The novel adjuvant dmLT promotes dose sparing, mucosal immunity and longevity of antibody responses to the inactivated polio vaccine in a murine model

Elizabeth B. Norton a, David L. Bauer a, William C. Weldon b, M. Steven Oberste b, Louise B. Lawson a, John D. Clements a,⁎

a Department of Microbiology and Immunology, Tulane University School of Medicine, 1430 Tulane Avenue (SL38), New Orleans, LA 70112, USA
b Division of Viral Diseases, Centers for Disease Control and Prevention (CDC), 1600 Clifton Ave, Atlanta, GA 30333, USA

A R T I C L E   I N F O

Article history:
Received 20 November 2014
Received in revised form 5 February 2015
Accepted 26 February 2015
Available online 9 March 2015

Keywords:
dmLT
Polio
Vaccination
Adjuvant
Mucosal immunity

A B S T R A C T

One option for achieving global polio eradication is to replace the oral poliovirus vaccine (OPV), which has the risk of reversion to wild-type virulence, with the inactivated poliovirus vaccine (IPV) vaccine. Adjuvants and alternate routes of immunization are promising options that may reduce antigen dose in IPV vaccinations, potentially allowing dose sparing and cost savings. Use of adjuvants and alternate routes of immunization could also help promote mucosal immunity, potentially mimicking the protection against intestinal virus shedding seen with OPV. In the current study, we examined the impact of combining the novel adjuvant dmLT with trivalent IPV for dose sparing, induction of mucosal immunity and increasing longevity of anti-poliovirus (PV) responses in a mouse model following either intradermal (ID) or intramuscular (IM) delivery.

We found that non-adjuvanted ID delivery was not superior to IM delivery for fractional dose sparing, but was associated with development of mucosal immunity. Vaccination with IPV + dmLT promoted serum anti-PV neutralizing antibodies with fractional IPV doses by either IM or ID delivery, achieving at least five-fold dose sparing above non-adjuvanted fractional doses. These responses were most noticeable with the PV1 component of the trivalent vaccine. dmLT also promoted germinal center formation and longevity of serum anti-PV neutralizing titers. Lastly, dmLT enhanced mucosal immunity, as defined by fecal and intestinal anti-PV IgA secretion, when included in IPV immunization by ID or IM delivery. These studies demonstrate that dmLT is an effective adjuvant for either IM or ID delivery of IPV. Inclusion of dmLT in IPV immunizations allows antigen dose sparing and enhances mucosal immunity and longevity of anti-PV responses.

© 2015 The Authors. Published by Elsevier Ltd. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

1. Introduction

Polio is a highly infectious disease with no reservoir outside of its human host and is, therefore, a target for global eradication. Poliovirus spreads through contaminated food and water and person-to-person contact, infecting susceptible populations (e.g., children < 5 years) where intestinal virus replication and shedding occur over a period of weeks [1]. While the majority of infections are asymptomatic or cause mild disease, ~0.5% of infections result in acute onset of flaccid paralysis leading to permanent loss of muscle function, limb use, and, in severe cases, respiratory control and death [1]. Vaccination and a global eradication effort have successfully dropped the burden of disease from an estimated 350,000 cases of paralytic polio worldwide in 1988 to 416 cases in 2013 [1] and polio now remains endemic in only three countries [2]. Vaccination is key to virus control, as polio disease is rapidly re-established after low or disrupted vaccination coverage. For instance, disease outbreaks may occur during political disturbances and then spread into adjacent communities, such as those in the horn of Africa (2008–2013) and in Syria (2013–2014) [2,3].

Immunization programs use either the live, attenuated oral polio vaccine (OPV) or a formalin-inactivated polio vaccine (IPV), both trivalent for serotypes 1, 2 and 3. Most of the developed
world uses the injectable IPV vaccine which costs ~US$1.00 per dose in GAVI eligible countries. However, OPV is preferred for developing countries because it is less expensive, at ~US$0.15 per dose, and easier to administer in less advanced health-care settings. As a live attenuated vaccine, OPV replicates in the intestines of vaccine recipients and promotes intestinal resistance to poliovirus re-infection (i.e., mucosal immunity) [4]. Herd immunity also increases with IPV, including expanding community coverage beyond vaccinated individuals, presumably through OPV fecal shedding [5]. However, OPV strains can replicate in areas with incomplete vaccine coverage, enabling step-wise reversion to wild-type virulence and transmissibility (vaccine-derived poliovirus or VDPV) as seen most frequently for type-2 OPV. IPV can also replicate and be shed in the feces of immunocompromised hosts creating poliovirus reservoirs from which strains with wild-type virulence can later emerge [2]. While bivalent OPV lacking the type-2 strain has been developed to help address type-2 VDPV, use of OPV should be discontinued in order to completely eradicate polio.

A major part of current global polio eradication efforts is to reduce the cost of IPV and improve the existing IPV formulation through dose sparing, addition of appropriate adjuvants, and induction of mucosal immunity to reduce intestinal virus replication and subsequent fecal shedding [6–9].

The adjuvant LT(R192G/L211A), or dmlT, is a detoxified version of the heat-labile enterotoxin of Escherichia coli, with two mutations in its A-subunit [10] that remove the enterotoxocity but preserve the adjuvancy of the molecule. dmlT appears to induce a cAMP-dependent danger signal combined with a B-subunit GM1-binding signal to activate the immune system. Unlike its parent molecule, dmlT has no observable gastrointestinal toxicity in either animals or humans, but is still an effective mucosal adjuvant [10–13]. Vaccines including dmlT have improved immune responses to bacterial and viral antigens following oral, sublingual, transcutaneous, intradermal and intramuscular delivery [12–23]. Notably, we have also recently shown increased viral neutralization titeres with a full dose of IPV delivered sublingually within a thermoresponsive gel including dmlT [19]. The objective of the current study was to evaluate whether intradermal (ID) or intramuscular (IM) delivery of IPV in combination with dmlT could reduce the required dose or number of doses of an existing IPV vaccine and whether this novel adjuvant could promote mucosal immunity following parenteral immunization.

2. Materials and methods

2.1. Virus and adjuvant reagents

Inactivated viral stocks Brunnhilde PV1, MEF-1 PV2 and Saukett PV3 (977, 1260 and 1860 D-antigen units (DU)/ml), respectively, and premixed trivalent IPV (327 Brunnhilde PV1, 70 MEF-1 PV2, and 279 Saukett PV3 DU/ml) were obtained from Statens Serum Institut (SSI, Copenhagen, DK). Individual PV-types were used for immunological analysis. All immunizations were performed with premixed trivalent IPV as supplied by SSI so the ratio of PV-types remains the same when different doses are administered. dmlT was prepared under GMP conditions by Walter Reed Army Institute of Research (Washington, DC) and obtained from PATH-EVI.

2.2. Animals, immunizations and sample collections

Female BALB/c mice aged 6–8 weeks were purchased from Charles River Laboratories and housed in filter-top cages under sterile conditions. All animal studies were approved by the Tulane University Institutional Animal Care and Use Committee. Groups of 7–10 animals each were immunized by ID or IM injection with 50 μl of formulations in M199 (Gibco) or saline. Immunizations were performed 1–3 times at 3-week intervals prior to CO2 euthanasia for sample collection, including serum, spleens, colon-derived fecal pellets, and Peyer’s patches (PP). Fecal pellets were weighed, homogenized in 1.5 ml PBS – 0.05% Tween 20 containing a protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN), and the supernatants collected. PP were excised after rinsing the intestine with 10 ml cold saline. After weighing, PP were plated with 400 μl 10% RPMI in a 24-well tissue culture plate and incubated at 5% CO2 at 37 °C. After 7 days, supernatants were harvested and stored at –20 °C.

2.3. Antibody ELISAs

Antigen-specific IgG and IgA ELISAs were performed similar to methods previously described [10,12] using Linbro/Titertek 96-well U-shaped plates (MP Biomedical, Santa Ana, CA, 76-341-05) coated at 1:50 with PV antigens or dilutions of purified mouse standards, specifically IgG1-κ (Sigma M9269) or IgA-κ (Sigma M1421). Detection by IgG ELISAs was performed using AKP-conjugated anti-mouse IgG (Sigma A1902). Detection by IgA ELISAs was performed using HRP-conjugated anti-mouse IgA (SouthernBiotech, Birmingham, AL, 1040-05) and TMB Substrate (KPL 50-76-00). Results were expressed as ELISA units/ml (EU/ml) using an average of three sample dilutions closest to the midpoint of the standard curve.

2.4. Poliovirus neutralization

Neutralizing antibody titeres against Brunhilde PV1, MEF-1 PV2 and Saukett PV3 were measured as described previously [19,24]. Following this assay ranges from 2.5 log2, or “negative,” to 10.5 log2, as highest tested value; a log2 titer of ≥3 is considered protective.

2.5. Flow cytometry

Spleens were homogenized using C-tubes (Invitrogen) and filtered into suspension, stained and analyzed by flow cytometry as previously described [12]. Staining antibodies included anti-mouse CD3-eFlour450, GL7-FITC (eBioscience, San Diego, CA), CD4-BV510 (BioLegend, San Diego, CA), and CD19-PE-Texas Red (Invitrogen).

2.6. Immunoblot

Blots were made using a slot blot apparatus (Hybri-slot vacuum apparatus, Life Technologies, Grand Island, NY) coated with 10 DU of PV1, 2 or 3 on a PVDF membrane and blocked with 5% skim milk in PBS. Fecal samples (200 μl) were diluted in 800 μl PBS-0.05% Tween 20 with 5% skim milk and added to an 8-rectangular well plate containing a test blot. Following overnight incubation at 4 °C, each blot was washed then treated with anti-mouse IgA-HRP. Blots were developed with ECL substrate (Invitrogen) and imaged with ImageQuant LAS-4000 imaging system (GE). Images were quantified for band integrated density units (IDU) per ml using ImageJ, an open source image processing and analysis software [25].

2.7. Data analysis

Statistical analysis was performed using Prism (GraphPad Software v6, La Jolla, CA). Data were analyzed by Spearman’s Rank Order Correlation Coefficient (r) analysis and one-way ANOVA analysis with Tukey’s multiple comparison post hoc test or Dunn’s multiple comparison post hoc test for nonparametric data.
3. Results

The trivalent IPV vaccine is traditionally delivered via three IM injections containing 40 DU PV1, 8 DU PV2 and 32 DU PV3 each and usually reported based on type-1 content with the understanding that the ratio of the constituents remains constant (40:8:32). Dose-sparing strategies can either reduce the number of doses or the amount of antigen delivered by each immunization. ID delivery has been suggested as the best parenteral route to achieve dose sparing due to the density of antigen-presenting cells in the skin (compared to muscle tissue) available to promote vaccine immunity [8]. Promising clinical results have shown dose sparing of several human vaccines, including IPV, by switching to ID from IM or subcutaneous delivery [26–28]. For these reasons, in our studies we compare both IM and ID routes.

3.1. Dose sparing and adjuvanticity

We first evaluated the antibody responses following vaccination with fractional doses of IPV comparing IM and ID delivery. For these purposes, 5 DU IPV based on PV1 content was set as a “full” mouse dose for comparative purposes. This dose was chosen based on experiments that showed mid-point antibody titers with IPV at this dose compared to 0.1, 1, 10 and 15 DU of IPV (Fig. 1A). Our goal then was to demonstrate non-inferiority of any fractional doses compared to 5 DU IPV. Groups of BALB/c mice were immunized twice (days 0 and 21) by either the ID or IM route with IPV (0.1–15 DU) alone or admixed with dMLT (0.1–1 µg). Two weeks after the final immunization, serum was collected and analyzed for anti-PV IgG by ELISA (Fig. 1A). A subset of these groups was also tested for virus neutralizing antibodies by cell culture assay using Brunhilde PV1, MEF-1 PV2, and Saukett PV3 (Fig. 1B). The neutralization assay is considered the gold standard for assessing vaccine efficacy [24], and a log₂ titer of ≥3 is considered protective. Our results revealed that un-adjuvanted fractional doses provided consistent levels of serum IgG and neutralization titers by both the ID and IM routes except at the lowest doses (<0.5 DU). Un-adjuvanted ID delivery did not appear to be superior to un-adjuvanted IM delivery at any dose tested by either ELISA or virus neutralization; however, inclusion of dMLT enhanced both serum IgG and neutralization titers following ID immunization (dMLT was not included with the IM immunizations for this analysis). Specifically, anti-PV1 responses induced by 1 DU IPV administered ID with dMLT exceeded the responses to the control group receiving un-adjuvanted “full” dose of 5 DU IPV administered IM. Moreover, the neutralization titers with 10 DU IPV + dMLT were significantly higher than any level

Fig. 1. Poliovirus serum IgG and neutralization titers following IM or ID immunization. Animals (n = 7–10) were immunized twice (days 0 and 21) with the indicated IPV dose (DU) via IM delivery or ID delivery with or without dMLT (µg). Serum collected 2 weeks after the last immunization was analyzed by ELISA (A) or in vitro virus neutralization (B) against each vaccine poliovirus strain: Brunhilde type-1 (top), MEF-1 type-2 (middle) and Saukett type-3 (bottom). Serum IgG values are mean ± SEM with significance indicated as *P < 0.05, **P < 0.01 or ***P < 0.001 versus naïve control for each virus serotype or as *P < 0.05 versus 5 DU IM group using Tukey’s multiple comparison post hoc test. Virus neutralization titers were determined in triplicate using median cell culture infectious dose (CCID50) measurements with the final titer estimated by the Spearman–Kärber method. Detection by this assay ranges from 2.5 log₂, or “negative,” to 10.5 log₂, as highest tested value, and is shown as median bars with interquartile range with significance indicated as above except using Dunn’s multiple comparison post hoc test. Dotted line placed at the median titer in response to 5 DU IPV, or 1/8th of current human dose.
achieved with un-adjuvanted 10 DU by either the IM or ID route. Throughout, groups receiving IPV + dmLT developed either higher antibody responses or were not statistically different from the 5 DU IM group (i.e., non-inferiority was achieved). The production of serum neutralizing titers to Brunhilde PV1 is particularly important since PV1 is the least immunogenic component of IPV and this strain is the most prominent cause of wild-type polio [2]. These results indicate that dmLT can promote dose-sparing by reducing the IPV dose required for immunogenicity by at least five-fold, compared to the IM and ID full-dose and fractional-dose controls. Individual animal responses are shown in Supplemental Fig. S1.

Supplementary Fig. S1 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2015.02.069.

3.2. Kinetics and longevity

We next evaluated the kinetics and longevity of the virus neutralization response to trivalent IPV (1 DU based on PV1 content) alone or adjuvanted with 0.1 μg dmLT by either the IM or ID route. Brunhilde PV1 neutralization titers developed more rapidly and were maintained over a longer time period in animals immunized with IPV + dmLT compared to un-adjuvanted groups (Fig. 2). By contrast, MEF-1 PV2 and Saukett PV3 neutralization titers developed rapidly and were essentially equivalent through day 56 in this model. However, as with PV1, neutralization titers against PV2 and PV3 were maintained or even higher 4- or 6-months post-final immunization (e.g., day 179 and 243) when dmLT was included in the vaccine. These results once again indicate that dmLT enhances PV1 neutralizing responses following either IM or ID delivery. In addition, longevity of all PV neutralizing responses was evident in dmLT-adjuvanted groups. Individual animal responses are shown in Supplemental Fig. S2.

Supplementary Fig. S2 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2015.02.069.

3.3. Germinal center marker expression

Our kinetic response studies demonstrated that dmLT impacts the peak and decay of the neutralizing antibody responses following IPV immunization, suggesting altered development of antibody-secreting cells. Vaccine responses are generated in secondary lymphoid organs where antigen is presented to activated T-cells and B-cells within germinal centers expressing the GL7 marker [29,30]. To determine if dmLT enhances germinal center formation, we evaluated splenic CD4+ T-cells and B-cells for GL7 expression in mice immunized IM with 1 DU IPV alone or ID with 1 DU IPV with or without dmLT. Our results indicate that while all immunization groups have detectable expression of GL7+ T-cells and B-cells, only in groups receiving formulations containing dmLT was this significantly higher than in naive mice (Fig. 3).

3.4. Mucosal antibodies

Because mucosal immunity can protect against intestinal poliovirus replication [31,32], we next evaluated induction of fecal anti-PV IgA after vaccination. For these studies, we first analyzed fecal samples from the IM and ID groups shown in Fig. 1 by ELISA against all three serotypes using the trivalent vaccine. Analysis revealed that dmLT enhanced the number of group responders (40%) following ID immunization compared to un-adjuvanted groups immunized IM or ID (0–29%, Fig. 4A); however, we observed poorer detection sensitivity than seen previously in our laboratory with IgA ELISAs for other antigens, implying interference in IPV antibody detection. For instance, fecal extracts from naive (unimmunized) mice blocked detection of anti-IPV antibodies in IPV-positive serum (1 or 2) tested by anti-IPV IgG ELISA compared to PBS dilution buffer. These results indicate that detection of fecal anti-PV antibodies by ELISA is complicated by non-specific interactions (Fig. 4B).

We next explored a modified immunoblot assay to test for anti-PV fecal antibodies as an alternative to the ELISA. In this assay, pre-loaded PV1, PV2 and PV3 antigens are detected upon incubation of immunoblots with individual fecal samples diluted 1:5 with blocking buffer in 1 ml total volume followed by anti-mouse IgA. Immunoblot results showed the presence of IgA antibodies in individual samples without the interference seen in the ELISA (Fig. 5A). Using this method, we quantified (band integrated band density (IDU) per ml sample) anti-PV IgA in fecal samples of mice immunized twice with 1 DU of IPV alone or with dmLT by each route. Our results revealed that fecal IgA responses were not detectable following IM immunization without adjuvant. However, un-adjuvanted ID immunization with IPV did elicit detectable anti-PV IgA in fecal pellets. The adjuvant dmLT both enhanced the fecal anti-PV response following ID immunization and facilitated

![Image](http://dx.doi.org/10.1016/j.vaccine.2015.02.069)
Induction of fecal responses following IM immunization (Fig. 5A and B).

We next confirmed that fecal antibody responses were due, at least in part, to local antibody production (and not rodent hepatobiliary recirculation of serum IgA into the intestines [33]) by evaluating different groups for ex vivo secretion of antibodies by the intestinal secondary lymphoid tissue, PP. This assay has been used in other polio vaccine studies [34] to indicate that antibody presence is due to secretion from PP-associated plasma cells and not derived directly from the serum. In our tests, no detectable anti-PV antibodies appeared until at least 3 days incubation, but we were able to demonstrate anti-IPV IgA in the supernatants of PP tissue cultured for 7 days ex vivo and correlate that with fecal IgA immunoblot results (Fig. 5B–D). Taken together, our mucosal analyses indicate significant mucosal responses following parenteral immunization by either the IM or ID route when dmLT is included as an adjuvant in the formulation.

4. Discussion

Global polio eradication is close to being achieved thanks to the efforts of widespread vaccination programs; however, the existing live-attenuated oral vaccine will soon be replaced by a non-replicating vaccine. Unfortunately, the use of IPV is cost-prohibitive based on the current full dose schedule. In order to reduce the cost of IPV immunization, strategies to reduce the required vaccine dose are being investigated [6,7,28,34–36]. In addition, OPV provides a certain level of mucosal immunity [5,32] that has not yet been achieved by a parenteral immunization with IPV. The objective of the current study was to evaluate whether ID or IM delivery of IPV in combination with the adjuvant dmLT could reduce the required dose or number of doses of an existing IPV vaccine and whether this novel adjuvant could promote mucosal immunity following parenteral immunization.

We first compared un-adjuvanted fractional dose IPV by both ID and IM delivery. Fractional doses of IPV delivered either ID or IM promoted virus-neutralizing titers for PV2 and PV3, but not PV1. In our study, ID immunization with IPV alone was not superior to IM for induction of serum anti-IPV antibodies, in contrast to earlier reports by other investigators [28,36]. We held the volume of IM and ID injection constant (50 μL) to avoid the variable of delivery volume. This volume may not be optimal for ID delivery in mice, as antigen loss may occur from higher dermal pressure (with higher volumes). We also utilized the Mantoux technique, although other ID delivery devices and technology show promise for promoting immune responses over this classic ID technique [26,27] and warrant further study for ID delivery of IPV and other vaccines. In contrast to serum responses, un-adjuvanted ID delivery did promote increased fecal anti-PV IgA that was not seen with IM delivery.

We further demonstrated that dmLT enhances serum anti-PV neutralizing antibodies with fractional IPV doses by either IM or ID delivery. Using 5 DU for an equivalent “full” mouse dose, dmLT achieved at least five-fold dose sparing above non-adjuvanted fractional doses. These responses were most noticeable with the PV1 strain of the vaccine, likely because PV1 is the least immunogenic serotype in IPV and exhibits lower neutralizing antibody responses even with higher DU doses than PV2 and PV3 (which develop robust D35 responses with unadjuvanted fractional doses). However, when longevity of response was evaluated, dmLT-adjuvanted groups displayed higher levels of PV1, PV2 and PV3 neutralizing titers (out to 243 days), dmLT also induced higher levels of mucosal immunity, as defined by fecal IgA and intestinal anti-PV IgA secretion, when included in IPV immunization by either the ID or IM route. This finding has potential implications for reformulating a fractional dose IPV vaccine, as increasing intestinal IgA correlates with decline in intestinal virus replication [32] and pre-existing IgA levels in elderly humans better protects against virus fecal shedding after challenge [31]. Moreover, in contrast with OPV, intramuscular injection with IPV does not normally induce mucosal antibodies, so these findings are particularly important given the role intestinal immunity plays in reduction of disease transmission in the community in addition to reducing viral replication in the individual. Our findings correlate with other immunization studies that show dmLT

![Fig. 3. Germinal center marker expression by splenocytes following IM or ID immunizations. Animals were immunized twice (days 0 and 21) with 1 DU trivalent IPV via IM or ID delivery route with or without 1 μg dmLT. Two weeks after the final immunization, spleens were harvested and stained for T-cells (CD4+ CD3+), B-cells (CD19+), and germinal center (GL7+) markers. Stained cells were analyzed by flow cytometry and % GL7+ B-cells (left panel) or CD4+ T-cells (right panel) were graphed. Values are individual animals (symbols) or group means (bars). Significance is shown as *P ≤ 0.05 and **P < 0.01 versus naive control using Tukey’s multiple comparison post hoc test.](image1)

![Fig. 4. Poliovirus fecal IgA following IM or ID immunizations. Animals were immunized twice (days 0 and 21) with the indicated dose of trivalent IPV via IM (5 animals per group) or ID (7 animals per group) delivery route with or without dmLT adjuvant (0.1 μg). (A) Fecal supernatants collected 2 weeks after the last immunization were analyzed for anti-poliovirus IgA antibodies by ELISA against the trivalent IPV vaccine. Values are ELISA units per ml (EU/ml) for individual animals (n = 4–7 per group) with 5% group responders indicated. (B) Poliovirus antibodies by IgG ELISA using positive mouse serum samples (serum 1 or serum 2) pre-incubated with dilution buffer or fecal supernatants from naive animals.](image2)
by skin administration helps achieve mucosal immunity; dmLT in a transcutaneous vaccine against *Haemophilus influenzae* otitis media improved clearance of ongoing bacterial biofilms and promoted mucosal immune responses in the inner ear in a chinchilla model [16,17]. Similarly, dmLT addition to an ID vaccine against Shigella promoted IgG in pulmonary secretions and protection from lethal pulmonary challenge in a mouse model [15]. Germinal center formation is key for development of T-dependent immunity and optimal antibody responses [29,30]. Germinal center development was initially characterized using cholera toxin [37] and GL7-induction has been found in related, cholera toxin-derived adjuvants [38]. In our study, we were able to demonstrate that dmLT enhances germinal center formation in immunized mouse spleens, shown as increased GL7 expression on T-cells and B-cells. These observations help explain how this adjuvant improves magnitude and duration of immunity after IPV vaccination.

### 5. Conclusion

Our study revealed that dmLT is a potential adjuvant for either IM or ID delivery of IPV. Inclusion of dmLT in IPV immunizations allows antigen dose sparing and enhances mucosal immunity and longevity of anti-PV responses following either IM or ID delivery. Future expansion of these studies to other models (rodent, NHP) or human clinical studies could further demonstrate the potential of dmLT as an adjuvant in a modified IPV vaccine strategy and thereby take another step toward achieving the global eradication of polio.

### Acknowledgements

Support for this project was provided by the Bill and Melinda Gates Foundation (Grant No. OPP1031440). The authors would like to thank Monica Czapla, Carl Thompson, Rachel Hahn and Zaid Mahdi for their help performing these studies, as well as Debra Moore, Yiting Zhang, Sharla McDonald, William Hendley, Larin McDuffie, Patricia Mitchell, and Mario Nicolas for performing the serology tests. Robin Baudier is also thanked for writing assistance and proofreading manuscript drafts.

**Conflict of interest:** The findings and conclusions in this report are those of the author(s) and do not necessarily represent the official position of the Bill and Melinda Gates Foundation or Centers for Disease Control and Prevention. The authors have no conflicts of interest.

### References


