Staphylococcus aureus β-toxin Production is Common in Strains With the β-toxin Gene Inactivated by Bacteriophage

Wilmara Salgado-Pabón, Alfa Herrera, Bao G. Vu, Christopher S. Stach, Joseph A. Merriman, Adam R. Spaulding, and Patrick M. Schlievert

Department of Microbiology, University of Iowa Carver College of Medicine, Iowa City

Background. Staphylococcus aureus causes life-threatening infections, including infective endocarditis, sepsis, and pneumonia. β-toxin is a sphingomyelinase encoded for by virtually all S. aureus strains and exhibits human immune cell cytotoxicity. The toxin enhances S. aureus phenol-soluble modulin activity, and its activity is enhanced by superantigens. The bacteriophage φSa3 inserts into the β-toxin gene in human strains, inactivating it in the majority of S. aureus clonal groups. Hence, most strains are reported not to secrete β-toxin.

Methods. This dynamic was investigated by examining β-toxin production by multiple clonal groups of S. aureus, both in vitro and in vivo during infections in rabbit models of infective endocarditis, sepsis, and pneumonia.

Results. β-toxin phenotypic variants are common among strains containing φSa3. In vivo, φSa3 is differentially induced in heart vegetations, kidney abscesses, and ischemic liver compared to spleen and blood, and in vitro growth in liquid culture. Furthermore, in pneumonia, wild-type β-toxin production leads to development of large caseous lesions, and in infective endocarditis, increases the size of pathognomonic vegetations.

Conclusions. This study demonstrates the dynamic interaction between S. aureus and the infected host, where φSa3 serves as a regulator of virulence gene expression, and increased fitness and virulence in new environments.

Keywords. bacteriophage; β-toxin; infective endocarditis; pneumonia; sepsis; Staphylococcus aureus.
superantigens [16], may contribute to pathology associated with infections.

The majority of human S. aureus isolates, excluding those of the CC30/ST36/USA200 clonal group, are reported not to express β-toxin due to integration of the bacteriophage φSa3 into the β-toxin structural gene hlb [24, 25]. φSa3 encodes for accessory virulence genes reported to be involved in immune evasion, including superantigens, staphylokinase (SAK), chemotaxis inhibitory protein (CHIP), and staphylococcal complement inhibitor (SCIN) [26–29]. Given the specificity of these accessory gene products for human cells and the high incidence of φSa3 among human versus animal isolates [30], it has generally been accepted that φSa3 phage-encoded virulence factors provide a greater advantage to S. aureus for human colonization and survival than β-toxin production.

Recent evidence demonstrates that φSa3 inactivation of β-toxin is not indefinite [31]. S. aureus strains from cystic fibrosis (CF) or bacteremia patients show evidence of phage excision compared to isolates from nares of healthy individuals [32, 33]. S. aureus isolates from CF and bacteremia patients comprise a heterogeneous population of phage positive and negative, and in a few of the CF isolates, the phage excised and translocated to atypical sites on the chromosome [33]. Phage excision with β-toxin production was also documented in S. aureus grown in culture with ciprofloxacin and trimethoprim, antibiotics used to treat CF patients [34]. These observations suggest that during chronic infections, host pressure and/or antibiotic treatment favors phage excision with consequent production of β-toxin, possibly benefiting S. aureus survival and disease progression.

The community-associated, methicillin-resistant S. aureus (CA-MRSA) strain MW2 (USA400 clonal group) was isolated from a child succumbing to staphylococcal pneumonia and toxic shock syndrome (TSS) [35, 36]. S. aureus MW2 is exceptional at causing pneumonia, infective endocarditis, and sepsis in rabbits [37, 38]. MW2 carries φSa3 and thus thought not to produce β-toxin. However, during our infective endocarditis studies, we observed β-toxin-producing MW2 colonies recovered from rabbit vegetations [39]. β-toxin production was also observed in vitro when MW2 was heavily streaked onto sheep blood agar plates (SBAPs) from frozen stocks. Based on these observations, we investigated whether phage excision was common in S. aureus clonal groups containing φSa3, and addressed this dynamic both in vitro and in vivo in rabbit models of infection.

**MATERIALS AND METHODS**

**Growth Conditions and Frequency Counts**

CA-MRSA MW2 was utilized from stocks of low passage. MW2 hyper-β was isolated from the original MW2 wild-type stock through 3 round passage at 37°C on SBAPs with selection for single-colony isolates producing β-toxin zones. Overnight cultures of MW2 were grown at 30°C, 37°C, and 42°C at 220 revolutions/minute shaking in Todd-Hewitt (TH; Difco Laboratories, Detroit, MI) or beef heart (BH) broth [40], diluted, and plated on SBAPs at the above-mentioned temperatures for frequency counts of β-toxin-producing colonies. Frequency counts were determined by identifying β-toxin-producing colonies on SBAPs: (1) individual colonies with inner zones of hemolysis + large dark outer zones with resultant hot-cold hemolysis, or (2) discrete, strongly lytic patches on lower dilutions, then divided by the total colony forming units (CFUs). β-toxin colonies were similarly identified from homogenized rabbit tissues: blood, infarcted spleens, ischemic livers, kidney abscesses, and hemorrhagic/necrotic lungs following pneumonia and aortic vegetations from rabbits with infective endocarditis and sepsis. Bacterial dilutions were plated onto SBAPs and incubated at 37°C.

**Sheep Erythrocyte Lysis Inhibition Assay**

Microscope slides were coated with 4 mL of 1% agarose (Sigma Aldrich, St. Louis, MO) containing sheep erythrocytes. Sheep blood was washed by centrifugation (400 × g, 10 minutes) 3 times with phosphate-buffered saline (PBS; 0.005 M NaPO4, 0.15 M NaCl), and 50 μL of packed cells were added to 10 mL of 1% agarose to coat microscope slides and allowed to solidify. For assays, 4-mm-diameter wells were punched in the agarose and filled with 20 μL of PBS, β-toxin hyperimmune antiserum, α-toxin hyperimmune antiserum, or β-toxin antiserum + α-toxin antiserum. The plates were incubated at 37°C until the PBS or serum was absorbed into the agarose. Supernates from overnight S. aureus cultures were treated with 4 volumes of absolute ethanol to precipitate β-toxin, the toxin from each sample was resuspended in water to 1/10th its original concentration, and 20 μL of the concentrates were placed in the wells. Slides were incubated overnight at 37°C followed by 4 hours’ incubation at 4°C. Lysis diameters were measured and analyzed with the National Institutes of Health ImageJ program [41].

**Gene Detection**

Uninterrupted hlb was detected by polymerase chain reaction (PCR) with primer set β-toxin.F (5′-ATGGTGAAAAAAA CAAAATCCAA-3′) and β-toxin.R (5′-CCTACAAAAGCC TATAGTAAATAG-3′). Phage detection was done with primer sets int-F (5′-CTGCTGACTAGACAAGTTAAATGAG-3′) and int-R (5′-GAGTGTCTTAAATGGCTGG-3′), specific to integrase gene, and Scn-1/Scn2 specific to SCIN (previously described [30]).

**Rabbit Model of Pneumonia and Infective Endocarditis/Sepsis**

New Zealand white rabbits were anesthetized with ketamine (25 mg/kg) and xylazine (25 mg/kg) (Phoenix Pharmaceuticals,
Burlingame, CA). Pneumonia model was performed as previously described [37]. Rabbits were administered $1 \times 10^9$ bacteria (in 0.3 mL volumes) via intratracheal inoculation. Experiments proceeded for up to 7 days. The infective endocarditis/sepsis model was performed as previously described [39]. Briefly, hard plastic catheters were introduced through the left carotid artery, left in place for 2 hours, removed, and incision closed. An amount of $1-2 \times 10^7$ bacteria were injected through the marginal ear vein. Experiments proceeded for up to 4 days. At the time of death or euthanasia, heart vegetations were dissected, weighed, homogenized, and plated to determine frequency of β-toxin+ colonies and bacterial CFUs per total vegetations within a single heart. CFUs were also determined from venous blood. Animal experiments were performed according to guidelines and a protocol approved by the University of Iowa Institutional Animal Care and Use Committee (Protocol 1106140).

**Statistical Analyses**
Statistical significance in survival experiments was determined using the log-rank, Mantel–Cox test, and significance across means was carried out using the Mann–Whitney test (GraphPad Prism Software).

**RESULTS**

*S. aureus* MW2 and LAC Produce β-toxin Variants During In Vitro Growth
The 5′ end of β-toxin converting phages is conserved, including the integrase and attachment site [30]. φSa3 encodes for virulence factors at both ends. Staphylococcal enterotoxin such as (SEl)-K and SEl-Q are encoded on the 5′ end; SEA, SAK, and SCIN are encoded on the 3′ end (Figure 1A). Phage excision restores an intact hlb and leads to β-toxin production. CA-MRSA MW2 grown overnight in liquid culture and plated onto SBAPs at high density resulted in hyperlytic patches after overnight incubation at 37°C (Figure 1B). Restreaking colonies from the hyperlytic patches yield isolated colonies with either a narrow zone of hemolysis or a narrow zone of hemolysis + a large outer dark zone (Figure 1C). Upon overnight incubation at 4°C, the blood cells in the outer dark zones lysed, indicative of β-toxin production. Colonies with an ellipse zone of hemolysis were also noted, suggesting low-level phage excision and secretion of β-toxin within the growing colonies (Figure 1C, left panel). MW2 wild-type and the β-toxin-producing (phage cured) variant, termed MW2 (hyper-β), were compared with a Christie Atkins Munch-Petersen (CAMP) test for synergism with PSMs. In the CAMP test, MW2 wild-type produces a clear zone of hemolysis only in the area where MW2 PSMs meet with β-toxin produced by control strain RN4220 streaked vertically across the plate. A thin and diffuse zone of hemolysis is observed around the MW2 wild-type streak (Figure 1D, left panel) indicating only low-level or no production of β-toxin. However, a clear hemolytic zone is observed completely around MW2 hyper-β, indicating β-toxin production (Figure 1D, right panel).

Four additional *S. aureus* USA400 clinical isolates were grown in liquid culture and plated onto SBAPs to determine if β-toxin-producing variants were present. Hyperlytic patches were observed in all 4 strains at varying numbers (Supplementary Figure 1A). We evaluated USA300 strain LAC (Fitzgerald strain), also with a previously inactivated α-toxin gene (*hla*), for β-toxin hemolysis when plated on SBAPs; the hyperlytic
patches are difficult to observe in the presence of high levels of α-toxin, as produced by LAC. In this strain, too, lytic patches were observed (Supplementary Figure 2, left panel). Recovery of β-toxin-producing variants was also possible from the wild-type LAC strain (Supplementary Figure 2, right panel). Since it became evident that β-toxin-producing variants are present in strains of different clonal lineages, we screened for and detected the presence of the intact β-toxin gene across multiple USA clonal groups (Figure 2). While the β-toxin gene is intact across multiple clonal groups, the phage remains prevalent highly prevalent, β-toxin-producing variants are present among S. aureus isolates of multiple lineages.

**β-toxin is Produced at Variable Levels in USA100–USA400 Strains**

We performed a CAMP test on several isolates from the USA100–USA400 clonal groups to test for β-toxin production. A clear zone of hemolysis around the streak is suggestive of β-toxin production. Lysis was observed in several strains from all clonal groups (Supplementary Figure 3A–D). However, the arrow shape that forms between cross streaks indicates production of α-toxin, making it difficult to draw conclusions regarding β-toxin production. Similarly, zones of incomplete hemolysis in the absence of clear lysis indicate production of α- and β-toxin, as β-toxin’s activity antagonizes that of α-toxin [42]. Therefore, we tested culture supernate concentrates for lytic activity on sheep blood cell agarose slides in the presence or absence of β-toxin-specific serum. The anti-β-toxin serum inhibited hemolysis in the MW2 hyper-β strain (8-fold reduction of lysis zone), demonstrating hemolysis is due to β-toxin (Figure 3A). β-toxin was also detected in MW2 wild-type, the USA200 strains MN8 and CDC587, and in very low levels in the USA400 strains IA96 and PSGN (Figure 3B). The S. aureus laboratory strain RN4220 (which secretes β-toxin in large quantities) provided evidence of the specificity of the β-toxin serum and of the role of β-toxin in the hemolytic zone that results after treatment with culture supernate concentrates (Figure 4A).

Strains in the USA100 and USA300 clonal groups produce high levels of α-toxin, some of which remains active in the supernate concentrates. Hence, for these strains, wells were treated with either anti-α toxin serum alone or in combination with anti-β toxin serum. The USA100 strains IA209 and IA116 produced zones of hemolysis; however, lysis resulting from IA209 concentrates was largely due to β-toxin, while lysis from IA116 was largely due to α-toxin (Figure 4B). USA300 strains LAC and IA1012 produced β-toxin at low levels (Figure 4C). Additionally, we tested the frequency of β-toxin-producing variants in selected USA100–300 isolates. In liquid culture, β-toxin colony frequencies ranged from 1/2000–1/20 (USA100), 1/500–1/31 (USA200), and 1/380–1/260 (USA300) (Supplementary Figure 3E).

**β-toxin Production in MW2 Exacerbates Pneumonia Pathology**

USA200 strains are considered the predominant clonal group associated with β-toxin production. These strains can cause

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**Figure 2.** Intact β-toxin structural gene is present among different S. aureus clonal groups. PCR screen for the full-length β-toxin gene in USA100–400 clonal groups and nontypeable strains. Abbreviation: PCR, polymerase chain reaction.

**Figure 3.** Quantification of β-toxin production via antibody hemolysis-inhibition assays. A, hemolysis inhibition assays of strains MW2 (hyper-β), MN8, and MW2 wild-type showing culture concentrate hemolysis compared to culture hemolysis inhibited by anti-β toxin serum. B, Area of lysis measured in cm from hemolysis inhibition assays of USA200 and USA400 strains; black bar represents area of lysis from culture concentrates. Gray bar represents area of lysis when inhibited by anti-β toxin serum.

**Figure 4.** β-toxin Production in MW2 Exacerbates Pneumonia Pathology

USA200 strains are considered the predominant clonal group associated with β-toxin production. These strains can cause
S. aureus pneumonia and lethality, where lethality results from TSS toxin-1 systemic effects and development of TSS [43]. The contribution of β-toxin to S. aureus pneumonia is not well defined. Initial studies suggest the toxin contributes to lung injury [44]. MW2 was the causative agent of pneumonia and TSS in a child from the Midwest [36]. Because MW2 hyper-β produces 4–8 times more β-toxin in liquid culture than the parent strain, we evaluated whether or not increased levels of β-toxin correlates with increased lung injury and pneumonia exacerbation in a rabbit model.

Bacteria were inoculated intratracheally and infection allowed to proceed for ≤7 days. Under these experimental conditions, 1 rabbit infected with the wild-type strain succumbed to infection at day 1, while all other infected rabbits (wild-type and hyper-β) survived. Lungs from MW2 wild-type-infected rabbits showed classical signs of S. aureus pneumonia, including hemorrhagic pleural effusion, diffuse or hemorrhagic consolidation, and caseating granulomas (Figure 5A). Strikingly, increased expression of β-toxin by the MW2 (hyper-β) strain results in the formation of larger caseous granulomas. Lungs from animals infected with the MW2 (hyper-β) strain also exhibited severe hemorrhagic consolidation and pleural effusion (Figure 5B).

These results provide evidence that β-toxin contributes to lung pathology.

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**Figure 4.** Hemolysis inhibition assays of USA100 and USA300 strains. A. Image of hemolysis inhibition assay of S. aureus RN4220 culture concentrate alone, with anti-β toxin serum, with anti-α toxin serum, or with both anti-β and anti-α toxin serum to demonstrate the hemolysis due to each toxin; areas of lysis measured in centimeters from hemolysis inhibition assays of (B) S. aureus RN4220, USA100 strains, and (C) USA300 strains. Black bar represents area of lysis from culture concentrates, dark gray bar represents area of lysis when inhibited by anti-β toxin serum, gray bar represents area of lysis when inhibited by anti-α toxin serum, and light gray bar represents area of lysis when inhibited by both anti-β and anti-α serum.

**Figure 5.** MW2 and MW2 (hyper-β) in a rabbit model of pneumonia. Lungs dissected from rabbits infected with (A) MW2 wild-type or (B) MW2 (hyper-β). Dotted lines highlight areas of granulomas.
β-toxin Production Contributes to Vegetations in a Rabbit Infective Endocarditis/Sepsis Model

β-toxin has biofilm ligase activity, inducing the formation of covalent nucleoprotein complexes [23] to help establish biofilms. β-toxin contributes to vegetation formation by MRSA COL in a rabbit model of infective endocarditis [23]. It would then be expected that increased levels of β-toxin production could lead to increased biofilm formation on heart valves, resulting in larger vegetations.

To test this, rabbits were infected with MW2 wild-type and hyper-β variant for ≤4 days. The dose used was selected because it results in similar survival curves between strains, ensuring vegetation size is not confounded by survival time differences (Figure 6A) [39]. Bacterial counts recovered from the blood of infected animals were not significantly different between the strains, indicating vegetation formation was not due to differences in bacterial bloodstream survival (Figure 6B). All infected rabbits developed vegetations. Vegetations in rabbits infected with the wild-type MW2 averaged 25 mg, while vegetations in rabbits infected with MW2 hyper-β averaged 80 mg (Figure 6C). Similar CFUs were recovered from vegetations (Figure 6D).

β-toxin+ Colony Frequency Increases in Infective Endocarditis Vegetations, Liver, and Kidney

We tested if the frequency of β-toxin-producing variants changed as a result of environment or host pressure exerted upon the bacterial population. MW2 was cultured overnight in TH and BH broths at 30°C, 37°C, and 42°C and plated onto SBAPs. At 30°C, a suboptimal temperature for S. aureus growth, β-toxin-producing variants averaged 1/500 total colonies in both broths (Figure 7A). At 37°C (body temperature) and 42°C (high fever), β-toxin colony frequencies increased from 1/500 to 1/200 when grown in TH broth, and from 1/200 to 1/100 when grown in BH broth. The β-toxin colony frequencies also significantly increased in BH broth, from 1/500 at 30°C to 1/200 at 37°C.

Knowing that inoculation of MW2 in the rabbit model of infective endocarditis/sepsis leads to both vegetation formation and metastatic abscesses and tissue ischemia, we tested how growth in different tissues affects β-toxin colony frequencies. Rabbits were infected with MW2 after mechanical damage to the aortic valves [39], and bacteria were tested directly for β-toxin production from vegetations, kidney abscesses, ischemic livers, hemorrhagic lungs, infarcted spleens, and blood (Figure 7B). β-toxin colony frequencies in the blood and spleen resembled frequencies following growth in BH broth at 37°C (1/200) and 42°C (1/100), respectively (Figure 7). Colonies recovered from vegetations and ischemic livers had the highest frequencies (1/30), followed by kidney abscesses (1/40) and hemorrhagic lungs (1/80) (Figure 7B). These results demonstrate the dynamic nature of phage φSa3, allowing β-toxin production in a subset of bacterial populations under different environmental and host pressures.

DISCUSSION

β-toxin is encoded in most S. aureus strains regardless of host origin. However, the β-toxin gene inactivating phage, φSa3, is widely distributed among strains of human origin [25, 26].

Figure 6. MW2 and MW2 (hyper-β) in a rabbit model of infective endocarditis and sepsis. A, The percent survival of rabbits from the MW2 and MW2 (hyper-β) infected groups each day postinfection until termination of the experiment at day 4. B, CFUs/mL recovered from the blood at death for each experimental group. C, Mean mass of vegetations in milligrams recovered from the heart valves of each experimental group. D, Mean total CFUs recovered per vegetation from each experimental group. Abbreviation: CFUs, colony forming units.

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β-toxin production is considered a characteristic of *S. aureus* strains belonging only to the USA200 lineage, common colonizers of mucosae [45, 46]. φSa3 is present in 77%–93% of *S. aureus* isolates in defined strain collections [30, 47, 48]. These studies suggest that most human *S. aureus* strains do not produce β-toxin, and therefore, the toxin plays no role in *S. aureus* pathogenesis.

We show strains carrying φSa3 exist as heterogeneous populations containing a subset producing β-toxin. Intact β-toxin genes were amplified from the chromosomes of strains of USA100–USA400 clonal groups and nontypeable strains. β-toxin-producing variants were directly detected in these strains, as determined by the presence of hyperlytic patches on SBAPs, and β-toxin hemolysis detected on sheep erythrocyte slides. Phage excision resulting in β-toxin production was dependent on culture conditions. In vitro, growth of MW2 in BH broth at 42°C induced the highest frequency of colonies producing β-toxin (1/100), while growth at 37°C in TH broth resulted in a frequency of 1/500. Because growth at 42°C in BH broth may more closely resemble the human environment during infection with fever, we directly addressed how growth in rabbits affects phage induction and appearance of β-toxin-producing colonies. In the blood and the spleen of infected animals, β-toxin-producing colonies increased in frequency compared to laboratory growth conditions. The frequencies were similar to those after growth in BH broth at 37°C–42°C. The highest frequencies were found in bacteria recovered from endocarditis vegetations (1/30), ischemic livers (1/30), and kidney abscesses (1/40). These results indicate that phage induction is favored during growth in particular niches and β-toxin contributes significantly to tissue disease progression.

The β-toxin contribution in *S. aureus* human nasal colonization was demonstrated, comparing *S. aureus* NCTC 8325-4 (containing an intact β-toxin gene) with a derivative carrying φSa3 [49]. The β-toxin-producing strain persisted 14 days, while the φSa3-carrying strain persisted 4 days. Hence, β-toxin production, as in the USA200 clonal lineage, significantly contributes to *S. aureus* capacity to colonize mucosal surfaces. Strains of the USA200 group are also associated with pneumonia, and β-toxin may contribute to disease in the lungs [23]. The USA400 strain MW2, a lethal pneumonia human isolate, produces variants that lost φSa3 and secrete β-toxin in large quantities, similar to strains NCTC 8325-4 and RN4220 (which produce up to 500 μg/mL) [23]. These variants (referred to as MW2 hyper-β), when inoculated intratracheally in rabbits, induce formation of large granulomatous pulmonary lesions containing viable bacteria that cavitate, causing massive hemorrhagic pleural effusions and consolidation. These observations indicate that β-toxin contributes significantly to the pathology of *S. aureus* pneumonia.

Two studies documented β-toxin-producing variants in the sputum of CF patients [32, 33]. These patients have chronic S.
aureus lung infections and are regularly treated with antibiotics. Phage excision and genomic alterations have resulted from the selective pressure of antibiotic therapy and host responses [34]. Staphylococcal endocarditis is an infection of the heart, predominantly the valves, frequently resulting in septic emboli and metastatic abscesses [5]. In our rabbit model, vegetations develop within 4 days, and selective pressure comes strictly from the host environment. Studies with S. aureus strain COL demonstrated that β-toxin is essential for vegetation formation [23]. We provide further evidence for the role of β-toxin in endocarditis, as the MW2 hyper-β variant produces vegetations larger than those produced by MW2 wild-type. While vegetations are larger in the hyper-β variant, they do not contain higher bacterial loads. We predict the β-toxin ligase activity, which catalyzes the formation of nucleoprotein complexes in the extracellular matrix necessary for vegetation growth, induces host factor aggregation independently of the rate at which S. aureus multiplies. β-Toxin-producing variants increase in frequency within vegetations, kidney abscesses, and ischemic liver during infection with parent MW2. If β-toxin expression (in the absence of Sa3; MW2 hyper-β) results in secretion of 500 μg/mL of β-toxin, we estimate that within the vegetations, β-toxin levels could be 15 μg/mL, and in blood, 3 μg/mL.

S. aureus is often considered an opportunist in both humans and animals. The β-toxin converting phage, Sa3, is widely distributed uniquely among S. aureus strains of human origin compared to bovine strains [34]. Sa3 encodes for superantigens (SEl-K, SEl-Q, and SEA) and proposed innate immune evasion factors (SAK, CHIP, and SCIN) [26, 28, 29, 50]. It has been assumed, that because Sa3 inactivates the β-toxin gene, Sa3 acquisition is more advantageous to S. aureus than β-toxin production, and therefore, β-toxin does not significantly contribute to pathogenesis. We provide evidence that β-toxin variants occur within strains carrying Sa3, and that Sa3 induction occurs during active infection and is differentially induced dependent on the environmental niche. Our study advances the understanding of the role of β-toxin in disease and underscores the dynamic interaction between S. aureus and the host, where Sa3 provides a novel regulatory mechanism for virulence gene expression and increased fitness.

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 copyrighted. 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

Financial support. This work was supported by the University of Iowa Carver College of Medicine Startup to P.M.S. W.S.P. was supported by the US Public Health Service (USPHS) training grant T32AI007511 from the National Institute of Allergy and Infectious Diseases.

Potential conflicts of interest. All authors: No reported conflicts.

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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