Measles-specific Neutralizing Antibodies in Rural Mozambique: Seroprevalence and Presence in Breast Milk

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Abstract. In Mozambique, as in many sub-Saharan countries, measles remains a public health problem. We conducted cross-sectional surveys in which we assessed measles-specific antibodies in serum and breast milk by plaque reduction neutralization (PRN) assay and measles secretory IgA in breast milk by enzyme-linked immunosorbent assay. A total of 151 persons <1 month to 23 years of age were surveyed; 81 (53.6%) of 151 had PRN titers equal to or above the protective level (≥200 mIU/mL). We found many serosusceptible persons, including 20.5% in whom no PRN antibody was detected. Almost all (96%) infants 6–8 months of age had non-protective PRN titers. Overall, 20.7% (6 of 29) of persons known to have received measles vaccine had non-protective titers. The geometric mean titer (GMT) of breast milk PRN antibodies was 41.6 mIU/mL (95% confidence interval [CI] = 34.0–51.0 mIU/mL) and the secretory IgA GMT was 227.6 (EU/mL) (95% CI = 179.1–289.1 EU/mL). The PRN titers of breast milk tended to increase with age. A notable proportion of the population in Manhiça, Mozambique apparently remains susceptible to clinical measles despite recent mass vaccination campaigns.

INTRODUCTION

Measles remains a major health problem worldwide, especially in developing countries. It is responsible for an estimated 420,000 annual deaths worldwide including 220,000 in Africa among children less than five years of age.1 In Mozambique, a single dose of measles vaccine is administered at nine months of age through the Expanded Program of Immunization (EPI). Measles vaccine was fully incorporated into the EPI in 1981.2 However, routine national measles vaccination policy was preceded by an extensive national campaign of vaccination with a high coverage rate (98.6%), which started in northern provinces (Niassa, Cabo Delgado, and Nampula) in 1976 and ended in Maputo City in February 1978 (Martins H, unpublished data). This campaign also initiated the implementation of measles vaccination through the EPI. In 2003, Mozambique had a large measles outbreak (approximately 23,000 cases) that affected children and young adults. In the Manhiça District, despite a high reported vaccination coverage rate, 265 cases of measles were reported during 2001–2004 with 8 deaths during July 2001–September 2004 (Mandomando I, unpublished data).

Immunity induced by natural measles infection is considered to be lifelong, and it was initially assumed that attenuated measles vaccine would confer long-term, perhaps lifelong immunity.3 However, various measles epidemics in the 1990s affected large numbers of vaccinated persons, and this led to the introduction of two doses of vaccine in most industrialized countries.4,4 In African countries, a sizable proportion of measles cases occur among young infants during the window of vulnerability when placentally transferred antibody diminishes below protective levels (at approximately 4–8 months of age), but the infant has not yet received measles vaccine.3,7,8 There is growing evidence that measles vaccine–induced antibody levels wane over time, raising a concern that such a decrease in antibody levels could affect maternal passive immunity when vaccinated women reach childbearing years.9,10 Thus, the window of vulnerability of an infant may be even greater in vaccinated women than in women with natural measles infection.

In adults, the presence of measles-specific antibodies in serum is indicative of natural measles infection or vaccination. However, in infants less than nine months of age, these antibodies usually reflect placental antibodies transferred from the mother to the child. Detection of measles-specific antibodies is usually performed by enzyme immunoassays11,12 or by a plaque reduction neutralization (PRN) assay, a technique that measures measles antibodies with the biological capacity to neutralize measles virus in vitro. The PRN assay is considered the gold standard for clinically relevant detection of measles-specific antibody.13–15 Previous studies have determined that a PRN titer ≥120 units/mL (generally accepted to correspond to 120–200 mIU/mL by comparison with the World Health Organization [WHO] measles serum standard) is protective against clinical measles disease and illness.13,15,16

In addition to placentally transferred antibodies, human breast milk is thought to play an important role in protection of young infants from infections.17–19 Human milk is rich in protective proteins, including secretory IgA (sIgA).20 Secretory antibodies protect the host by excluding antigens from accessing the mucosal surfaces and by intracellular virus and toxin neutralization within the epithelial barrier.20 It has been shown that sIgA protects infants and young children against gastrointestinal and respiratory pathogens.18,19 such as Shigella, respiratory syncytial virus, and Bordetella pertussis.21–24 However, little is known of the role, if any, of sIgA in contributing to protection of young infants from measles during the window of vulnerability when placentally transferred maternal antibodies are waning and prior to vaccination. There are few data on the prevalence of measles-specific antibodies in breast milk.25

There has been much discussion on the necessity of a second dose of measles vaccine and of new strategies to immunize newborns earlier than at nine months of age. Evaluating

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new vaccine approaches requires gathering more information on the seroprevalence of measles antibodies in African children and young adults. Thus, to assess antibody levels in a putatively well-vaccinated population, we conducted two cross-sectional studies in the Manhiça District, a rural area of southern Mozambique (June 2001 and April 2002). We investigated the prevalence of neutralizing measles antibody in serum from children and young adults <1–23 years of age and in breast milk of lactating women 14–47 years of age. We also investigated levels of measles-specific secretory IgA in breast milk.

MATERIALS AND METHODS

Study area and population. This study was conducted in the Manhiça District, a rural area of Maputo Province, in southern Mozambique. The characteristics of the area have been described in detail elsewhere. Briefly, the climate is subtropical with two distinct seasons: a warm, rainy season between November and April; and a generally cool and dry season during the rest of the year. The district has an estimated population of 140,000 inhabitants, who are mostly subsistence farmers, as well as workers in two large sugar- and fruit-processing factories.

In this area, the Manhiça Health Research Center (Centro de Investigação em Saúde da Manhiça [CISM]) has since 1966 conducted a continuous demographic surveillance system for vital events and migrations and currently includes approximately 82,000 people in the demographic surveillance system. The CISM is adjacent to semi-urban Health Center and District Hospital, 110-bed health facility. Since 1997, the hospital and CISM have jointly operated a round-the-clock surveillance of all pediatric visits at the outpatient department and admission to the wards. The national EPI in Mozambique includes bacillus Calmette-Guérin (BCG) vaccination (at birth), polio vaccine (at birth, 8, 12, and 18 weeks of age), diphtheria-tetanus-whole cell pertussis-hepatitis B vaccine (8, birth), polio vaccine (at birth, 8, 12, and 18 weeks of age), and measles vaccine (9 months of age). The global measles vaccine coverage rate in the country since the late 1990s and the most recent campaign was carried out in 2005.

Subjects and sample collection. In June 2001, a seroprevalence cross-sectional study was conducted with children and young adults (<1–23 years of age) in a random sample of population stratified by age group (0–11 months, 1–4 years, 5–9 years, 10–14 years, 15–19 years, and 20–23 years of age). A list of potential eligible subjects was produced from the demographic surveillance system database of the study area, and a field worker visited the home of the randomly selected subjects to explain the study and offer consent forms. Households visited twice without success were replaced by the others from the same area. After obtaining informed consent, blood samples (1 mL of whole blood) were collected in EDTA-microtainer tubes and transported to the laboratory in a cool box. A questionnaire was completed and information on vaccine status was collected when a vaccination card was available.

In April 2002, a cross-sectional study on breast milk was conducted from a sample of 50 lactating women 14–47 years of age living in this community and attending the maternal child clinic for EPI. Women who met the criteria (living in the study area, lactating, and agreed to provide the consent form) were consecutively enrolled in the study. Informed consent was obtained and breast milk samples (5 mL) were collected. Samples were transported to the Laboratory at CISM in a cold box. A brief questionnaire was completed during the sample collection for the studies. After blood centrifugation, plasma, pellet, and milk samples were stored at –20°C until shipment to the Center for Vaccine Development at the University of Maryland (Baltimore, MD) for processing. This study is part of the measles surveillance study protocol and was reviewed and approved by Mozambican Ethic Committee, the Hospital Clinic of Barcelona Institutional Review Board (IRB), and IRB of University of Maryland School of Medicine (Baltimore, MD) in 2001.

Laboratory procedures. PRN. PRN titers were measured as previously described. Briefly, plasma samples in serial dilutions were incubated with 100 plaque-forming units of wild-type measles virus Edmonston strain for 1 hour at 37°C in an atmosphere of 5% CO2 and plated onto confluent Vero cells (no. CCL-81; American Type Culture Collection, Manassas, VA) in 12-well plates. After incubation, cells were overlaid with 2 mL of agar per well and incubated for 5 days. Wells were stained with neutral red (Invitrogen Life Technologies, Carlsbad, CA), incubated overnight, and plaques were counted. The WHO measles serum standard 66/202 and internal controls calibrated against WHO standards were tested in parallel with the samples, thereby allowing PRN titers to be expressed as milli-international units per milliliter. Titers ≥ 200 mIU/mL were considered protective, as previous described.

ELISAs to measure total IgA and measles-specific IgA in milk. For detection of total IgA, Immulon II plates (Thermo-Labsystems, Franklin, MA) were coated for 3 hours at 37°C with 1 µg/mL of α chain specific anti-human IgA (Jackson Immuno Research Laboratories, West Grove, PA) in phosphate-buffered saline (PBS). For detection of measles-specific IgA, plates were coated with measles virus lysate at a concentration of 5 µg/mL (Advanced Biotechnology, Elliott, IL) in carbonate buffer, pH 9.0. Plates were washed with PBS containing 0.05% Tween 20 (PBST) and blocked overnight at 4°C with 10% dried milk (Nestle USA Inc., Glendale, CA) in PBS. Milk samples were tested in serial two-fold dilutions in PBST containing 10% dried milk (PBSTM) starting at 1:1,000 for measurement of total IgA and at 1:2 for measles-specific IgA. Plates were incubated for 1 hour at 37°C and washed as described above. Total IgA was detected using horseradish peroxidase (HRP)-labeled goat anti-human IgA (Jackson Immuno Research Laboratories) diluted 1:15,000 in PBSTM (plates were incubated for 1 hour at 37°C). Measles-specific IgA was detected with biotin-labeled goat anti-human IgA (Jackson Immuno Research Laboratories) diluted 1:2,000 in PBSTM followed by a 30-minute incubation at room temperature with HRP-avidin (Sigma, St. Louis, MO) diluted 1:400 in PBS. In both assays, the substrate used was 3,3',5,5'-tetramethylbenzidine (Kirkgaard and Perry Laboratories, Inc., Gaithersburg, MD), and the reaction was stopped after...
incubation for 15 minutes by addition of 100 μL of 1 M H₃PO₄. Absorbance values at 450 nm were measured. Samples were run in duplicate; appropriate controls were included in each assay.

Total IgA levels were calculated by interpolation of the regression-corrected optical density values for serum samples into a standard IgA curve of purified human IgA (dose range = 0.15–10 ng/mL; Calbiochem Corp., La Jolla CA) and results were reported in micrograms per milliliter. Measles-specific IgA titers were calculated as end point titers through linear regression as the inverse of the serum dilution that produces an absorbance value of 0.2 above the blank (ELISA units per milliliter). Measles IgA titers were divided by the concentration of total IgA to adjust for variation in the IgA content in samples in different persons.

Definitions. Protective titer is defined as a plaque reduction neutralization titer ≥ 200 mIU/mL. Non-protective titer is defined as a plaque reduction neutralization titer < 200 mIU/mL. The limit of detection of the assay is 6 mIU/mL. Samples were considered to have undetectable antibody levels when PRN titers were < 6 mIU/mL.

Data management and statistical analysis. Data were entered into Excel® (Microsoft, Redmond, WA) and transferred to the Statistical Package for the Social Sciences (SPSS Inc., Chicago, IL) by stat transfer version 6.0. Analysis was performed using SPSS version 11.0. The chi-square test was used for analysis of categorical variables. When the expected frequency in a cell was < 5, the Fisher exact test was used. The Wilcoxon rank sum test was used for analysis of non-parametric data. Antibody titers were expressed as geometric mean titer (GMT) and 95% confidence interval (CI). Pearson correlations were performed on original or log transformed variables with normal distribution. A P value < 0.05 was considered significant.

RESULTS

Serum levels of measles-neutralizing antibodies in infants and young adults. One hundred sixty infant and young adult blood samples were collected and the samples sent to the Applied Immunology Laboratory at the Center for Vaccine Development at the University of Maryland School of Medicine (Baltimore, MD) for measles antibodies measurement by PRN assay. One hundred fifty-one (94.4%) samples with adequate volume were assessed for neutralization titers and analyzed. Approximately 53.6% (81 of 151) of the population had protective titers of measles-specific neutralizing antibody (PRN titer ≥ 200 mIU/mL) (Table 1). The GMT of neutralizing antibodies were significantly different according to age, being the highest in persons ≥ 20 years of age and in children 1–4 years of age (Table 1). This finding was also reflected in that 94% of those ≥ 20 years of age and all children 1–4 years of age had protective PRN titers, whereas in infants 6–8 months of age, only 4% (1 of 25) had a protective titer (Table 1). Because 200 mIU/mL is a theoretical threshold, the data were stratified according to the proportion of persons with titers < 100 mIU/mL (Table 1). This stratification showed that most titers below the protective threshold were actually < 100 mIU/mL (Table 1), and there were a surprisingly high number of volunteers with undetectable titers (20.5%, 31 of 151).

Measles vaccination status was assessed in children greater
than nine months of age by examination of vaccination cards; 43 of 108 persons (approximately 40%) had vaccination cards. Overall, 29 of these 43 persons (67.4%) had received measles vaccine (29 of 108 persons, 26.9%), and 14 of the 43 persons (32.6%) with vaccination cards had no record of having received measles vaccine (14 of 108 persons, 13%). Vaccinated persons showed a higher GMT of PRN antibodies than non-vaccinated persons greater than nine months of age (GMT = 559, 95% CI = 258–1,097 versus GMT = 26, 95% CI = 8–48; \( P < 0.001 \)), but this finding was not significantly different from those with unknown vaccination status (GMT = 559, 95% CI = 258–1,097 versus GMT = 505, 95% CI = 336–759; \( P = 0.62 \)).

The proportion of persons with protective PRN titers was also significantly higher among measles-vaccinated persons than unvaccinated persons (\( P < 0.001 \)). Among the population previously immunized, 20.7% (6 of 29) had non-protective PRN titers.

**Vaccine status of infants and young adults with non-protective titers of measles-neutralizing serum antibodies.** The 70 persons with non-protective titers of the 151 tested were then analyzed according to vaccination status. Excluding infants less than nine months of age, 18.2% (6 of 33) had been previously immunized against measles, 36.4% (12 of 33) had not received immunization, and the remaining persons (45.5%) had unknown vaccination status. When age group was taken into consideration, among children 9–11 months of age with non-protective PRN titers, 29.4% (5 of 17) had been previously immunized and the remainder was not vaccinated. Among children 5–9 years of age, one child had been previously vaccinated, and the remaining persons had unknown vaccination status. We were unable to quantify this finding in children and young adults greater than 10 years of age because of the lack of vaccine records.

**Measles-neutralizing antibodies and IgA in breast milk of lactating women.** Table 2 shows the GMT and 95% CI of breast milk PRN and measles-specific IgA according to age. From the 50 samples processed, the neutralizing antibody GMT was 41.6 mIU/mL (95% CI = 34.0–51.0) and IgA GMT was 227.6 EU/mL (95% CI = 179.1–289.1). A poor correlation (\( r = 0.245 \), lnIgA versus PRN, \( P = 0.086 \)) was observed between measles-specific IgA (natural log transformed) and PRN-neutralizing antibodies in breast milk.

There was a trend towards higher titers of measles-specific neutralizing antibodies in breast milk with age; however, it was not statistically significant. Furthermore, GMT of measles-specific IgA was slightly lower in women 14–24 years of age than in women 24–47 years (GMT = 35.6, 95% CI = 27.2–46.6 versus GMT = 48.1, 95% CI = 35.3–65.4; \( P = 0.053 \)) (Table 2).

**DISCUSSION**

This seroepidemiologic study describes the prevalence and titers of measles antibody in infants and young adults in rural southern Mozambique and assesses measles PRN and IgA antibody titers in breast milk from lactating women. Although the reported measles vaccine coverage is high in this area (94.4% in 2001), a considerable proportion (46.4%) of the study population was found to have titers of measles PRN antibody titers below the protective cutoff (< 200 mIU/mL), and many had undetectable levels of PRN antibody.

**TABLE 2**

Characterization of measles-specific neutralizing antibodies and IgA from breast milk in Mozambique*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>No. (%)</th>
<th>PRN titer (mIU/mL), GMT (95% CI)</th>
<th>ELISA (EU/mL), GMT (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall</td>
<td>50 (100)</td>
<td>41.6 (34.0–51.0)</td>
<td>227.6 (179.1–289.1)</td>
</tr>
<tr>
<td>Age, years</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14–24</td>
<td>24 (48.0)</td>
<td>35.6 (27.2–46.6)</td>
<td>242.1 (172.1–340.6)</td>
</tr>
<tr>
<td>&gt; 24</td>
<td>26 (52.0)</td>
<td>48.1 (35.3–65.4)</td>
<td>214.9 (150.4–307.2)</td>
</tr>
</tbody>
</table>

* PRN = plaque reduction neutralization; GMT = geometric mean titer; ELISA = enzyme-linked immunosorbent assay.

Most infants less than 12 months of age had non-protective titers of measles-specific antibody, which was likely caused by the window of vulnerability prior to vaccination when placentally transferred maternal antibodies are waning. This phenomenon may be accentuated in babies born of vaccinated mothers who potentially have lower titers of measles antibodies than do mothers who had natural infections. A decrease in vaccine-induced antibody titers with age has been well documented, and is likely to affect transfer of maternal antibody. Moreover, human immunodeficiency virus (HIV) status of the mothers has been shown to affect transplacental transfer of measles antibodies. The HIV prevalence in women and pregnant women in the Manhiça district was approximately 10% and 21% in 1999 and 2004, respectively (Menendez C, unpublished data).

The non-protective PRN titers observed among vaccinated infants (approximately 21%) can be related to several factors. Among recently vaccinated infants, their inability to achieve protective titers may be due in part to vaccine failure or to HIV status. For all persons, except for one child, sufficient time had elapsed to generate an antibody response. However, this 21% of non-protective is consistent with the generally accepted dogma that 15% of children have primary vaccine failure when vaccinated at nine months of age.

The low prevalence of measles PRN titers in infants documented in this study is of public health significance because it suggests that a large number of infants may be highly susceptible to measles infections. However, the role of cell-mediated immunity (CMI), which was not assessed in this study, should be taken into consideration because previous studies have shown early initiation of CMI in the absence of antibodies and in patients with agammaglobulinemia recover from infection and remain protected.

A significant number of persons 10–19 years of age had non-protective PRN titers. However, interpretation of these results is difficult because vaccine records were not available for most of those persons. Cases of vaccinated persons with non-protective PRN titers at this age could be related to waning of immunity over time, although our data do not permit this assessment. Higher antibody titers observed among persons greater than 20 years of age could be caused by enhanced ability of naturally acquired immunity to persist or to exposure to natural boosting by circulating wild measles virus. One of the limitations of the study is that we were unable to discriminate the possible causes for the lack of protective antibody levels, including failure to vaccinate, primary or secondary vaccine failure, or concurrent HIV-1 infection.

Breastfeeding is important in disease protection in infants because breast milk contains antibodies, lysozyme, lactoferrin, and bifidus factor, among others. However, there are scarce data evaluating the presence and neutralization capaci-
ity of measles-specific antibodies in breast milk. In this study, we quantified measles-specific IgA and measles PRN antibody in breast milk and provided a basic set of data for comparisons for further research into the role of these antibodies in protection against measles in young infants. We found a poor correlation between titers of measles PRN antibodies and measles-specific IgA (r = 0.24, P = 0.086) in breast milk. Although IgA predominates in mucosal secretions, we cannot rule out the contribution of other immunoglobulins in the observed virus neutralizing activity because measurements were performed in whole breast milk samples as opposed to purified IgA. It has recently been shown that most of the neutralization activity in serum is caused by IgG1 and IgG3 with little contribution from IgG2 and IgG4.15,38 However, there are examples of neutralizing activity of sIgA efficiently inhibiting epithelial colonization and invasion of pathogens through microbial agglutination and virus neutralization.20 Furthermore, the effect of polymeric IgA and sIgA is superior in comparison with monomeric antibodies.6 In addition, anti-rotavirus neutralizing activity in milk containing IgA but not specific IgG or IgM has been reported,30 and comparable levels of neutralizing activity against rotavirus serotypes SA11 (G3) and Wa (G1)39 have been found in serum and transitional milk.

A PRN titer of 120–200 mIU/mL has been widely accepted as the correlate of protection in serum antibody but there is no known correlate of protection for breast milk antibodies. It is possible and perhaps likely that the presence of measles-specific neutralizing activity in breast milk contributes to protection. However, it is unknown what titer of neutralizing activity in breast milk is necessary to exert a protective effect per se.

When breast milk PRN titers were stratified by the woman’s age (< 24 and ≥ 24 years), higher titers of neutralizing antibodies were observed in women ≥ 24 years of age than in women < 24 years of age (P = 0.053). This finding could potentially be caused by a difference in vaccinated women versus women who had natural measles infection. Women greater than 24 years of age were born before the introduction of measles vaccine in Mozambique in 1981. Despite the national vaccination campaign in 1976, at that time the probability of acquiring natural measles infection was high because the measles EPI was not well established. In contrast, most women less than 24 years of age were born after the measles vaccine was well established in the EPI program, although there was a period during the civil war where the coverage rate was low in the whole country (< 40%, 1986–1988).27 Women whose immunity derives from natural measles exposure are likely to have generated mucosal sIgA antibodies, including breast milk sIgA, in addition to serum antibodies, consequent to the wild virus entering by the respiratory tract. In contrast, attenuated vaccine is administered subcutaneously and the mucosal sIgA titer may be lower. As is the case for waning of serum antibodies,9,36,40 vaccinated women reaching childbearing age may have lower titers of breast milk antibodies. In our study, the group 14–23 years of age was likely to be a mixture of vaccinated women and women having had natural measles. In a homogeneous population of vaccinated women, mean breast milk titers may be even lower. It will be important to further define the titers of measles-neutralizing breast milk antibodies in vaccinated women to understand their potential role in protection.

Our study provides insightful data on prevalence of measles immunity in a rural area of southern Mozambique. Results from this study suggest that a high proportion of infants below the recommended vaccination age are susceptible to infection and possibly to clinical measles disease. This finding highlights the public health paradox whereby vaccination decreases the incidence and severity of measles in the general population and creates other pockets of vulnerability, particularly in young infants. With the highly contagious nature of measles and high mobility of populations, improvement of vaccination strategies for vulnerable populations is needed. Prior to 2000, control of measles in developing countries largely depended on vaccination of infants nine months of age through the EPI. However, this strategy was not effective and in 1998 there were still approximately 800,000 deaths attributed to measles, mostly in Sub-Saharan Africa and in rural India. However, innovative strategies were proposed as adjuncts to EPI delivery of routine immunization, including mass immunization campaigns and a second dose of measles vaccine (to capture that 5–10% who failed to respond to the first dose), which should be generalized to many African countries. In parallel with development of safe and efficacious new vaccines to protect infants less than nine months of age, future studies should also establish levels of breast milk and serum antibodies during pregnancy, which successfully protect the infant during the first months of life.

Received March 5, 2008. Accepted for publication July 6, 2008.

Acknowledgments: We thank the parents and guardians of study participants for cooperation, the Manhiça Health District Authorities for assistance, Samira Sirage (Centro de Investigación en Saúde da Manhiça) for sample collection, and Mardi Reymann and Yu Lim (Applied Immunology Section, Center for Vaccine Development, University of Maryland School of Medicine) for excellent technical support and shipment of samples.

Financial support: This study was supported by grants from the Bill & Melinda Gates Foundation.

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