Current Vitamin D Status in Healthy Japanese Infants and Young Children

Satoshi NAKANO1, Mitsuyoshi SUZUKI1,*, Kei MINOWA1, Saeko HIRA1, Noriyuki TAKUBO1, Yoko SAKAMOTO2, Munenaki ISHIHMA3, Eri HOSHINO4, Akifumi TOKITA5 and Toshiaki SHIMIZU1

1Department of Pediatrics, Juntendo University Faculty of Medicine, Tokyo 113–8421, Japan
2Department of Orthopedic Surgery, Juntendo University Nerima Hospital, Tokyo 177–8521, Japan
3Department of Orthopedics Surgery, Juntendo University Faculty of Medicine, Tokyo 113–8421, Japan
4Graduate School of Public Health, St. Luke International University, Tokyo 104–0044, Japan
5Clinic Bambini, Tokyo 108–0071, Japan

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Summary This study aimed to characterize serum 25-hydroxyvitamin D (25OH-D) values among Japanese children aged ≤48 mo. The study included 290 healthy infants and young children aged 0–48 mo (males/females=166/124) living in Shizuoka or Tokyo. The subjects were divided into three groups by age (Low Age: 0–5, Middle Age: 6–15, High Age: 16–48 mo). The vitamin D deficient state was defined as 25OH-D <12 ng/mL, the insufficient state as 12–20 ng/mL, and the sufficient state as ≥20 ng/mL. The seasonal variation of serum 25OH-D levels was also analyzed. The median serum 25OH-D levels in each group were: Low Age (n=50), 19 ng/mL; Middle Age (n=94), 30 ng/mL; and High Age (n=146), 30 ng/mL. The serum 25OH-D level was significantly lower in the Low Age group than in the other groups (p<0.01). Serum 25OH-D levels in summer and autumn (n=149) were significantly higher than in winter and spring (n=141) (33 vs. 25 ng/mL, p<0.01). In the Low Age group, there was a significant difference in serum 25OH-D levels between breast-fed infants (n=26) and formula-fed or mixed-fed infants (n=19) (12 vs. 32 ng/mL, p<0.01). However, there were no significant differences in 25OH-D levels between the two season classifications in either breast-fed or formula-fed and mixed-fed infants. Although clinical symptoms were not available, more than 75% of the breast-fed infants and 14.6% of infants and young children to whom food had been introduced were defined as having a vitamin D deficient or insufficient state. Breastfeeding seems one of the contributing factors to lower serum 25 OH-D levels among infants ≤5 mo of age.

Key Words vitamin D deficiency, 25-hydroxyvitamin D, sunlight exposure, breast feeding

Vitamin D is an essential nutrient for the maintenance of serum calcium levels. Vitamin D deficiency (VDD) causes rickets and hypocalcemia, which may lead to tetany or seizures in children (1). By the end of the 19th century, some reports estimated that more than 80–90% of children in the industrialized cities of North America and Europe suffered from rickets. After Sniadecki et al. suggested in 1,822 that sunbathing was the most important method for prevention and treatment of rickets, the incidence of this disease decreased dramatically, especially in North America and Europe (1, 2). However, children with rickets are still found today in every country, including the industrialized countries (3). This situation is conjectured to be influenced by maternal VDD, prolonged breastfeeding without appropriate complementary feeding from 6 mo, high latitude, season (winter and spring), dark skin pigmentation, restricted sun exposure, predominantly indoor living, and a low vitamin D diet (4).

Unification of the various definitions of VDD (the cutoff values of serum 25OH-D) has only recently occurred after a long-time period. In 2013, the Japanese Society for Pediatric Endocrinology (JSPE) issued guidelines for 25OH-D levels in which VDD was defined as levels ≤20 ng/dL (5). Global consensus recommendations were also released in 2016 (4), with the goal of becoming the universal standard. The consensus defined the cutoff values of 25OH-D as follows: <12 ng/mL (30 nmol/L) for VDD, 12–20 ng/mL (30–50 nmol/L) for insufficiency, and >20 ng/mL (50 nmol/L) for sufficiency. These values correspond to the standards of the American Academy of Pediatrics (AAP) (6) and the Institute of Medicine (IOM) (7). Many previous studies have suggested serum 25OH-D reference levels in healthy children (8–14). However, measurement of serum 25OH-D levels was not covered by health insurance in Japan; therefore, serum bone type alkaline phosphatase (BAP) and/or intact parathyroid hormone (iPTH) were used as diagnostic indicators of VDD until July 2016, and reference levels for serum 25OH-D in the Japanese population, especially in children ≤48 mo of...
The present study aimed to characterize the serum 25OH-D values among healthy Japanese children, focusing on those 6–15 mo of age. At the same time, the relationship between serum 25OH-D values and infant feeding methods was investigated.

### MATERIALS AND METHODS

#### Study population
A total of 290 healthy children aged 0–48 mo, living in Shizuoka (rural) or Tokyo (urban), Japan, were enrolled in this study (Table 1). The study group consisted of 166 males and 124 females. The participants underwent physical examinations and blood tests at Tokita Genki Clinic, Shizuoka, Japan (located at 35.2˚N, 138.4˚E) or Juntendo University Hospital, Tokyo, Japan (35.4˚N, 139.4˚E) from May 2011 to July 2012. All children were term infants and had no chronic diseases, including parathyroid or cholestatic disease, or any history of vitamin D supplementation. Blood samples of the study subjects were taken when children or their parents requested blood group or allergy tests; when it was necessary to screen for breast milk jaundice by blood examinations; or when children with minor surgical conditions such as inguinal hernia or cryptorchidism required blood examinations before surgery. According to the support guide for breastfeeding/weaning provided by the Japanese Ministry of Health, Labour and Welfare in 2005, most infants more than 6 mo old (91.7%) have started eating baby foods (http://www.mhlw.go.jp/shingi/2007/03/dl/s0314-17.pdf). At the same time, many children (80.3%) have completed weaning by 15 mo of age. Therefore, the participants were divided into three groups according to age (Low Age [0–5 mo], a group that relies on breast milk and/or formula as their main nutritional source; Middle Age [6–15 mo], a group taking baby foods in addition to...
breast milk and/or formula; and High Age [16–48 mo], a group in which the main nutritional source has shifted from baby foods to children’s diet.

Definitions. The vitamin D deficient state (<12 ng/mL = 30 nmol/L), insufficient state (12–20 ng/mL = 30–50 nmol/L), and sufficient state (>20 ng/mL = 50 nmol/L) have been defined according to clinically accepted ranges (4). According to the publication “Diagnosis guidance for vitamin D deficient rickets and hypocalcemia” issued in 2013 by the JSPE, VDD is classified into either vitamin D deficient rickets or vitamin D deficient hypocalcemia (http://jspe.umin.jp/medical/files_vitaminD.pdf). This guideline also states that patients with these two diseases must have some clinical symptoms, such as lower limb deformity (including bowlegs and X-legs), limping, excruciate, craniotabes, large fontanel openings, rachitic rosary, recessed Harrison’s groove, arthrocele, pathological fracture, or failure to thrive. Therefore, we use two similar, but different terms in this report. One is “VDD”, and the other is “vitamin D deficient state”. VDD refers to children who are diagnosed as having vitamin D deficient rickets or hypocalcemia; these children have some clinical symptoms together with low serum 25OH-D values. The term “vitamin D deficient state” refers to children without any clinical symptoms who solely have low serum 25OH-D values.

The four seasons are defined according to the Japan Meteorological Agency as follows: the spring period is from March to May, the summer period is from June to August, the autumn period is from September to November, and the winter period is from December to February. It is well known that serum 25OH-D levels are influenced by the amount of solar irradiation. The division of the year into the four seasons reflects the amount of sun exposure well. Serum 25OH-D levels tend to fall in the winter and spring, and rise in the summer and autumn (10, 11, 15). Hence, the relationship between serum 25OH-D levels and seasons at the times when the participants underwent blood examinations in the present study was also analyzed.

Assay. Within 2 h of collection, blood samples were centrifuged for 10 min at 3,000 rpm to separate the serum, and they were then stored at −20°C until measurement. Recipient vitamin D status was measured by assessing circulating levels of 25OH-D in serum samples that had been stored frozen, having never been previously thawed, using a radioimmunoassay (RIA) (25-hydroxyvitamin D 125I RIA Kit, Diasorin, Saluggia, Italy; SRL, Inc., Tokyo, Japan). This assay is a non-chromatographic quantification of circulating 25OH-D using an 125I-labeled reporter. Since an antigen used in this assay would generate an antibody that is co-specific for two different vitamin D distinct forms, 25OH-D2 (ergocalciferol) and 25OH-D3 (cholecalciferol), this RIA kit recognizes 25OH-D2 equally as well as 25OH-D3 and is unable to distinguish them (16, 17). Blood sample values below the measurement sensitivity of this kit (with 25OH-D < 5 ng/mL) were regarded as 5 ng/mL. Serum BAP and iPTH were measured by a chemiluminescent immunoassay (CLIA) (SRL, Inc., Tokyo, Japan). Serum albumin (Alb), alanine aminotransferase (ALT), creatinine (Cre), calcium (Ca), phosphorus (Pi), magnesium (Mg), and alkaline phosphatase (ALP) levels were measured in our institute; the accuracy of these tests was assured by the Japanese Society of Laboratory Medicine. The normal ranges of these serum biochemical markers were those previously described (18).

Statistical analysis. The serum value of each blood measurement is expressed as the median, and the interquartile range (IQR) is shown. The Kruskal-Wallis test was used to compare continuous variables in the three age groups, and the chi-squared test was used for binary outcomes. The Mann-Whitney U test compared continuous variables in the two feeding method groups and the two season classifications. The chi-squared test was used to compare binary variables. Post hoc multiple comparisons were made using Dunn’s test for continuous variables. All statistical analyses were performed using Stata version 14.1 (Light Stone, Inc., Tokyo, Japan). p-values <0.05 were considered significant.

Ethical considerations. This study was approved by the institutional review board at Juntendo University (approval number 24-174). Informed consent was obtained from the parents of each subject prior to enrollment in the study. The study was also in compliance with the 1964 Helsinki Declaration and its later amendments (as revised in Edinburgh 2000) or comparable ethical standards.

RESULTS

Serum 25OH-D levels by age group

Among all children, 21 (7.2%) were in a vitamin D deficient state, 40 (13.8%) were in an insufficient state, and 229 (79.0%) were in a sufficient state (Table 1). The median (IQR) 25OH-D levels in each age group were 19 (25) ng/mL in the Low Age group, 30 (17) ng/mL in the Middle Age group, and 30 (13) ng/mL in the High Age group. The median 25OH-D level was significantly lower in the Low Age group than in the other groups (p<0.01).

Among the 240 infants and young children in the Middle and High Age groups to whom food had been introduced, 25OH-D levels were at the deficient state in 4 (1.7%), the insufficient state in 31 (12.9%), and the sufficient state in 205 (85.4%). In contrast, in the Low Age group, the number of children in the deficient state was 17 (34%), in the insufficient state was 9 (18%), and in the sufficient state was 24 (48%). The number of children in the deficient state was significantly lower in the Low Age group than in the other groups (p<0.01).

Differences in serum 25OH-D levels according to feeding method in the 0–5 mo age group

The relationship between serum 25OH-D levels and feeding method in the 0–5 age group has been validated. Unfortunately, 5 infants in the Low Age group were excluded from this analysis because there was not enough information about their feeding methods. In the remaining 45 infants in the Low Age group, 26 infants received breastfeeding, and 19 infants received formula
or mixed feeding (Table 2). There were no significant differences between these two groups in their background characteristics except serum creatinine and phosphorus levels. The median 25OH-D level was significantly lower in the breast-fed infants (12 ng/mL) than in the formula or mixed-fed infants (32 ng/mL; \( p, 0.01 \)). At the same time, the number of children in the deficient state was significantly higher in the breast-fed infants (13/26, 50%) than in the formula or mixed-fed infants (2/19, 10.5%; \( p, 0.01 \)).

### DISCUSSION

The present study showed that the prevalence of the vitamin D deficient (\(<12\) ng/mL) and insufficient (\(\geq 12\) to 20 ng/mL) states was 21% (61/290), and revealed that the incidence of the vitamin D deficient state among infants \(\leq 5\) mo of age was 34% (17/50). In par-

### Table 2. Distribution of serum 25OH-D levels in infants aged 0–5 mo.

| Demographics | Total (n=45) | Breast-fed (n=26) | Formula/Mixed-fed (n=19) | \( p\)-value |
|--------------|-------------|-------------------|--------------------------|-------------|
| Age (months), median (IQR) | 3 (3) | 3 (3) | 2 (4) | 0.91 |
| Sex (male), n (%) | 26 (57.8) | 17 (65.4) | 9 (47.4) | 0.23 |
| Area (Shizuoka), n (%) | 21 (46.7) | 10 (38.5) | 11 (57.9) | 0.20 |
| Body weight (kg), median (IQR) | 5.7 (2.0) | 5.6 (1.8) | 5.8 (2.4) | 0.76 |

| Season | Total (n=45) | Breast-fed (n=26) | Formula/Mixed-fed (n=19) | \( p\)-value |
|--------|-------------|-------------------|--------------------------|-------------|
| Summer and Autumn, n (%) | 22 (48.9) | 15 (57.7) | 7 (36.8) | 0.17 |

| Clinical parameter | Total (n=45) | Breast-fed (n=26) | Formula/Mixed-fed (n=19) | \( p\)-value |
|-------------------|-------------|-------------------|--------------------------|-------------|
| Alb (g/dL), median (IQR) | 4.3 (0.7) | 4.2 (0.6) | 4.4 (0.7) | 0.14 |
| ALT (IU/L), median (IQR) | 28 (16) | 29 (19) | 27 (15) | 0.92 |
| Cre (mg/dL), median (IQR) | 0.20 (0.10) | 0.17 (0.09) | 0.21 (0.06) | <0.05 |
| Ca (mg/dL), median (IQR) | 10.5 (0.6) | 10.5 (0.6) | 10.2 (0.7) | 0.35 |
| Pi (mg/dL), median (IQR) | 6.0 (1.0) | 5.8 (0.5) | 6.2 (1.2) | <0.05 |
| Mg (mg/dL), median (IQR) | 2.3 (0.3) | 2.3 (0.2) | 2.4 (0.3) | 0.08 |
| ALP (IU/L), median (IQR) | 1.134 (612) | 1.160 (666) | 1.095 (413) | 0.67 |
| BAP (mg/L), median (IQR) | 95.8 (53.9) | 96.0 (65.4) | 94.3 (31.6) | 0.28 |
| iPTH (pg/mL), median (IQR) | 16 (20) | 18 (23) | 16 (15) | 0.69 |
| 25OH-D (ng/mL), median (IQR) | 19 (23) | 12 (13) | 32 (14) | <0.01 |

Area: Shizuoka (35.2˚N, 138.4˚E) or Tokyo (located at 35.4˚N, 139.4˚E).
Seasons: Spring: from March to May; Summer: from June to August; Autumn: from September to November; and Winter: from December to February. Season represents times when the participants underwent blood examinations in the present study.
IQR: interquartile range, Alb: albumin, ALT: alanine aminotransferase, Cre: creatinine, Ca: calcium, Pi: phosphorus, Mg: magnesium, ALP: alkaline phosphatase, BAP: bone type alkaline phosphatase, iPTH: intact parathyroid hormone, 25OH-D: 25-hydroxyvitamin D.

The Mann-Whitney U test compared continuous variables in the two feeding method groups and the chi-squared test was used for binary variables.
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Table 3. Differences in serum 25OH-D levels by season.

|                          | Summer and Autumn | Winter and Spring | p-value |
|--------------------------|-------------------|-------------------|---------|
| Total [0–48 mo (n=290)  |                   |                   |         |
| 25OH-D level (ng/mL), median (IQR) | 33 (15)           | 25 (14)           | <0.01   |
| Age                      |                   |                   |         |
| Low Age [0–5 mo (n=50)]  |                   |                   |         |
| 25OH-D level (ng/mL), median (IQR) | 19 (22)           | 20 (25)           | 0.59    |
| Middle Age [6–15 mo (n=94)|                   |                   |         |
| 25OH-D level (ng/mL), median (IQR) | 34 (13)           | 25 (18)           | <0.01   |
| High Age [16–48 mo (n=146)|                   |                   |         |
| 25OH-D level (ng/mL), median (IQR) | 35 (12)           | 26 (9)            | <0.01   |
| Feeding methods among infants of Low Age [0–5 mo] |                   |                   |         |
| Breast-fed (n=26)        |                   |                   |         |
| 25OH-D level (ng/mL), median (IQR) | 14 (15)           | 10 (7)            | 0.12    |
| Formula/Mixed-fed (n=19) |                   |                   |         |
| 25OH-D level (ng/mL), median (IQR) | 32 (19)           | 33 (13)           | 0.73    |

Seasons: Spring: from March to May; Summer: from June to August; Autumn: from September to November; and Winter: from December to February.

IQR: interquartile range. 25OH-D: 25-hydroxyvitamin D.
The Mann-Whitney U test compared continuous variables in the two season classifications.

ticular, 50% (13/26) of breast-fed infants showed a vitamin D deficient state. The vitamin D deficient state does not continue throughout infancy, since the 25OH-D level rises after weaning at the appropriate time. In considering child development and growth, the nutrition of infants ≤5 mo of age depends mostly on breast milk or formula (http://www.mhlw.go.jp/shingi/2007/03/dl/s0314-17.pdf). Yorifuji et al. focused on 1-mo-old normal infants with craniotabes and reported that serum 25OH-D levels were <10 ng/mL in 37.3% of infants, but especially in 56.9% of breast-fed infants (19). A global study including children from the USA, China, and Mexico also demonstrated that formula feeding is an independent predictor of a higher serum 25OH-D concentration status in 6-mo-old infants (20).

For preventing rickets, it is generally important for children to receive not only vitamin D, but also calcium (4). Infants 0–6 mo of age should receive 200 mg/d of calcium, which is based on the calcium content of breast milk (4). However, few data exist regarding how much calcium infants of age 0–6 mo should receive, at least to prevent rickets. Considering the present study in which there was no child with rickets in the entire study population, although breast milk contains a small amount of vitamin D and about half the amount of calcium compared to formula (21), breast milk is considered to probably contain enough calcium to prevent the development of rickets. We also consider that most breast-fed infants can recover from a vitamin D deficient state without symptoms if they are weaned at the appropriate time, because the development of rickets is gradual. Breastfeeding is important in promoting attachment between mother and child and in helping children gain immunity (21–24); however, breastfeeding is also a risk factor for VDD. Therefore, an awareness of the possible development of VDD and rickets, especially in breast-fed infants and their families, is considered necessary.

It is well known that there are seasonal variations in 25OH-D levels (10, 11, 15). Since the incidence of rickets increases when serum 25OH-D levels are <12 ng/mL, the consensus advocates the importance of maintaining serum 25OH-D levels >20 ng/mL, because seasonal variations in 25OH-D range from 5.2–9.6 ng/mL (4). In the present study, the serum 25OH-D level difference between summer/autumn and winter/spring was 8 ng/mL (33 vs. 25 ng/mL, p=0.01), providing support for seasonal variations in the 25OH-D range. Interestingly, in the infants aged 0–5 mo, there were no significant differences between 25OH-D levels in the summer season and in the winter season, either in the breast-fed or formula/mixed-fed infants. Younger children usually have less opportunity to go outside (for exposure to sunlight) than older children; therefore, the serum 25OH-D levels in younger children would be more affected by oral intake than exposure to sunlight, especially in infants aged 0–5 mo.

The limitation of this study is that the dietary and lifestyle habits of the participants and their mothers were not investigated. There were no infants who received vitamin D supplementation, and there were no obvious differences in participants’ backgrounds between breast-fed and formula or mixed-fed infants. Therefore, breastfeeding seems one of the contributing factors to lower serum 25OH-D levels among infants ≤5 mo of age.

Isotope-dilution liquid chromatography-tandem mass spectrometry (isotope-dilution LC-MS/MS) has become the gold standard (25) that allows quantification of 25OH-D₂ and 25OH-D₃ separately, and it has less measurement error than other methods (26, 27). However,
the LC-MS/MS method has some problems with reproducibility of measuring vitamin D, since each laboratory uses its original standard reference material (28), requiring high technical capabilities and cost (17). On the other hand, the RIA method, which is still widely used and affordable (16), quantifies both 25OH-D$_2$ and 25OH-D$_3$. Since most of the circulating vitamin D fraction in serum is 25OH-D$_3$ (17), direct measurement of 25OH-D$_3$ by chemiluminescent enzyme immunoassay (CLIA) is approved by Japan’s health insurance, and this method has a good correlation with the RIA method (30). The data of the present study can serve as the basis for reference serum 25OH-D values in young Japanese children. Further study is needed to clarify the actual status of VDD in relation to 25OH-D levels.

**CONCLUSION**

In the present study, half of the breast-fed infants were classified as being in the vitamin D deficient state, and 14.6% of the infants and young children aged 6–48 mo to whom food had been introduced were classified as being in the vitamin D deficient and insufficient state. These results suggest that subclinical VDD in children ≤48 mo of age is not rare in Japan.

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