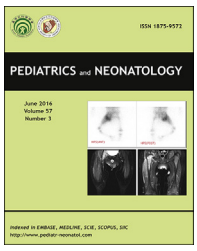
Pediatrics and Neonatology (2016) 57, 213–218

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: http://www.pediatr-neonatol.com



ORIGINAL ARTICLE

Immunoglobulin Concentrations in Plasma and Saliva During the Neonatal Period

Sandra Pineda-Martínez [a,b], José Luis Hernández-Islas [a], Mónica Patricia Escobedo-Torres [c], Iris Evelin Paredes-Alonzo [c], Carlos López-Candiani [a], Dolores Correa [a], Marcela Vela-Amieva [a,*]

[a] *Instituto Nacional de Pediatría, Secretaría de Salud, México, D.F., Mexico*
[b] *Universidad Nacional Autónoma de México, Programa de Maestría y Doctorado en Ciencias Médicas, Odontológicas y de la Salud, México, D.F., Mexico*
[c] *Hospital General Dr. Manuel Gea González, México, D.F., Mexico*

Received Mar 2, 2015; received in revised form May 18, 2015; accepted Jun 5, 2015
Available online 22 October 2015

Key Words
IgA;
IgE;
IgM;
newborn;
plasma;
saliva

Background: Screening for infectious diseases in newborns using immunoglobulin (Ig)A-, IgM-, and IgE-specific antibodies is expensive and impractical. To determine if total levels of these Igs can be used for screening purposes, thus simplifying the process, their basic levels in the 1st month of extrauterine life need to be determined. Additionally, the ability to simplify screening by using saliva also needs to be determined. The aim of this study was to determine IgA, IgM, and IgE concentrations in plasma and saliva in newborns, correlation between the samples, and relationship between Ig levels and newborn age.

Methods: We enrolled 53 apparently healthy newborns, paired samples of plasma and saliva were collected, and total IgA, IgM, and IgE concentrations determined by capture enzyme-linked immunosorbent assay. The correlation between plasma and saliva values was calculated by Spearman's rank correlation coefficient and the IgA, IgM, and IgE distributions were analyzed by the Shapiro-Wilk test. We also determined the level of each Ig concentration according to age.

Results: IgA and IgM levels in plasma and IgA levels in saliva increased significantly during 1st month of life, especially in the 2nd week and 3rd week, with a good correlation of IgA between plasma and saliva. IgE levels in both plasma and saliva and IgM levels in saliva were very low or absent.

* Corresponding author. Laboratorio de Errores Innatos del Metabolismo y Tamiz, Instituto Nacional de Pediatría, Avenida Insurgentes Sur 3700-C Colonia Insurgentes Cuicuilco, C.P. 04530, México, D.F., Mexico.
E-mail address: dravelaamieva@yahoo.com (M. Vela-Amieva).

http://dx.doi.org/10.1016/j.pedneo.2015.06.005

Conclusion: These results suggest that Igs in saliva could be good biomarkers for newborn screening programs during the 1st week of life. This study established reference values for Igs according to age in the neonatal period.

1. Introduction

Immunoglobulins (Igs) are humoral immunity molecules produced in response to internal and external antigens.[1] These proteins are synthesized *in utero* from the 1st week of gestation in response to maternal or other substances that cross the placenta, or as result of an active infection of the fetus.[2] Different Ig types are present in neonatal blood, with IgG being a mixture of antibodies synthesized by the fetus and those of the mother transferred by the chorionic villi FcRn receptor.[3] Conversely, maternal IgA, IgM, and IgE antibodies do not cross the placenta, therefore, these concentrations reflect the fetal immune response.[4]

Abnormal IgM, IgA, or IgE levels have been found as a result of fetal or newborn response to congenital infections, sepsis, allergies, stress, or exposure to environmental toxins (i.e., lead). Therefore, the detection of abnormal levels of these Igs could be useful for identification of health risks in the neonate.[5–9] In fact, postnatal screening programs for congenital infectious diseases, such as toxoplasmosis, have been implemented based on the search for specific antibodies associated with these Igs as primary biomarkers.[9,10]

Immunoglobulins are usually measured in venous or capillary blood, however, alternative samples, including saliva, have also been used for young children, given the non-invasive nature and ease of collection.[11] Specific antibodies against *Trypanosoma cruzi*, *Toxoplasma gondii*, and hepatitis B and C have been found in adult saliva, however, studies in newborns or infants using this sample as a biological matrix are scant.[12–15] Although total IgM and IgA levels have been measured in serum from the umbilical cord and 1-month-old infants, there are few data concerning their concentrations within the neonatal period.[16] Moreover, IgM and IgE levels in newborn saliva are practically unknown.[17–23] Therefore, the objective of this study was to determine total IgA, IgM, and IgE concentrations in the plasma and saliva of newborns, the correlation between samples, and the relationships between these levels and age.

2. Methods

2.1. Studied population and data collection

The study included a subset of newborns who participated in the large multicenter project, "Prenatal and neonatal screening for congenital infections in Mexico City". The study protocol was approved by the Research and Ethics Reviewing Board of Instituto Nacional de Pediatría, México, D.F., Mexico (registry number 002/2008). All procedures were explained to the parents or guardians, who signed an informed consent form. Apparently healthy children born at the Hospital General Dr. Manuel Gea González in Mexico City, Mexico were enrolled in this study. Only those cases with effective paired blood and saliva samples were included for the analysis. The exclusion criteria were the presence of maternal chronic disease, as well as a low Apgar score at birth ($<$ 6 at 1 minute or 5 minutes), a congenital abnormality, suspected infection, or transfusion with blood derivatives between birth and the day of sample collection.

Information on gestational age, health of mother and of child, and type of newborn feeding was obtained by an interview with the guardian and clinical data from the hospital.

2.2. Sample collection

All samples were collected from newborns following a 2 hour fast. Whole unstimulated saliva (100 μL) was obtained using sterile polypropylene transfer pipettes and placed in tubes with 3×10^{-4} g protease-inhibitor cocktail (Sigma P2714; Sigma-Aldrich, St. Louis, MO, USA). Samples were transported on ice to the laboratory where they were centrifuged at 176 g for 5 minutes and the supernatants stored at −80°C until analysis.

Blood samples were obtained by venopuncture using Becton Dickinson devices (Sigma-Aldrich). Blood was initially sampled in a heparinized Vacutainer tube (BD Bioscience, Franklin Lakes, NJ, USA), plasma separated by centrifugation, and aliquots prepared, frozen, and stored at −80°C until use.

2.3. Immunoglobulin quantification

Total IgA, IgM, and IgE concentrations in saliva and plasma were determined by capture enzyme-linked immunosorbent assay (ELISA). Briefly, 96-well plates (Maxisorb Nalge Nunc International, Rochester, NY, USA) were coated with 100 μL of the capture antibodies (anti-IgA, 2 μg/mL; anti-IgM, 0.125 μg/mL; and anti-IgE, 3 μg/mL) in 0.01M borate buffer (pH 8.0) and incubated at 4°C overnight. At each reaction step, three 5-minute washes with 200 μL 0.01M phosphate-buffered saline (PBS; pH 7.2) with 0.5% Tween 20 (PBS-T) and two 5-minute washes with PBS only were undertaken. The nonspecific-binding free sites were blocked with 1% albumin diluted in PBS-T (200 μL/well) by incubation at 37°C for 30 minutes.

Samples were processed by two-fold serial dilutions starting at ratios of 1:160, 1:500, and 1:40 for IgA, IgM, and

IgE, respectively, for plasma, and 1:50 for IgA and 1:5 for IgM and IgE in the case of saliva, and samples (100 μL/well) were incubated for 2 hours at 37°C. Then, 100 μL/well of peroxidase-conjugated anti-IgA (1:4000), anti-IgM (1:2000), or anti-IgE (1:1000) were added and incubated under the same conditions. The presence of labeled antibodies was revealed using 100 μL/well of chromogenic substrate solution prepared with 5 mL sodium citrate, 5 mL citric acid, 4 μL hydrogen peroxide, and 10 mg O-phenylenediamine dihidrochloride (Sigma-Aldrich). The reaction was stopped by adding 50 μL/well 0.1 N sulfuric acid and the absorbance measured in a Turner Microplate Biosystems Modulus ELISA plate reader (Turner Biosystems Inc., Sunnyvale, CA, USA) at 490 nm. IgM, IgA, or IgE standard curves were obtained for each plate to determine the concentration of each sample by absorbance interpolation and multiplication by the dilution factor.

2.4. Statistical analysis

Correlation between plasma- and saliva-sample concentrations was determined by Spearman's rank correlation coefficient. Descriptive statistics (mean values) and 95% confidence intervals were calculated. Each Ig-concentration distribution was analyzed by Shapiro-Wilk test using the statistical program Stata version 11.1 Statistics (StataCorp., College Station, TX, USA). Data were graphed in order to visualize the dispersion of each Ig concentration in relation to age. Comparison of Ig concentrations among samples from children fed exclusively with breast milk or a combination of formula and breast milk was made by Mann–Whitney *U* test.

3. Results

3.1. Population data

Paired plasma and saliva samples from 53 apparently healthy newborns were obtained, with 31 males (58.5%) and 22 females (41.5%). Mean gestational age at birth was 38.6 weeks [standard deviation (SD), 1.42; range, 34.2–41.6]. Mean birth weight was 2976.3 g (SD, 496.4; range, 857.0–3918 g), while mean height was 48.8 cm (SD, 2.5; range, 38.0–53.0 cm). Age at sample collection was 15.4 days (SD, 6.75; range, 1–29 days). Forty-two children (79.2%) were full term, five children (9.5%) were pre-term, and six children (11.3%) were post-term.

Almost half (48.9%) of the newborns were exclusively breastfed, while the rest (51.1%) received a combination of breast milk and formula. Five of the newborns received formula exclusively.

3.2. Immunoglobulin levels

The Ig concentrations in plasma and saliva are shown in Table 1. None of the Ig levels presented normal distributions in plasma or saliva. IgM values in saliva were null, and IgE in both plasma and saliva samples were almost undetectable.

A significant positive relationship between age and total IgA and IgM levels in plasma and IgA levels in saliva was observed during the 1st month of life, with very low levels observed in the 1st week (Figures 1 and 2). Conversely, IgE concentrations were unrelated to age in the neonatal period (Figure 3). Statistically significant correlations between plasma and saliva samples were observed only for IgA ($r_s = 0.6$; $p < 0.001$).

IgM values in saliva were slightly higher in those infants fed with a combination of breast milk and formula (mean, 28.1 μg/mL) as compared to those exclusively undergoing breast feeding (mean, 19.7 μg/mL, $p = 0.026$).

4. Discussion

Following birth, the newborn is exposed to a very different environment charged with antigens, including normal flora microorganisms, vaccines, and breast or formula milk. The exposure to these immunogenic substances may cause an immune response and the creation of antibodies, which is likely to be reflected by changes in total Ig values. Few studies have examined total concentration of various Ig classes in umbilical cord or peripheral blood from infants. Jolliff et al[16] followed a cohort monthly up to the 1st year of life and annually for 12 years, however, variations observed within the 1st month were not studied. This period represents an important adaptation phase of the individual to the environment involving multiple physiological changes, therefore, variations in Ig levels are to be expected.[21–25] The main contribution of this study was our demonstrated significant correlation between age and IgA levels in both plasma and saliva, as well as IgM levels in

Table 1 Mean IgA, IgM, and IgE concentrations in newborn plasma and saliva ($n = 53$).

	Sample type	Mean ± SD	95% CI	Minimum	Maximum	Median
IgA	Plasma	54.1 ± 63.8	36.5–71.7	0.3	317.4	36.1
	Saliva	24.5 ± 33.2	15.3–33.6	0.0	132.0	6.7
IgM	Plasma	641.3 ± 616.0	471.5–811.1	3.2	2929.9	419.5
	Saliva	3.6 ± 12.4	0.2–7.1	0.0	80.5	0.0
IgE*	Plasma	0.5 ± 1.2	0.1–0.8	0.0	5.0	0.0
	Saliva	0.5 ± 0.7	0.2–0.7	0.0	2.7	0.1

Data are presented as concentrations (μg/mL).
* IgE was determined only in 43 newborns because the remaining samples were insufficient for analysis.
CI = confidence interval; Ig = immunoglobulin; SD = standard deviation.

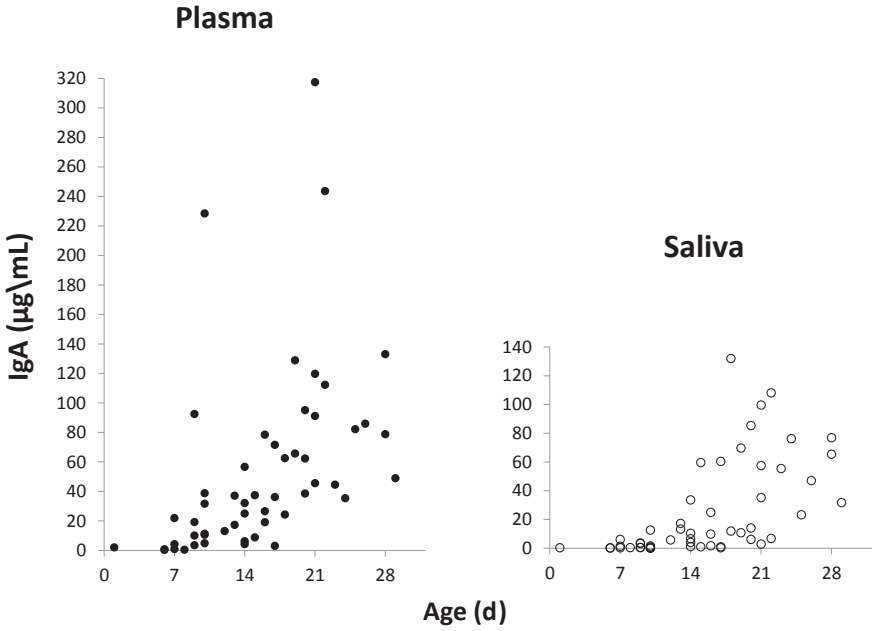


Figure 1 IgA concentration in plasma and saliva of neonates at different ages. Each dot represents one newborn ($n = 53$). IgA = immunoglobulin A.

plasma, within the 1st month of life, although this tendency was not observed for either IgM in saliva or for IgE in plasma or saliva. It is important to note that IgM and IgE values in saliva samples from the 1st week of life were practically null, which could make them useful markers indicating *in utero* exposure to allergens, infections, or other toxins.[4–9]

The concentrations of IgA in plasma and saliva and of IgM in plasma were much higher in neonates older than 2 weeks. These findings are important for establishing Ig neonatal reference values for the design of newborn screening programs based on these Igs as biomarkers. They may also be useful for establishing proper sampling times, which are likely between the 1st day and 7th day of life.

Since it is a secretory protein of mucosa, we confirmed our expectations to observe IgA in saliva. Previous reports involving Latin American populations from Chile and Venezuela showed IgA concentrations of 32.9 µg/mL and 30.0 µg/mL, respectively, with variations similar to those reported here.[19,20]

The correlation of IgA values between plasma and saliva observed here support the use of the latter as a good alternative for screening studies, given that the sample collection is simple, low-cost, non-invasive, and acceptable

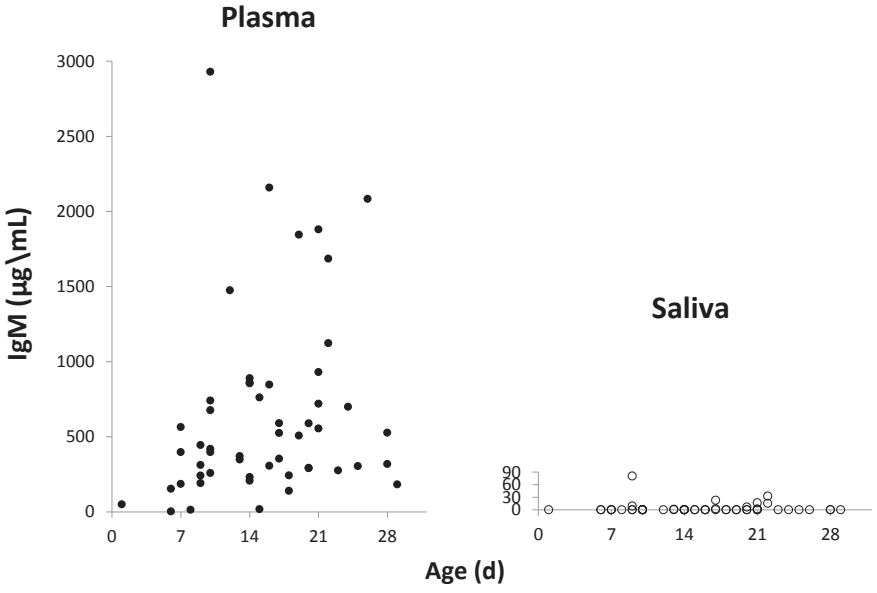


Figure 2 IgM concentration in plasma and saliva of neonates at different ages. Each dot represents one newborn ($n = 53$). IgA = immunoglobulin A.

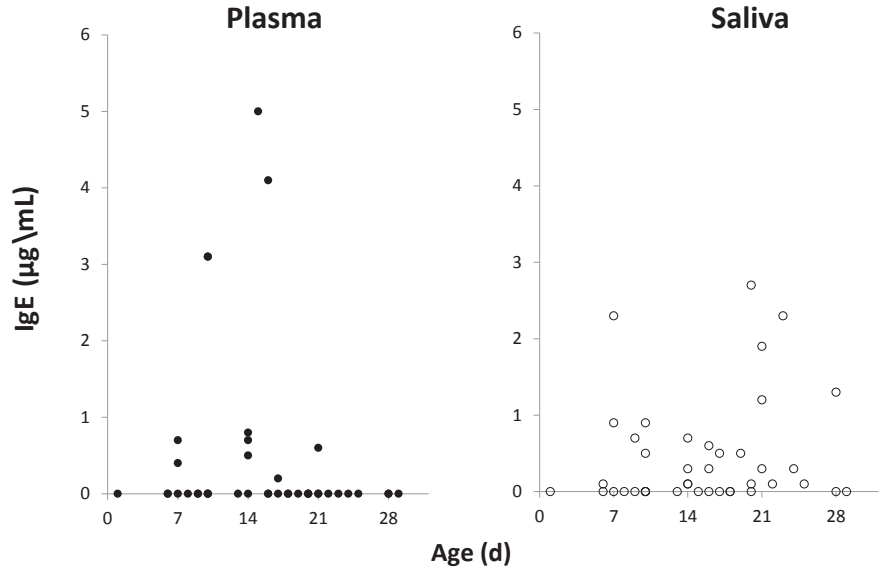


Figure 3 IgE concentration in plasma and saliva of neonates at different ages. Each dot represents one newborn ($n = 43$). IgA = immunoglobulin A.

to parents. Unlike previous studies, we found no effect of the feeding type on IgA levels. Additionally, these studies examined antigen-specific antibodies, while we measured the total IgA concentration.[24,25] Interestingly, total IgM appeared correlated to formula feeding. Although this correlation was not statistically significant, this finding could be biologically relevant and should be taken into account to determine reference levels.

There is a need to establish normal ranges for IgA, IgM, and IgE levels in blood and saliva samples in early infancy, given their potential use as biomarkers for the diagnosis of diverse diseases. In this study conducted in apparently healthy newborns, we found cases with very high plasmatic levels of IgA (> 200 μg/mL) or IgM (> 1000 μg/mL). These children were not harboring an infectious disease associated with TORCH (Toxoplasma, rubella, cytomegalovirus, herpes simplex and other agents) syndrome, such as syphilis, HBV, or HBC, as determined by laboratory and clinically specific diagnosis. High levels of Igs can also be related to other factors, such as maternal immunization just prior to or during pregnancy, substance abuse, allergies, or hyper-immunoglobulin syndromes.[5–9,26] Unfortunately, we did not take these factors into account, therefore, they must be included in future studies on this topic. Furthermore, the small sample size necessitates study of larger groups to definitively establish relationships between high levels of Igs to specific biological or pathological features.

We observed significant increases in levels of IgA and IgM in serum and saliva during the 1st month of life, especially in the 2nd week and 3rd week. Our study contributes to establishing reference values according to age in the neonatal period. IgA determination in saliva may be a good biomarker for possible newborn screening programs, with the ideal sampling age being from Day 1 to Day 7 of life. Conversely, both samples exhibited low IgE levels, indicating the possibility that high levels of this Ig should be examined carefully by clinicians, although further studies regarding this finding are needed.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

The authors of this work are indebted to Blanca-Luz Tecuátl-Herrada and Liliana Hernández Luengas for their invaluable support. This work was partially funded by grant No. 69666 from the Consejo Nacional de Ciencia y Tecnología of Mexico (CONACYT).

References

1. Delves J, Roitt IM. The immune system. First of two parts. *N Engl J Med* 2000;**343**:37–49.
2. Correa D, Cañedo-Solares I, Ortiz-Alegría LB, Caballero-Ortega H, Rico-Torres CP. Congenital and acquired toxoplasmosis: diversity and role of antibodies in different compartments of the host. *Parasite Immunol* 2007;**29**:651–60.
3. Simister NE. Placental transport of immunoglobulin G. *Vaccine* 2003;**21**:3365–9.
4. McMurray DN, De Aly AC, Rey H. Serum IgM as an indicator of intrauterine infection in Colombian newborns. *Bull Pan Am Health Organ* 1980;**14**:376–85.
5. Andersen NJ, Mondal TK, Preissler MT, Freed BM, Stockinger S, Bell E, et al. Detection of immunoglobulin isotypes from dried blood spots. *J Immunol Methods* 2014;**404**:24–32.
6. Peters JL, Cohen S, Staudenmayer J, Hosen J, Platts-Mills TA, Wright RJ. Prenatal negative life events increases cord blood IgE: interactions with dust mite allergen and maternal atopy. *Allergy* 2012;**67**:545–51.
7. Annesi-Maesano I, Pollitt R, King G, Bousquet J, Hellier G, Sahuquillo J, et al. *In utero* exposure to lead and cord blood total IgE. Is there a connection? *Allergy* 2003;**58**:589–94.
8. Seydel LS, Petelski A, van Dam GJ, van der Kleij D, Kruize-Hoeksma YC, Luty AJ, et al. Association of *in utero* sensitization to *Schistosoma haematobium* with enhanced cord blood

IgE and increased frequencies of CD5—B cells in African newborns. *Am J Trop Med Hyg* 2012;**86**:613—9.
9. Paul M, Petersen E, Szczapa J. Prevalence of congenital *Toxoplasma gondii* infection among newborns from the Poznań region of Poland: validation of a new combined enzyme immunoassay for *Toxoplasma gondii*-specific immunoglobulin A and immunoglobulin M antibodies. *J Clin Microbiol* 2001;**39**: 1912—6.
10. Gómez-Marin JE, de la Torre A, Angel-Muller E, Rubio J, Arenas J, Osorio E, et al. First Colombian multicentric newborn screening for congenital toxoplasmosis. *Plos Negl Trop Dis* 2011;**5**:e1195.
11. Nogueira RD, Sesso ML, Borges MC, Mattos-Graner RO, Smith DJ, Ferriani VP. Salivary IgA antibody responses to *Streptococcus mitis* and *Streptococcus mutans* in preterm and fullterm newborn children. *Arch Oral Biol* 2012;**57**:647—53.
12. Moretti E, Basso B, Gil P, Vaca B, Josefina J, Yasenzaniro P. Detection of antibodies to Trypanosoma cruzi and Toxoplasma gondii in oral mucosal transudate. *Acta Bioquím Clín Latinoam* 2004;**38**:159—63. [Article in Spanish].
13. Zmuda JF, Wagoneer B, Liotta L, Whiteley G. Recognition of multiple classes of hepatitis C antibodies increases detection sensitivity in oral fluid. *Clin Diagn Lab Immunol* 2001;**8**:1267—70.
14. Loyola AM, Durighetto Jr AF, Silva DA, Mineo JR. Anti-*Toxoplasma gondii* immunoglobulins A and G in human saliva and serum. *J Oral Pathol Med* 1997;**26**:187—91.
15. Taylor CE, Toms GL. Immunoglobulin concentrations in nasopharyngeal secretions. *Arch Dis Child* 1984;**59**:48—53.
16. Jolliff CR, Cost KM, Stivrins PC, Grossman PP, Nolte CR, Franco SM, et al. Reference intervals for serum lgG, IgA, IgM, C3, and C4 as determined by rate Nephelometry. *Clin Chem* 1982;**28**:126—8.
17. Seidel MB, Schulze B, Kiess W, Vogtmann C, Borte M. Determination of secretory IgA and albumin in saliva of newborn infants. *Biol Neonate* 2000;**78**:186—90.
18. Seidel MB, Schubert S, Schulze B, Borte M. Secretory IgA, free secretory component and IgD in saliva of newborn infants. *Early Hum Dev* 2001;**62**:159—64.
19. Tomasello E, Arévalo QF, Fuenzalida ME, Torres Kameid E. Secretory immunoglobulin A in salivary secretions of healthy children. *Rev Chil Pediatr* 1981;**52**:227—32 [Article in Spanish].
20. Chacón MR, Flores ME, Rodríguez D, Valles L, Petrola C, Torres A, et al. Levels of secretory immunoglobulin A in saliva in a healthy population of the State of Carabobo, Venezuela. *Salus online* 2004;**1**:8—16. Available at http://salus-online.fcs.uc.edu.ve/t1_igas_saliva.pdf. Accessed November 23, 2015. [Article in Spanish].
21. Stephens S. Development of secretory immunity in breast fed and bottle fed infants. *Arch Dis Child* 1986;**61**:263—9.
22. Weemaes C, Klasen I, Göertz J, Beldhuis-Valkis M, Olafsson O, Haraldsson A. Development of immunoglobulin A in infancy and childhood. *Scand J Immunol* 2003;**58**:642—8.
23. Tapia C, Quiroga T. Secretory IgA in saliva of healthy children. *Rev Chil Pediatr* 1998;**69**:72—6. [Article in Spanish].
24. Avanzini MA, Plebani A, Monafo V, Pasinetti G, Teani M, Colombo A, et al. A comparison of secretory antibodies in breast-fed and formula-fed infants over the first six months of life. *Acta Paediatr* 1992;**81**:296—301.
25. Cheng MM, Huang CF, Yang LY, Lin YG, Peng HJ, Chang CY, et al. Development of serum IgA and IgM levels in breast-fed and formula-fed infants during the first week of life. *Early Hum Dev* 2012;**88**:743—5.
26. Nandan D, Nag VK, Trivedi N, Singh S. X-linked hyper-IgM syndrome with bronchiectasis. *J Lab Physicians* 2014;**6**:114—6.