Perfluorocarbon Emulsion Therapy Attenuates Pneumococcal Infection in Sickle Cell Mice

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Impaired immunity and tissue hypoxia-ischemia are strongly linked with Streptococcus pneumoniae pathogenesis in patients with sickle cell anemia. Perfluorocarbon emulsions (PFCEs) have high O2-dissolving capacity and can alleviate tissue hypoxia. Here, we evaluate the effects of intravenous PFCE therapy in transgenic sickle cell (HbSS) mice infected with S. pneumoniae. HbSS and C57BL/6 (control) mice intravenously infected with S. pneumoniae were treated intravenously with PFCE or phosphate-buffered saline (PBS) and then managed in either air/O2 (FiO2 proportion, 50%; hereafter referred to as the PFCE-O2 and PBS-O2 groups) or air only (hereafter, the PFCE-air and PBS-air groups) gas mixtures. Lungs were processed for leukocyte and bacterial counts and cytokine measurements. HbSS mice developed severe pneumococcal infection significantly faster than C57BL/6 mice (Kaplan–Maier analysis, P < .05). PFCE-O2–treated HbSS mice had significantly better survival at 72 hours than HBSS mice treated with PFCE-air, PBS-O2, or PBS-air (P < .05). PFCE-O2–treated HbSS mice also had significantly lower pulmonary leukocyte counts, lower interleukin 1β and interferon γ levels, and higher interleukin 10 levels than PFCE-air–treated HbSS mice. Clearance of S. pneumoniae from lungs of HbSS mice or C57BL/6 mice was not altered by PFCE treatment. Improved survival of PFCE-O2–treated HbSS mice infected with S. pneumoniae is associated with altered pulmonary inflammation but not enhanced bacterial clearance.

Keywords. antisyickling agents; Streptococcus pneumoniae; anemia.

Sickle cell anemia is the most common form of sickle cell disease [1]. Individuals with homozygous sickle cell disease (HbSS) produce a variant hemoglobin that aggregates under conditions of low oxygen [2, 3]. Red blood cells (RBCs) with HbSS aggregates deform to a characteristic sickle shape and are significantly less plastic [2–4]. The alteration in mechanical properties predisposes sickled RBCs to sticking within capillaries, thereby causing obstruction to blood flow and hypoxic-ischemic organ damage [4].

Clinically, sickle cell anemia is a chronic disease punctuated by acute crises [4–9]. Vasoocclusive crises cause a variety of symptoms and syndromes [5–9]. Individuals with HbSS are also prone to bacterial sepsis, particularly Streptococcus pneumoniae infection [10, 11]. Streptococcus pneumoniae infection can lead to hypoxemia and precipitate vasoocclusive events in tissues, to produce a prognostically poor clinical scenario as, for example, in acute chest syndrome [5, 6, 8–11]. Initial treatment of acute crises such as acute chest syndrome includes O2 therapy, intravenous fluids, and broad-spectrum antibiotics [5, 8–11]. Individuals who do not respond promptly or who deteriorate are treated with top-up or exchange blood transfusions [12], which, while effective and lifesaving, are associated with adverse reactions, including transfusion-related acute lung injury [13]. Hence, management of life-threatening vasoocclusive/infective crises remains a challenge, and better treatments are needed to improve outcomes [4–13].

In HbSS disease, blood transfusion therapy reduces the proportion of circulating RBCs containing variant hemoglobin [12]. However, it does not readily improve oxygenation and/or perfusion of tissues whose capillary bed is occluded by sickled RBCs prior to transfusion [4–9]. Here, cellular injury continues until perfusion is restored fully.

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Oxycyte (Oxygen Biotherapeutics, Morrisville, North Carolina), a third-generation perfluorocarbon emulsion (PFCE), is composed of particles (diameter, 0.2 μm) that have a hydrophobic organofluorine (perfluorotert-butylcyclohexane, C₁₀F₂₀) core and a hydrophilic outer coat (egg yolk phospholipids) suspended in a water-based solution [14–16]. It has high gas-dissolving capacity and, unlike hemoglobin, has a linear relationship between oxygen partial pressure (pO₂) and oxygen content [14–16]. Although whole blood has a higher absolute oxygen content than PFCE, O₂ release from PFCE is almost complete in the presence of a high PO₂ gradient and usually exceeds the amount released by hemoglobin [16]. Presently, Oxycyte is undergoing evaluation in a phase 2 study of patients with traumatic brain injury at risk of hypoxic-ischemic brain damage [17]. Although PFCE is often suggested as an alternative to blood transfusion, little is known about its therapeutic potential in HbSS. In this proof-of-concept study, we set out to determine whether intravenous PFCE therapy alters the clinical course of S. pneumoniae infection in transgenic sickle cell (HbSS) mice.

**MATERIALS AND METHODS**

**Ethics Approval, Animals, and Bacterial Strains**

All animal experiments were performed at a designated facility in the University of Leicester and in accordance to United Kingdom Home Office guidelines with ethics approval from The University of Leicester Ethics Committee. HbSS mice (Hbatm1-Paz Hbbtm1Tow Tg(HBA-HBBs)41Paz/J) and background strain C57BL/6 mice [18] were purchased from The Jackson Laboratory (United States), and C57BL/6 (control) mice were purchased from Charles River (United Kingdom). Genotypes of mice bred from transgenic mating pairs were established by polymerase chain reaction (PCR) analysis. Briefly, DNA from an ear biopsy specimen was prepared using the QiaPrep DNeasy Blood and Tissue Kit (Qiagen, West Sussex, United Kingdom). Genomic DNA was then amplified using MyTel Red DNA polymerase (Bioline, London, United Kingdom) and reverse and forward sequence complementary DNA primers for HbB and HbS (Eurofins Genetic Services, London, United Kingdom). The amplified products were visualized using agarose

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**Figure 1.** Figure 1 outlines the experimental schema. Transgenic sickle cell (HbSS) mice were bred, genotyped and grown to maturity before experiments. HbSS and C57 mice were placed into one of three groups (n = 6 from each strain) at point [E] in the schema. Group (a) consisted of mice that were neither infected with Streptococcus pneumoniae nor injected with phosphate-buffered saline (PBS) or perfluorocarbon emulsion (PFCE). Group (b) consisted of mice treated with PBS or PFCE but not infected with S. pneumoniae. Group (c) consisted of mice infected with S. pneumoniae and treated with PBS or PFCE in air or O₂. Mice were euthanized at 72 hours after entry into an experiment.
electrophoresis and ethidium bromide as described by Sambrook et al [19]. *S. pneumoniae* serotype 2, strain D39, was prepared as described previously [18].

**Experimental Design**

The experimental design is illustrated in Figure 1. Mice were infected with *S. pneumoniae* by injecting 100 µL of inoculum (2–6.4 × 10^5 colony-forming units [CFU]/mL) into the tail vein of anesthetized animals. Three hours after infection, mice were reanesthetized and given intravenous doses (3 mL/kg) of phosphate-buffered saline (PBS) or PFCE (Oxycyte) via the tail vein and recovered in ambient-air chambers or chambers supplied with supplemental oxygen (FiO2 proportion, 50%). Three hours following PBS or PFCE injection, mice receiving supplemental oxygen were transferred to ambient air conditions. Animals were euthanized 72 hours after infection or immediately on developing severe lethargy as previously described [20]. Lungs of euthanized mice were processed for histologic analysis, white blood cell counts, cytokine analysis, and determination of *S. pneumoniae* colony counts. Blood specimens were aspirated via a cardiac puncture for colony count determinations.

**Lung Histological Analysis**

The left lungs of mice were fixed in formalin, processed into paraffin wax, cut (4–6 µm thick), mounted onto slides, and stained with hematoxylin–eosin. Histopathologic analysis was performed using low-power (×10 and ×20) and high-power (×50 and ×100) objective lenses and a graticule. Lung injury was assessed by quantifying healthy alveoli and alveoli with exudate, hemorrhaging, or inflammatory cells (macrophages, neutrophils, and lymphocytes were identified using nuclear morphology). The overall percentage of alveoli in a lung section with the histologic abnormality was converted to a linear score (range, 0–5), referred to as the airspace score, with 0 indicating absence of the variable and 5 indicating inflammation present in 81%–100% of alveoli. Three lung sections from each were used to determine an average score. Each lung section was scored separately by 2 of the authors (N. H. and H. P.), and the average was used as the final airspace score.

**Lung White Blood Cell Counts**

Lung differential white blood cell counts were performed using cell homogenates of right lung and the Calibur FACS system with Cell Quest Pro software (Becton Dickinson, Cowley, United Kingdom), as previously described [21]. Lung cells were incubated with fluorophore-conjugated antibodies directed against mouse neutrophil (Gr-1/Ly-6G-phcoerythrin-cy7), macrophage (F4/80-allophycocyanin), T-cell (CD3-phcoerythrin), and B-cell (CD19-FITC) antigens (all purchased from Biolegend, San Diego, California).

**Estimation of S. pneumoniae Recovered From Lung Tissue and Blood and Lung Cytokine Assays**

*Streptococcus pneumoniae* were recovered from whole-blood specimens and from the right lung homogenized in PBS. Colony counts were performed as previously described [22, 23]. Cytokine quantifications were performed using homogenized right lung and sandwich enzyme-linked immunosorbent assay (Qia- gen, Valencia, California).

**RBC Sickling in HbSS Mice**

The percentage of sickled RBCs in HBSS mice was determined using glass slide blood smears stained with Giemsa stain (Sigma, Poole, United Kingdom).

**Effect of PFCE on S. pneumoniae In Vitro**

*Streptococcus pneumoniae* was cultured in chemically defined medium (CDM) [23] at 37°C and then transferred into tubes containing varying concentrations of PFCE (0%–4% v/v) in CDM. Culture samples were removed at hourly intervals for 10 hours and plated overnight on blood agar for colony counting [23, 24].

**Statistical Analysis**

All statistical analyses were performed using GraphPad Prism software (La Jolla, California). Kaplan–Meier survival curves were compared using the log-rank Mantel–Cox test. Between-group comparisons were performed using the Student *t* test with the Welch correction for different variances. A *P* value of < .05 was considered significant.

**RESULTS**

**PFCE-O2 Therapy Reduces the Susceptibility of HbSS Mice to S. pneumoniae Infection**

All HbSS mice infected with *S. pneumoniae* and treated with PBS-air developed signs of severe sepsis within 72 hours and were euthanized before the end of the experiment. Pneumococcus-infected C57BL/6 mice treated with PBS-air survived significantly better than HBSS littermates, with only 50% of animals euthanized before 72 hours (*P* < .05, by Kaplan–Meier analysis; Figure 2A). C57B6/L mice treated with PBS-air also survived significantly better than HbSS mice treated with PFCE-air (*P* < .05, by Kaplan–Meier analysis; Figure 2A). PFCE-air–treated C57BL/6 mice were more susceptible to *S. pneumoniae* than PBS-treated controls (*P* < .05, by Kaplan–Meier analysis; Figure 2A), whereas PFCE-air and PBS-air–treated HbSS mice had similar survival (*P* > .05, by Kaplan–Meier analysis).

HbSS mice treated with PFCE-O2 had significantly better survival than HbSS littermates treated with PFCE-air, PBS-O2, or PBS-air (*P* < .05 for all comparisons, by Kaplan–Meier analysis; Figure 2B). In contrast, pneumococcus-infected C57BL/6 mice treated with PFCE-O2 or PFCE-air had significantly worse
survival than infected C57BL/6 mice treated with PBS-O2 or PBS-air (P < .05 for all comparisons, by Kaplan–Meier analysis; Figure 2C).

**PFCE-O2 Therapy Does Not Attenuate Lung Injury in *S. pneumoniae*–Infected HbSS Mice**

Uninfected, untreated HbSS mice (the nil group; Figure 3A) had evidence of acute lung injury, characterized by increased inflammatory cells, hemorrhaging, and exudates (P < .05). These mice also had evidence of widespread lung fibrosis (data not shown). None of these features were present in uninfected, untreated C57BL/6 mice (Figure 3C).

*S. pneumoniae* infection of HbSS mice was associated with an increase in airspace exudates, inflammatory cells and hemorrhage, compared with infected C57BL/6 mice (Figure 3A and 3C). *S. pneumoniae*–infected HbSS mice treated with PFCE-O2 had lung inflammation scores similar to those treated with PFCE-air (P > .05; Figure 3B).

**PFCE-O2 Therapy Attenuates Pulmonary Leukocytosis in *S. pneumoniae*–Infected HbSS Mice**

Untreated, uninfected HbSS mice had higher pulmonary macrophage, neutrophil, T-cell, and B-cell counts than untreated, uninfected C57BL/6 mice (P < .05 for all comparisons; Supplementary Figure 3).

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**Figure 2.** Perfluorocarbon emulsion (PFCE)–O2 therapy improves survival of transgenic sickle cell (HbSS) mice but not C57BL/6 mice infected with *Streptococcus pneumoniae*. Survival over 72 hours among HbSS and C57BL/6 mice managed in air or oxygen and treated with phosphate-buffered saline (PBS) or PFCE 3 hours after infection with *S. pneumoniae*. A, PBS-air–treated C57BL/6 mice (white squares) survived significantly better than PBS-air–treated HbSS mice (black circles), PFCE-air–treated HbSS mice (white circles), and PFCE-air–treated C57BL/6 mice (black triangles). P < .05 for each comparison. B, PFCE-O2–treated HbSS mice (white triangles) died significantly later than HbSS mice treated with PBS-air (white squares), PFCE-air (white circles), or PBS-O2 (black circles). P < .05 for each comparison. C, C57BL/6 mice treated with PBS-air (black triangles) or PBS-O2 (white circles) survived longer than C57BL/6 mice treated with PFCE-air (black circles) or PFCE-O2 (white squares). P < .05 for each comparison. There were 6 animals in each treatment group.

**Figure 3.** Perfluorocarbon emulsion (PFCE)–O2 therapy does not attenuate lung injury in transgenic sickle cell (HbSS) mice infected with *Streptococcus pneumoniae* (SP). A, Peripheral airspaces of uninfected, untreated HbSS mice (NIL) were found to contain exudates, inflammatory cells, and hemorrhaging. Infected HbSS mice treated with phosphate-buffered saline (PBS)-air also had increased number of airspaces containing hemorrhaging, exudates, and inflammatory cells, compared with HbSS mice in the nil group (P < .05). B, Infected, PBS-air–treated HbSS mice had similar lung injury scores to infected PFCE-air–treated HbSS mice (P > .05). Infected, PFCE-O2–treated HbSS mice had similar lung injury scores to infected PFCE-air–treated HbSS mice (P > .05). C, In contrast, peripheral airspaces of uninfected, untreated C57B6/L mice (NIL) did not contain exudates or hemorrhage. C57B6/L mice infected with SP and treated with PBS had increased number of airspaces containing hemorrhaging, exudates, and inflammatory cells, compared with C57B6/L mice in the nil group (P < .05). Lung injury scores were not significantly different in pneumococcus-infected C57B6/L mice treated with PBS-air or PFCE-air (P > .05). There were 6 animals in each treatment group.
Infection with *S. pneumoniae* led to a significant increase in pulmonary macrophage, neutrophil, T-cell, and B-cell counts in both mouse strains (*P* < .05 for all comparisons; Supplementary Figure 1A–1D). There were no statistically significant between- or within-strain differences in white blood cell counts (all subsets) among PFCE- and PBS-treated mice (*P* > .05).

Infected HbSS mice treated with PFCE-O2 had significantly lower macrophage, neutrophil, T-cell, and B-cell counts in the lungs than PFCE-air or PBS-O2–treated HbSS mice (*P* < .05 for all comparisons; Figure 4A, 4C, 4E, and 4G). Infected C57BL/6 mice treated with PFCE-air or PFCE-O2 had significantly lower lung macrophage and neutrophil counts than PBS-air or PBS-O2–treated C57B6/L littermates (*P* < .05; Figure 4B and 4D). There were no differences in T-cell and B-cell counts in infected C57BL/6 mice in comparisons involving all treatments (*P* < .05; Figure 4F and 4H).

**PFCE-O2 Therapy Attenuates the Proinflammatory Cytokine Milieu in Lungs of HbSS Mice Infected with *S. pneumoniae***

Untreated, uninfected HbSS mice had significantly higher pulmonary levels of TNF-α, IL-1β, and IL-6 and significantly lower levels of IL-10 than untreated, uninfected C57B6/L mice (*P* < .05; Supplementary Figure 2). Among infected PFCE-treated mice managed in air, there were no significant differences in levels of all other assayed cytokines between the HbSS and C57B6/L groups (*P* > .05 for each comparison; Supplementary Figures 2).

At the time of death, infected HbSS mice treated with PBS-O2 had significantly higher levels of TNF-α and IL-6 than those treated PFCE-air or PFCE-O2 (*P* < .05 for each comparison; Figure 5A and 5B). However, TNF-α and IL-6 levels in HbSS mice treated with PFCE-O2 were not significantly different from levels in mice treated with PFCE-air (*P* > .05 for each comparison). Infected PFCE-O2–treated HbSS mice had significantly lower levels of pulmonary MIP-1α, IFN-γ, and IL-1β (Figure 5C–E) than either PFCE-air– or PBS-O2–treated littermates (*P* < .05 for each comparison). In addition, PFCE-O2–treated HbSS mice had higher IL-10 levels (Figure 5F) than PFCE-air– or PBS-O2–treated mice (*P* < .05 for each comparison; Figure 5D).

Infected C57BL/6 mice treated with PFCE-O2 and PFCE-air had significantly lower IL-6 (Figure 5B) and MIP-1α...
levels, compared with PBS-O2–treated mice (P < .05). There were no significant differences in levels of all other assayed cytokines in C57B6/L mice in comparisons of different treatments (P > .05 for each comparison; Figure 5A, 5C, and 5D–5F).

**CFU Counts From Blood and Lungs of *S. pneumoniae*–Infected HbSS and C57BL/6 Mice**
There were no significant differences in blood or lung CFU counts at the time of death in comparisons of PFCE and PBS treatment groups among HbSS or in C57BL/6 mice managed in air or O2 (P > .05; Figure 6A–6D).

**PFCE Does Not Enhance the *S. pneumoniae* Growth Rate In Vitro**
Over a 10-hour incubation period, *S. pneumoniae* cultures placed in CDM medium with or without PFCE exhibited a >10-fold increase in CFU counts (Table 1). The growth rate and generation times of cultures grown in CDM medium only and those containing PFCE were not significantly different (P > .05).

**PFCE Reduces RBC Sickling in HbSS Mice Infected with *S. pneumoniae***
Blood specimens from HbSS mice treated with PFCE and infected with *S. pneumoniae* had a lower percentage of sickled RBCs than blood specimens from HbSS mice treated with PBS (P < .05; Supplementary Figure 3). The percentage of sickled RBCs in blood specimens from HbSS mice treated with PFCE-O2 was not statistically different (P > .05) from the percentage of sickled RBCs in blood specimens from littermates treated with PFCE-air (Supplementary Figure 3A).

**DISCUSSION**
This study was designed to test the hypothesis that treatment with a third-generation PFCE, Oxycyte, attenuates *S. pneumoniae* infection in HbSS mice as a result of improved O2 delivery. Intravenous PFCE therapy is linked with immune system and complement activation [15, 16]. Hence, we compared the outcome of *S. pneumoniae* infection in PFCE-O2– and PFCE-air–treated mice. Our data show that intravenous PFCE therapy attenuates *S. pneumoniae* infection only in HbSS mice treated...
with supplemental oxygen, consistent with the original hypothesis. The improved survival of PFCE-O$_2$-treated HbSS mice was associated with a reduced pulmonary inflammatory response but no change in clearance of *S. pneumoniae* from lungs or blood. Since PFCE-O$_2$-treated C57BL/6 mice were more susceptible to infection, these observations suggest that tissue hypoxia plays a more significant role in the pathophysiology of *S. pneumoniae* infection in HbSS mice than in infected control mice.

The HbSS (Paszty) mice used in this study (background strain C57BL/6) express human α-, γ-, and βS-globin [18] and produce RBCs that are prone to sickling. As a consequence, the mice are chronically anemic and have a blood O$_2$ carrying capacity that is approximately 50% of that for wild-type mice [18]. At the time of death, lungs of uninfected HbSS mice were found to contain inflammatory cells, hemorrhages, and exudates, whereas lungs of uninfected C57BL/6 mice were completely normal (Figure 3 and Supplementary Figure 1). Moreover, compared with uninfected C57BL/6 mice, uninfected HbSS mice had higher pulmonary neutrophil, macrophage, T-cell, and B-cell counts (Figure 5) and higher levels of TNF-α, IL-1β, and IL-6 (Supplementary Figure 2 and 3). In contrast, pulmonary IL-10 levels were higher in uninfected C57BL/6 mice, compared with uninfected HbSS mice (Supplementary Figure 2). This constellation of features is commonly associated with acute pulmonary inflammation. However, uninfected HbSS mice had evidence of chronic disease, manifest as pulmonary fibrosis (data not shown), a finding also reported by other groups [25, 26]. We did not observe any outward signs of acute illness in uninfected HbSS mice. Taken together, the observations suggest that low-grade and persistent inflammatory processes contribute to the development of pulmonary fibrosis in HbSS mice.

Following infection with *S. pneumoniae*, HbSS animals died earlier than C57BL/6 mice, indicating, as in human HbSS, an increased susceptibility to pneumococcal infection. In both HbSS and C57BL/6 mice, infection with *S. pneumoniae* led to a significant influx of all leukocyte subsets and a proinflammatory cytokine milieu in lungs. The magnitude of each of the measured inflammatory markers was similar in both mice.

Figure 6. *Streptococcus pneumoniae* colony counts from cultured blood and lungs are unaffected by perflurocarbon emulsion (PFCE) therapy. Panels show *S. pneumoniae* colony counts from agar plates cultured overnight with blood (A and B) or lung homogenate (C and D) from transgenic sickle cell (HbSS) mice or C57BL/6 mice treated with phosphate-buffered saline (PBS) or PFCE and managed in air or O$_2$. Treatment with PBS or PFCE and mouse strain were not associated with significant differences in *S. pneumoniae* colony counts obtained from blood or lung samples taken at the time of death ($P > .05$). There were 6 mice in each treatment group. Abbreviation: CFU, colony-forming units.
Our data do not indicate that PFCE treatment affects the virulence of *S. pneumoniae* because lung homogenate and blood cultures from PBS-treated and PFCE-treated C57B6/L mice produced similar colony counts (Figure 6). In addition, the growth rate and generation time of *S. pneumoniae* cultured in the presence of PFCE were similar to the growth rate of bacteria grown in CDM alone (Supplementary Table 1).

In the absence of extra O$_2$, PFCE therapy was associated with a decreased percentage of sickled RBCs in pneumococcus-infected HbSS mice (Supplementary Figure 3). In addition, survival of pneumococcus-infected, PFCE-treated C57BL/6 mice was lower than that for PBS-treated infected littermates. There are few data to account for the actions of PFCE on RBC sickling in HbSS mice exposed to air. It is possible that the PFCE particles in Oxyce reduce RBC sickling by entering RBCs and then directly interacting with HbSS. An alternative explanation is that PFCE particles increase O$_2$ flux from RBCs to tissues without lowering the RBC pO$_2$ [15, 16]. Hence, in the presence of PFCE, RBCs transit capillaries (where sickling is most likely to occur) with a higher PO$_2$ than would otherwise be the case and, as a result, have a reduced sickling propensity. However, if operating, neither mechanism was sufficient to increase survival of PFCE-air–treated infected HBSS mice, compared to their PBS-air–treated littermates.

The data presented here suggest that that treatment with PFCE-O$_2$ improves tissue oxygenation in pneumococcus-infected HBSS mice. Future studies should focus on whether circulating PFCE particles loaded with O$_2$ improve tissue O$_2$ levels through stabilizing HbSS and decreasing RBC sickling or by increasing O$_2$ flux from RBCs to cells or through improved gas exchange in lungs. The effects of PFCE-O$_2$ on lung neutrophils and macrophages also need further investigation.

To our knowledge, this is the first study to assess the effects of intravenous PFCE therapy in a preclinical model of HbSS. It provides much needed data about the efficacy of PFCEs, as requested by regulatory agencies [30]. Our findings indicate that relief of tissue hypoxia occurs after treatment with PFCE and that supplemental O$_2$ improves outcomes of *S. pneumoniae* infection in HbSS mice. As such, the findings suggest that intravenous PFCE therapy may be a useful adjunct to managing life-threatening sickle crises.

 Supplementary Data

Supplementary materials are available at The Journal of Infectious Diseases online (http://jid.oxfordjournals.org). 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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