Clinical Assessment of a Recombinant Simian Adenovirus ChAd63: A Potent New Vaccine Vector

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Background. Vaccine development in human Plasmodium falciparum malaria has been hampered by the exceptionally high levels of CD8+ T cells required for efficacy. Use of potently immunogenic human adenoviruses as vaccine vectors could overcome this problem, but these are limited by preexisting immunity to human adenoviruses.

Methods. From 2007 to 2010, we undertook a phase I dose and route finding study of a new malaria vaccine, a replication-incompetent chimpanzee adenovirus 63 (ChAd63) encoding the preerythrocytic insert multiple epitope thrombospondin-related adhesion protein (ME-TRAP; n = 54 vaccinees) administered alone (n = 28) or with a modified vaccinia virus Ankara (MVA) ME-TRAP booster immunization 8 weeks later (n = 26). We observed an excellent safety profile. High levels of TRAP antigen–specific CD8+ and CD4+ T cells, as detected by interferon γ enzyme-linked immunospot assay and flow cytometry, were induced by intramuscular ChAd63 ME-TRAP immunization at doses of 5 × 1010 viral particles and above. Subsequent administration of MVA ME-TRAP boosted responses to exceptionally high levels, and responses were maintained for up to 30 months postvaccination.

Conclusions. The ChAd63 chimpanzee adenovirus vector appears safe and highly immunogenic, providing a viable alternative to human adenoviruses as vaccine vectors for human use.

Clinical Trials Registration. NCT00890019.

The induction of potent cellular immunity remains a central difficulty in vaccinology. Malaria is a disease widely regarded as an important potential target of improved T-cell–inducing vaccines. A method of immunoprophylaxis, such as a vaccine, would offer a valuable tool against both the morbidity and mortality caused by malaria [1]. Immunization of mice with irradiated sporozoites of murine Plasmodium falciparum provides protection against later challenge with murine malaria, and by transferring the CD8+ T lymphocyte clones specific to malaria surface antigens nonimmune mice can be protected [2–4]. High-level protective CD8+ T-cell responses can be induced by many vaccine types in small animals, but despite numerous attempts, there is no clear demonstration of the induction of very potent CD8+ T-cell responses in humans [5–7]. In animal models, such high levels are often required to induce protective immunity [8].

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Of the vaccination approaches assessed for induction of cellular immunity in humans, adenoviral vectors and heterologous prime-boost approaches have shown the most promise [9]. A series of phase I/IIa clinical studies at the University of Oxford have assessed prime-boost immunization strategies in healthy, malaria-naive adult human volunteers using plasmid DNA and the poxviruses modified vaccinia virus Ankara (MVA) and FP9 as vectors [7]. The most protective antigenic insert tested in these vectors was the T-cell multiple epitope string fused to the thrombospondin-related adhesion protein (ME-TRAP), which was more protective than the circumsporozoite protein or a polyprotein insert [7]. TRAP is a surface protein from the sporozoite stage of P. falciparum [10]. Several immunization regimes using these vectors with the ME-TRAP insert led to statistically significant delays in time to patent parasitemia, reflecting 80%–92% reductions in liver-stage parasite numbers emerging from the liver after experimental malaria infections [11]. However, these regimes induced predominantly CD4+ T-cell responses, and although T-cell responses correlated with vaccine efficacy, these approaches failed to induce protective immunity in the majority of vaccinees, suggesting a need for more potent vectors such as adenoviruses.

Adenoviral vectors suffered a setback with the failed human immunodeficiency virus type 1 (HIV-1) STEP vaccine trial, which showed a lack of efficacy and a nonsignificant trend toward increased HIV-1 infection in vaccinees [12]. However, antigen-specific responses in that trial were only of the order of 300 spot-forming cells (SFC) per million peripheral blood mononuclear cells (PBMCs), probably in part explaining the lack of efficacy. Moreover, the possibility that antivector immunity might have contributed to the suggested safety concern in the STEP trial has led to renewed interest in the use of nonhuman adenoviral vectors for several diseases [7].

Estimates suggest that, depending on the region, between 45% and 80% of adults carry AdHu5 neutralizing antibodies (nAb) [13]. Simian adenoviruses are not known to cause pathological illness in humans, and the prevalence of antibodies to chimpanzee-origin adenoviruses is <5% in humans residing in the United States. Prevalence in young children in Kenya, a target group for a malaria vaccine, is low, with only 4% of 1–6-year-old children in one study having high-titer nAb to chimpanzee adenovirus 63 (ChAd63), compared with 23% having high-titer nAb to AdHu5 [14]. When used in preclinical models, some simian adenoviruses showed similar levels of immunogenicity to the very potent human adenovirus AdHu5. In the preclinical P. berghei model, some simian adenoviruses were comparable to or appeared better than AdHu5 in terms of immunogenicity and protective efficacy; and in macaques, good T-cell immunogenicity was observed [15, 16].

Here, to our knowledge, we report the first-in-human clinical experience of a highly immunogenic nonhuman adenovirus vaccine vector.

**STUDY DESIGN**

This was an open-label phase I dose and route finding study from October 2007 to May 2010 to evaluate the safety and immunogenicity of ChAd63 ME-TRAP alone, and in a prime-boost regimen with MVA ME-TRAP. Participant flow and study design is summarized in Figure 1, and the vaccination regimens for each group are shown in Supplementary figure 1A and 1B.

**Figure 1.** CONSORT chart. Abbreviations: ChAd63, chimpanzee adenovirus; i.d., intradermal; i.m., intramuscular; MVA, modified vaccinia virus Ankara.
The dose of ChAd63 ME-TRAP was first escalated from $1 \times 10^8$ to $5 \times 10^{10}$ viral particles (vp) by the intradermal route (groups 1–4, n = 8 per group), then from $1 \times 10^{10}$ to $2 \times 10^{11}$ vp by the intramuscular route (groups 5–7, n = 4–10 per group; see Figure 1). Within these groups, 4 volunteers received a single immunization with ChAd63 ME-TRAP (“A” subgroups, n = 28), and 4 received ChAd63 ME-TRAP followed by MVA ME-TRAP 8 weeks later (“B” subgroups, n = 20). In the highest-dose group (2 $\times 10^{11}$ vp, group 7), 2 volunteers were boosted with intradermal MVA ME-TRAP 2 $\times 10^8$ plaque-forming units (PFU; group 7B), and 4 volunteers were boosted with intramuscular MVA ME-TRAP 2 $\times 10^8$ PFU (group 7C).

In addition, in May 2010, all volunteers in the prime-boost groups (n = 26) were invited to return for a late-boosting immunization with either ChAd63 ME-TRAP (5 $\times 10^8$ vp) or MVA ME-TRAP (2 $\times 10^8$ PFU) intramuscularly. In total, 11 of 26 eligible volunteers were screened for a third immunization and enrolled between June and July 2010. This group was termed group 8. Vaccine allocation was randomized 1:1, stratified by time interval since the last immunization (n = 5 ChAd63 ME-TRAP; n = 6 MVA ME-TRAP). Data for days 21–28 following reboost are presented.

RESULTS

Manufacturing Yield and Genetic Stability of ChAd63 ME-TRAP
The Clinical BioManufacturing Facility at the University of Oxford manufactured ChAd63 ME-TRAP [24] with high yield, producing approximately $3.7 \times 10^{14}$ vp from a single-bulk cell culture preparation. This was the yield obtained following cell lysis, disaggregation, centrifugation, and filtration. From 2 bulk harvest lots, 12,840 doses of ChAd63 ME-TRAP, with $1 \times 10^{11}$ vp per vial, were produced.

Genetic stability of ChAd63 ME-TRAP was tested by performing 8 passages of a batch of the virus in human embryonic kidney 293 (HEK293) cells and characterizing the resulting virus using a combination of polymerase chain reaction (PCR) and sequencing. No evidence of genetic instability was detected in either the passaged material or the clinical batch used in the clinical trial.

Safety
There were no serious adverse events. The 417 adverse events considered possibly, probably, or definitely related to vaccination were reported up to and including 28 days post ChAd63 ME-TRAP (184 local and 233 systemic). Local adverse events included pain, redness, swelling, scaling, itching, and warmth. Systemic symptoms solicited using diary cards included fever, feverishness (the sensation of fever without measurable pyrexia), malaise, fatigue, arthralgia, myalgia, headache, and nausea or vomiting. Over 90% of adverse events were mild in nature. A detailed breakdown of adverse events occurring postvaccination can be found in Supplementary figure 1G–K.

All subjects receiving ChAd63 ME-TRAP intradermally (groups 1–4) reported a local adverse event, most commonly redness (100%) and swelling (100%). Incidence of local adverse events was lower in those receiving intramuscular ChAd63 ME-TRAP (groups 5–7), with significantly fewer local adverse events reported per volunteer at comparable doses ($P < .005$; Figure 2A and 2B).

The most common systemic adverse events occurring were fatigue (87% of volunteers), malaise (69%), and feverishness (54%). In total, 69% of systemic adverse events were reported in the first 48 hours postvaccination, and 64% resolved in this timeframe. The median number of systemic adverse events experienced by a volunteer increased with vaccine dose regardless of route of administration (Figure 2C). In parallel, severity of reported systemic adverse events increased as the dose of ChAd63 ME-TRAP increased. There were 8 individual severe adverse events reported, 4 of which (feverishness, headache, malaise, and coryzal symptoms) occurred in the same volunteer who developed a coryzal illness 13 days postvaccination. Two other volunteers had 24 hours of symptoms immediately post vaccination (1 subject with coryzal symptoms and 1 with myalgia/arthralgia/feverishness).

The safety profile of MVA ME-TRAP was very similar to that reported previously (Figure 2B and 2D) [17, 18]. The preceding dose of ChAd63 ME-TRAP did not affect the intensity or duration of adverse events post-MVA ME-TRAP (data not shown). A similar acceptable safety profile was observed after the reboosting immunizations.

Immunogenicity

ELISPOT Responses
Ex vivo interferon γ (IFN-γ) enzyme-linked immunospot (ELISPOT) responses to the vaccine antigen were detectable in all groups after the priming vaccination with ChAd63 ME-TRAP (Figure 3). Unless stated otherwise, values reported represent total summed responses to TRAP pools and ME from the T9/96 P. falciparum strain. No significant difference between doses of ChAd63 ME-TRAP administered via the intramuscular and intradermal different routes was observed (Figure 3A), so these routes were pooled for further analysis.

Responses to ME-TRAP were detected 14 days after priming vaccination with ChAd63, with median ELISPOT responses per
dose group ranging from 61–915 SFC/10^6 PBMCs (hence SFC/M) with priming doses between 1 \times 10^8 and 2 \times 10^{11} vp (Figure 3B and 3D). Boosting with MVA ME-TRAP significantly augmented the immunogenicity with individual responses as high as 2465 SFC/M (medians ranging from 764 to 2063 SFC/M for 1 \times 10^8–2 \times 10^{11} vp) 7 days post-MVA ME-TRAP (Figure 3C and 3E). Response to the vaccine insert could be detected in all volunteers at their final visits, with excellent preservation of the effector immune response in the prime-boost volunteers, with medians of 246–1294 SFC/M across the different groups 3 months post boosting vaccination (Figure 3C).

Individual peak immune responses occurred either at 14 or 28 days post-ChAd63 ME-TRAP vaccination. Post priming vaccination, there was a trend toward higher responses at higher doses with a significant difference in ELISPOT response between 1 \times 10^8 and 2 \times 10^{11} vp ChAd63 ME-TRAP (Figure 3F; P = .005 Kruskal-Wallis test, medians ranging from 78.1–915 SFC/M). Post-MVA ME-TRAP immune responses were maximal at day 63 with a nonsignificant trend toward increasing responses as the priming dose increased.

To assess changes in the breadth of TRAP-specific T-cell responses by ELISPOT induced by both immunizations, the number of peptide pools (total of 6) in which responses per well were greater than a threshold of 100 SFC/M (after subtracting the background response) were summed for each individual, at every time point. Given the small number of samples for each dose group, data for all groups were pooled. A nonsignificant increase (Figure 3G) in the number of positive TRAP-specific peptide pools was observed following the first immunization (D14 mean, 1.0 [95% confidence interval {CI}, 0.4–1.6]), and this was significantly boosted by the second immunization (D63 mean, 3.0 [95% CI, 2.2–3.8] P < .001 compared with D14). This boosting effect of MVA ME-TRAP was maintained up to D140 (mean 1.4 [95% CI, 0.9–2.0] compared with D14, P < .05).

Cultured ELISPOT assay (cELISPOT) responses (Figure 3H) were present in 7 of 8 volunteers examined at day 140 but did not correlate significantly with peak ex vivo immune response (Pearson correlation r = −0.68, P = .2). Notably, cELISPOT responses in group 4, with a median cELISPOT response of 6638 SFC/10^6 original PBMCs (interquartile range [IQR], 2901–9073) are double the highest value previously recorded.

Figure 2. Adverse events. Local and systemic adverse events occurring post ChAd63 ME-TRAP (A–D) and MVA ME-TRAP (B and D). Median and interquartile range number of local (A) and systemic (C) adverse events reported per volunteer post ChAd63 ME-TRAP at different doses and routes. Panels B and D show percentage of volunteers reporting at least 1 local or systemic adverse event; shading indicates severity (highest severity of adverse events reported by volunteers is presented). (*P < .05; ***P < .001; analyzed by Kruskal-Wallis test with Dunn post test.) Abbreviations: ChAd63, chimpanzee adenovirus; i.d., intradermal; ME-TRAP, multiple epitope-thrombospondin–related adhesion protein; MVA, modified vaccinia virus Ankara.
Figure 3. ELISPOT assays. A, Comparison of median ELISPOT responses at comparable doses via intradermal and intramuscular routes. Error bars represent interquartile ranges (IQRs); no significant differences were observed. B and C, Median ELISPOT responses postvaccination in A groups and B groups. D and E, Median ELISPOT responses day 14 and day 63 grouped by priming dose ChAd63 ME-TRAP; error bars represent IQRs. F, Peak median ELISPOT response by priming dose ChAd63 ME-TRAP; error bars represent IQRs. G, Mean number of peptide pools recognized at different time points (data for all volunteers analyzed using 1-way analysis of variance with Bonferroni post test; error bars represent standard errors of the mean). H, Cultured ELISPOT responses compared with previous trials (data shown represents median and IQR, analyzed by Kruskal-Wallis test with Dunn post test. (*P < .05; x-axis displays regimes used in previous clinical trials: D = DNA ME-TRAP, F = FP9 ME-TRAP, M = MVA ME-TRAP). Abbreviations: ChAd63, chimpanzee adenovirus; ELISPOT, enzyme-linked immunospot; i.d., intradermal; i.m., intramuscular; ME-TRAP, multiple epitope-thrombospondin-related adhesion protein; MVA, modified vaccinia virus Ankara; PBMCs, peripheral blood mononuclear cells; vp, viral particles.
with a regime of DNA and MVA ME-TRAP given multiple times, one of the most immunogenic and protective regimes we have previously trialed (median, 2900; IQR, 140–4160) [19].

TRAP-specific antibody responses were identified by enzyme-linked immunosorbent assay (ELISA) in all recipients of ChAd63, and were significantly boosted by MVA ME-TRAP (data not shown).

**Flow Cytometry**

T-cell phenotypes (ie, CD4+ vs CD8+) and functional capacities of vaccine-generated T-cell responses (ie, the production of cytokines IFN-γ, interleukin 2, and tumor necrosis factor α in response to a pool of all 57 peptides spanning the TRAP antigen were evaluated using flow cytometry. Assays were performed on cryopreserved PBMCs from volunteers in groups 2B (n = 2), 3B (n = 4), and 4B (n = 2) (Figure 4). No significant differences were observed between the different groups in terms of the percentage of cytokine secreting cells; thus, all data were pooled for further analysis.

Vaccination with ChAd63 ME-TRAP induced an increase in the percentage of antigen-specific CD4+ and CD8+ T cells capable of secreting cytokines of interest with responses boosted by MVA ME-TRAP. TRAP-specific CD8+ T cells secreting IFN-γ underwent an 8-fold increase between baseline (mean, 0.014% [95% CI, 0–0.033%]) and day 63 (mean, 0.12% [95% CI: 0–0.167%]), P < .05. Boosting also induced a marked expansion of polyfunctional T cells up to 140 days (Figure 4B).

**Antivector Antibodies**

Serum samples were examined for the presence of nAb to ChAd63 in 50 of 54 volunteers. Initially, volunteers with any evidence of nAb to ChAd63 were excluded for group 1; then approval was given to allow recruitment in groups 2–6 of subjects with nAb titer, 1:200, and group 7 was subsequently approved for recruitment of volunteers with any nAb titer. Figure 5 shows the time course of nAb. In total, 35 of 50 (83%) subjects had no evidence of ChAd63 nAb at day 0,
including 8 subjects receiving $1 \times 10^8$ vp ChAd63 ME-TRAP (group 1), 4 of whom developed low levels after vaccination. Of the remaining subjects negative for nAb at day 0, >90% seroconverted after vaccination. ChAd63 dose correlated with peak nAb titer (Spearman rank correlation $r = 0.54$, $P < .0001$; $n = 50$), but no relationship was observed between baseline nAb and peak ELISPOT response (excluding volunteers from group 1 with no baseline nAb; $r = 0.031$, $P = .85$; Figure 5C, $n = 42$). In group 7, where any preexisting antibody titer was allowable, there was no evidence that higher baseline nAb titer reduced peak ($r = 0.21$, $P = .71$, $n = 6$) or day 140 ELISPOT response ($r = -0.51$, $P = .30$, $n = 6$).

**Reboosting**

No serious vaccine-related adverse events were reported after either reboosting immunization. The reactogenicity profile of both vaccines was similar (Figure 6A).

Stratification for time since last vaccination was effective, with a median of 236 days (IQR, 184.5–638) between last vaccination and reboost for the ChAd63 ME-TRAP recipients and 249 days (IQR, 172–606) for the MVA ME-TRAP recipients (Figure 6B and Supplementary figure 1B). ELISPOT responses were not significantly different prior to the reboosting immunization between those reboosted with ChAd63 and MVA ($P = .66$) and for both groups, the reboosting immunization resulted in a significant increase from pre-reboosting ELISPOT responses (median, 624 SFC/M; IQR, 294–839) prior to reboosting to a median of 1315 SFC/M [IQR, 1024–1991] at peak ($P = .001$; Figure 6C). There was no significant difference in the peak reboost responses induced by ChAd63 or MVA (1743 SFC/M [IQR, 994–2106] and 1280 SFC/M [IQR, 853–2060], respectively; $P = .7$). There was a correlation between reboosting interval and magnitude of the response to reboost by ELISPOT (Figure 6D).

Intracellular cytokine staining was performed on a subset of reboosted volunteers ($n = 3$ in each group) where cells were available. A comparable percentage of total antigen-specific CD8$^+$ IFN-$\gamma^+$ cells followed reboosting (ChAd63 median, 0.22% [range, 0–0.26%], MVA median, 0.410% [range, 0.009–0.142%], $P = 1.0$; Figure 6E).

**DISCUSSION**

ChAd63 ME-TRAP at doses between $1 \times 10^8$ and $2 \times 10^{11}$ vp has been used safely in this study and generates high levels of antigen-specific T cells which remain detectable for over 28 months post-MVA. Although other simian adenoviruses have shown promise in preclinical studies, this is the first report of the use of a nonhuman adenovirus as a vaccine vector in humans to our knowledge.

Vaccines have shown considerable promise as vaccine candidates due to their ease of generation, often low-cost manufacture, and their ability to induce significant cellular immunity. However, to date their development has been limited by several obstacles. Some viral vectors, even within the adenovirus family, are much more potent than others, and only a limited number of serotypes show good immunogenicity. Second, for several vectors, proprietary cell lines are required for large-scale manufacture, and these are not widely available. Other vectors have been found to show significant genetic instability when produced at large scale. Usage of HEK293 cells, or the alternative PER.C6 cell line, facilitates easy growth of ChAd63, thus providing a manufacturing process that can be easily scaled up for mass production. Several thousand doses of vaccine were produced in our manufacturing runs in a process that is now very efficient. Finally, certain viral vectors are limited by high levels of preexisting immunity in many human populations, whereas ChAd63 has limited preexisting immunity in European and African populations.

In this trial, ChAd63 ME-TRAP has been shown to have a good safety profile despite relatively stringent adverse analysis (all adverse events occurring up to 28 days postvaccination deemed possibly, probably, or definitely related were analyzed). Rates and types of adverse events are comparable to ongoing
trials using human adenovirus 5 as a vector. In a recent phase 1 trial of a human adenovirus 5 expressing glycoprotein (GP) from the Ebola virus species where 11 subjects received $2 \times 10^{10}$ vp of vaccine, 6 moderate or severe systemic adverse events were reported at this dose (6 of 29 total systemic adverse events) [20]. In comparison, we report 1 moderate or severe systemic adverse event occurring post-ChAd63 ME-TRAP at $1 \times 10^{10}$ vp (1 of 29 total systemic adverse events reported) making ChAd63 less reactogenic. Peiperl et al [21] assess safety and maximal tolerated dose of an adenoviral vaccine vector in volunteers without prior immunity, using a recombinant replication-defective adenovirus type 5 (rAd5) vaccine...
expressing HIV-1 Gag, Pol, and multiclade Env proteins. Here, 20 volunteers received $2 \times 10^{11}$ particle units of vaccine, and systemic adverse events were assessed for the 72 hours following vaccination. Fifteen volunteers report at least 1 moderate or severe systemic adverse event occurring postvaccination. In comparison, with ChAd63 ME-TRAP at the same dose, 10 volunteers were vaccinated and reported only 9 systemic adverse events postvaccination as moderate or severe.

MVA ME-TRAP is a well-characterized vaccine in terms of immunogenicity and once again is used safely in this trial. It has now been administered to >600 healthy volunteers in Oxford [22], The Gambia [23], and Kenya [24] without any serious adverse events with volunteers receiving between 1 and 3 intradermal doses of vaccine ($3–50 \times 10^7$ PFU per dose), at 3- to 4-week intervals.

The prime-boost vaccination regimen with ChAd63 and MVA ME-TRAP generated unprecedented levels of effector T-cell responses, as measured by ex vivo IFN-γ ELISPOT in comparison to previous malaria vaccine trials with the same antigenic insert. Responses here were both higher and different in quality. In contrast to average responses (at the peak time point) of approximately 450 SFC/M [18, 25], we now report average responses exceeding 2000 SFC/M. Second, by flow cytometry we observe that there are at least as many CD8$^+$ as CD4$^+$ gamma-interferon–secreting T cells induced at the peak time points, in contrast to previous prime-boost regimes using DNA and poxvirus-priming vectors, where predominantly CD4$^+$ T-cell responses were induced. This ability to induce both high-level CD8$^+$ and CD4$^+$ responses with simian adenovirus–MVA prime-boost regimes should broaden the potential utility of this approach.

This simian adenovirus vector used alone without an MVA boost also compares very favorably in terms of immunogenicity with reports of HIV vaccine trials using the AdHu5 vector, although different inserts prevent a definitive comparison. Over 90% of our volunteers had detectable ELISPOT responses 4 weeks after the priming vaccination, and after 3 months, all subjects receiving adenovirus alone had a detectable immune response compared with recently reported HIV-1 vaccine trials using human adenovirus 5 expressing HIV gag (Ad5 gag), where after 3 doses of $1 \times 10^{11}$ vp Ad5 gag at week 8 only 53% of volunteers had a detectable response on ELISPOT [26].

Neutralizing antibodies to ChAd63 were induced in all volunteers. But titers did not correlate with the level of vaccine-induced immune response to the malaria insert, as measured by ELISPOT, nor with the frequency or grade of adverse events (data not shown). As discussed above, ChAd63 nAb are rare in the general population but are clearly detectable prior to vaccination in some individuals. It is unclear whether this is caused by cross-reactivity to ChAd63 of antibodies induced by a closely related human adenovirus, or by a low prevalence of ChAd63 infections in humans. In group 7 where individuals with any titer of ChAd63 nAb were enrolled, there was no reduction in the vaccine-induced immune response in those with preexisting antibodies to the vector. Moreover, volunteers could be safely reboosted with either MVA ME-TRAP or ChAd63 ME-TRAP at 5–30 months after their first MVA immunization with no impairment of vaccine immunogenicity, suggesting that these viral vectors are suitable for repeated usage with such an interval.

**CONCLUSION**

This phase I clinical trial has shown that simian adenoviruses are safe when used alone or with an MVA boost with no evidence of dose limiting toxicity. Importantly, the cellular immunity induced to a full-length antigen by this vaccination strategy appears significantly greater than with any previously reported immunization regime.

**Supplementary Data**

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://www.oxfordjournals.org/our_journals/jid/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

**Notes**

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All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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