and conceptual advice. All authors read and approved the final manuscript.

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#### **Conflicts of interest**

Kimura M, Morichi S, Nishimata S, Yamanaka G, Kashiwagi Y, and Kawashima H declare that they have no conflicts of interest associated with this study.

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## Figure legends

### **Figure 1. Serum levels of 25-OH vitamin D in patients infected with RSV and controls**

Eight out of 10 subjects with RSV infection (80.0%) had serum 25-OH vitamin D levels of less than 20 ng/mL. Six out of the 10 age-matched hospitalized controls (60.0%) showed levels less than 20 ng/mL. The boxes in the graph indicate the first quartile and third quartile, and the bars indicate maximum, the second quartile, and minimum values. Statistical analysis was performed using the unpaired *t*-test. There was no statistically significant difference between the 2 groups. ( $p$  value = 0.538 >0.05)

### **Figure 2. Serum levels of NT-proBNP in patients infected with RSV**

No correlation was found between NT-proBNP and 25-OH vitamin D levels in patients infected with RSV. Two patients showed high levels NT-proBNP.

Table 1. Profile of the subjects who were infected RS virus at the age of 3 months or younger

Age (months)	Severity	Disease	Duration of admission	Underlying disease	Asthma after RS infection	Respiratory support	Birth weight (g)	Gestation	25-OH vitamin D (ng/mL)	NT-pro BNP (pg/mL)	Respiratory severity score
1	Mild	Bronchiolitis	5		-	None	3,028	40w3d	< 4	325	4
1	Moderate	Bronchiolitis	7		-	C-PAP →biphasic mode	2,970	40w1d	< 4	562	7
2	Mild	Bronchiolitis	3	Umbilical hernia, quiescent testes, fever at 1 month	-	None	2,978	38w2d	11.5	741	1
2	Mild	Pneumonia	7		-	None	3,184	38w3d	7.2	2,030	3
3	Mild	Bronchiolitis	5		-	None	2,850	39w	16.5	84	6
0	Severe	Bronchiolitis	12		+	Artificial ventilation	2,750	38w6d	17.8	4,090	11
1	Mild	Bronchiolitis	6		-	None	Unknown	Unknown	13	153	5
3	Mild	Bronchiolitis	8		-	O <sub>2</sub> mask	3,270	39w1d	29.8	137	6
2	Mild	Pneumonia	7	Umbilical hernia after TTN, and pneumothorax	-	O <sub>2</sub> mask	3,050	38w	25.5	272	3
1	Severe	Pneumonia	27		-	Artificial ventilation	2,874	36w4d	18.1	229	8

TTN: transient tachypnea of the newborn, CPAP:Continuous Positive Airway Pressure

Table 2. Pearson correlation coefficients and *p*-values

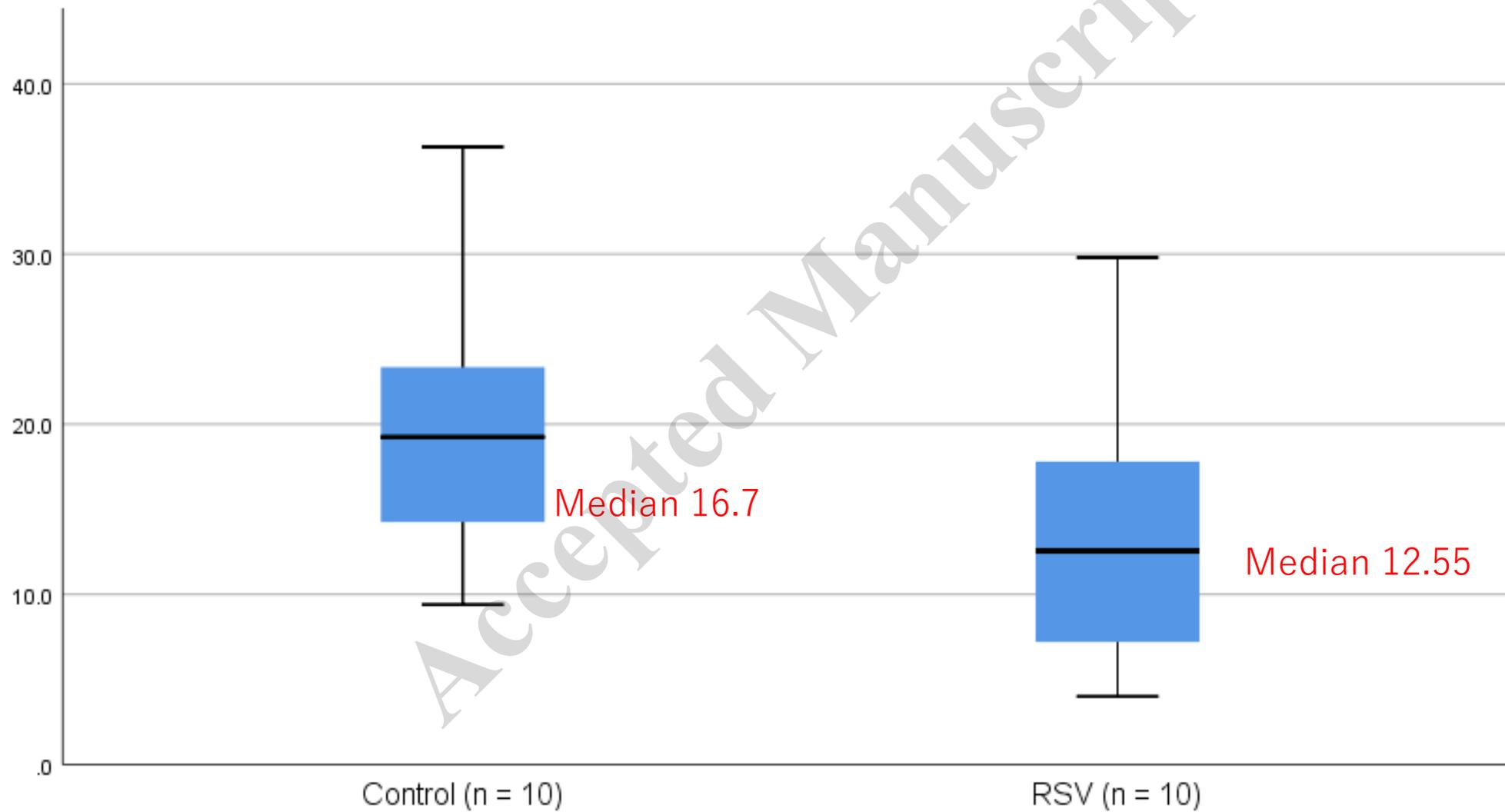
	Range (mean)		Age (months)	Bronchiolitis vs pneumonia	Duration of admission	Respiratory support	Severity score	Birth weight	RR	WBC	Hg	CRP	SpO <sub>2</sub>	PCO <sub>2</sub>	HCO <sub>3</sub>	25-OH vitamin D	NT-pro BNP
Duration of admission (days)	3–27 (6.66)	<i>r</i>	-0.356	0.5	1	<b>.771**</b>	0.565	-0.328	0.114	-0.382	0.315	<b>.871**</b>	-0.451	<b>.944**</b>	<b>.855**</b>	0.039	0.084
		<i>p</i>	0.313	0.141		0.009	0.088	0.388	0.755	0.276	0.375	0.001	0.262	0	0.002	0.915	0.818
Respiratory support	Unnecessary: 1 Required O <sub>2</sub> : 2 Under artificial support: 3	<i>r</i>	-0.447	0.251	<b>.771**</b>	1	<b>.760*</b>	-0.393	0.516	-0.205	0.119	0.553	-0.324	<b>.830**</b>	<b>.885**</b>	0.317	0.379
		<i>p</i>	0.195	0.483	0.009		0.011	0.296	0.127	0.57	0.743	0.097	0.433	0.003	0.001	0.372	0.28
Severity score	1–11 (5.11)	<i>r</i>	-0.496	-0.176	0.565	<b>.760*</b>	1	-0.556	0.494	-0.025	0.506	0.366	-0.521	<b>.709*</b>	<b>.708*</b>	0.115	0.463
		<i>p</i>	0.145	0.627	0.088	0.011		0.12	0.147	0.945	0.136	0.298	0.185	0.022	0.022	0.752	0.178
25-OH vitamin D (ng/mL)	< 4–29.8 (14.3)	<i>r</i>	0.459	0.064	0.039	0.317	0.115	0.219	0.087	0.532	-0.445	0.216	-0.399	-0.004	0.07	1	-0.054
		<i>p</i>	0.182	0.861	0.915	0.372	0.752	0.571	0.811	0.113	0.197	0.548	0.328	0.992	0.849		0.883
NT-pro BNP (pg/mL)	137–4,090 (932.6)	<i>r</i>	-0.523	-0.01	0.084	0.379	0.463	-0.36	0.575	0.079	0.028	-0.272	0.405	0.24	0.14	-0.054	1
		<i>p</i>	0.121	0.978	0.818	0.28	0.178	0.341	0.082	0.829	0.939	0.446	0.32	0.503	0.7	0.883	

\**P* < 0.01), \*\**P* < 0.05), *r* and *p* represent the Pearson *r*-correlation.

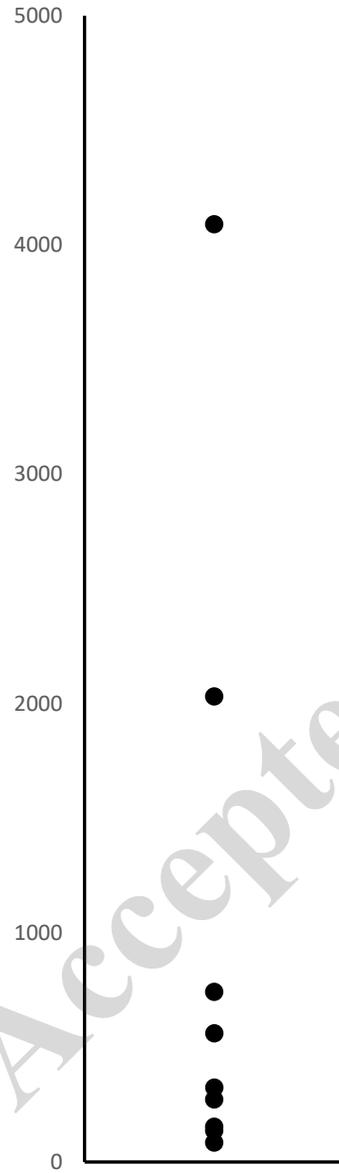
Bold values represent a statistically significant correlation between both markers or factors by SPSS.

# Serum 25-OH vitamin D

ng/mL



NT-proBNP  
(pg/mL)



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