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BRIEF REPORT

Interleukin-36γ Is Elevated in Cervicovaginal Epithelial Cells in Women With Bacterial Vaginosis and In Vitro After Infection With Microbes Associated With Bacterial Vaginosis

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In recent studies, the interleukin (IL)-36 cytokines were shown to be elevated in women with non-*Lactobacillus*-dominated vaginal microbiomes. In this study, we evaluated *IL36G* expression in clinical samples from women with and without bacterial vaginosis (BV) and a human 3-dimensional cervical epithelial cell model. *IL36G* expression was significantly elevated in cervicovaginal epithelial cells isolated from BV-positive women and corresponded with increased neutrophil counts relative to BV-negative women. In addition, specific BV-associated bacterial species as well as a polymicrobial cocktail significantly induced *IL36G* expression in vitro. These findings suggest that IL-36γ may exhibit an important function in the host response to BV and other sexually transmitted infections.

Keywords. *Atopobium vaginae*; *Gardnerella vaginalis*; *Lactobacillus crispatus*; *Prevotella bivia*; *Sneathia amnii*.

Bacterial vaginosis (BV) is the most prevalent vaginal bacterial infection worldwide, and it is associated with several obstetric and gynecologic sequelae, including enhanced sexually transmitted infection (STI) acquisition [1]. Bacterial vaginosis is characterized by a depletion of lactobacilli and an increase in colonization with diverse facultative and obligate anaerobes [2]. Despite the fact that BV is often associated with symptoms, including a thin discharge and fishy odor, many women are asymptomatic and remain undiagnosed [2]. The complex, polymicrobial nature of BV additionally presents a challenge in our understanding of the contribution of specific BV-associated bacteria to disease [3].

The cervicovaginal epithelium functions as a first line of defense against invading microbes through a variety of mechanisms, including the production immune mediators. The proinflammatory interleukin (IL)-1 family member IL-36γ is expressed in the female reproductive tract (FRT) epithelium. Interleukin-36γ has recently been shown to be upregulated in women with cervical cancer and induced by bacterial and viral products, including bacterial flagellin, a synthetic lipoprotein (FSL-1), and poly(I:C), as well as specific microorganisms, such as group B *Streptococcus* and herpes simplex virus 2 (HSV-2), indicating that IL-36γ exhibits an important function in host defense against bacterial and viral pathogens and pathobionts [4–8]. It is interesting to note that a proteomics report showed increased levels of the IL-36 cytokines in Rwandan sex workers with BV as defined by Nugent score, suggesting a potential link between the IL-36 cytokines and BV [9]. In this study, we sought to better understand (1) the host response to BV and (2) the contribution of BV-associated bacteria to IL-36γ induction.

METHODS

Participant Enrollment

Cervicovaginal lavage (CVL) and vaginal swab samples (n = 38) were collected from 23 premenopausal women aged 18–45 participating in a study in Atlanta, Georgia (2015–2017) as previously described [10]. A detailed description of enrollment criteria and participant demographics is provided in the Supplemental Methods and Supplemental Table 1. Women were evaluated for BV by Nugent scoring. Intermediate (4–6) or high scores (7–10) were grouped as BV-positive (BV^+) samples because only 2 samples scored as intermediate, and the subsequent samples from the same participants were scored as BV^+ (Supplemental Table 2).

Cervicovaginal Cell Collection and Analysis

Cervicovaginal lavages were collected, processed, and stored at each study visit as previously described [10]. In brief, epithelial cells were isolated by Percoll gradient centrifugation, enumerated by trypan blue exclusion, and stored in RNAlater (Invitrogen) at −80°C until analysis. Remaining cells in CVL were analyzed by flow cytometry as previously described [10].

16S Ribosomal Ribonucleic Acid Gene Sequencing

Deoxyribonucleic acid was extracted from vaginal swab samples using the DNeasy PowerSoil Isolation Kit (QIAGEN). The variable V3 and V4 regions of the 16S ribosomal ribonucleic acid (rRNA) gene were amplified per manufacturer's instructions

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(Illumina) and sequenced on an Illumina MiSeq (for details, see Supplemental Methods). 16S rRNA gene sequences were deposited in the National Center for Biotechnology Information (NCBI) database under Sequence Read Archive (SRA) submission number SUB6308695.

In Vitro Infection of Three-Dimensional Cervical Epithelial Cell Model With Vaginal Bacteria

Human 3-dimensional (3-D) cervical (A2EN) epithelial cells were cultured as previously described in the slow turning lateral vessel bioreactor (Synthecon) for 28 days [6, 11]. Fully differentiated 3-D aggregates were seeded into 24-well plates (1×10^5–5×10^5 cells/mL) for in vitro infections. Bacteria were cultured for 16–18 hours on specified media (see Supplemental Methods) at 37°C under anaerobic conditions, resuspended in sterile Dulbecco's phosphate-buffered saline (PBS), and used for in vitro infections. Aggregates were infected with individual bacterial strains (*Lactobacillus crispatus* VPI 3199 and JV-V01, *Atopobium vaginae* CCUG 38953, *Prevotella bivia* VPI 6822, *Gardnerella vaginalis* JCP8151B, *Sneathia amnii* Sn35) at a multiplicity of infection (MOI) of 10 for 24 hours under anaerobic conditions. In addition, 3-D aggregates were infected with the polymicrobial cocktail, consisting of MOI 2.5 of each of *A vaginae*, *S amnii*, *P bivia*, and *G vaginalis* for a total bacterial MOI of 10 for direct comparison to infection with individual bacteria. Aggregates were fixed and processed, and images were collected by scanning electron microscope as previously described [11].

Gene Expression Analysis

Gene expression in epithelial cells and 3-D aggregates was analyzed by quantitative polymerase chain reaction. Expression of *IL36G*, *IL8*, *CCL20* (*MIP3A*), and *TNF* genes was normalized to *GAPDH* (for details see Supplemental Methods). Hierarchical clustering analysis of gene expression was performed using ClustVis. Gene expression from 3 to 4 biological replicates were averaged. Values for each target were mean centered and variance scaled. Clustering of the heat map was based on Euclidean distance between rows and columns and average linkage cluster algorithm.

Statistics

Statistical analyses were performed in GraphPad Prism version 8 (GraphPad). Specific tests are described in figure legends. $P < .05$ was considered significant.

RESULTS

Participant Groupings

A total of 38 STI-negative samples were collected from 23 premenopausal women across 2 study visits (Visit 1 and Visit 2). Of the samples collected, 20 (52.6%) were from BV^+ women and 18 (47.3%) were from BV-negative (BV^-) women. Age, ethnicity and distribution of BV^- and BV^+ samples across study visits were not significantly different between the BV^- and BV^+ groups (Supplemental Table 1).

Women With Bacterial Vaginosis Exhibit Elevated *IL36G* Expression

To evaluate the potential link between IL-36γ and BV, *IL36G* expression was measured in epithelial cells isolated from BV^+ and BV^- women. Epithelial cells in the FRT are robust producers of the IL-36 cytokines, and particularly IL-36γ [8, 12]. *IL36G* expression was elevated in BV^+ women 6.1-fold at Visit 1 and 109.6-fold at Visit 2 relative to B^-women (Figure 1A). The BV^+ women exhibited significantly ($P = .0323$) elevated *IL36G* expression across study visits relative to BV^- women.

Through 16S rRNA gene sequencing, we profiled the vaginal microbiome (VMB) in study participants and explored the correlation between the abundance of specific vaginal bacteria and *IL36G* expression in epithelial cells. Spearman's correlation coefficients were computed for bacterial genera with an average relative abundance >1% and *IL36G* expression in epithelial cells for all collected samples (Figure 1B). Although *IL36G* expression was elevated in women with BV, none of the bacteria evaluated exhibited significant correlations (positive or negative) to *IL36G* expression.

Increased Neutrophil Counts in Cervicovaginal Lavages From Women With Bacterial Vaginosis

Interleukin-36γ has been shown to impact neutrophil infiltration in FRT [4], and, as such, we sought to evaluate the functional impact of elevated *IL36G* expression on immune cell infiltration in CVLs collected from participants. We measured a 2.2-fold increase ($P = .0243$) in neutrophil counts in BV^+ women relative to BV^- women, whereas there were no differences in the number of epithelial cells or antigen-presenting cells between BV^+ and BV^- women (Figure 1C). The increased neutrophil counts in BV^+ women corresponded with elevated *IL36G* expression levels in epithelial cells, and this provides a potential link between IL-36γ and neutrophil infiltration in the FRT.

Bacterial Vaginosis-Associated Bacteria Induce *IL36G* in Human Three-Dimensional Cervical Epithelial Cells

Using a robust human 3-D cervical epithelial cell model, we sought to evaluate the impact of specific BV-associated bacteria on *IL36G* in the FRT epithelium (Figure 2A). Scanning electron microscopy demonstrated bacterial colonization (Figure 2B). *Lactobacillus crispatus* did not induce *IL36G* expression in the model (Figure 2B). In contrast, infection with the highly prevalent BV-associated bacteria *G vaginalis*, *P bivia*, *A vaginae*, and *S amnii* [13] upregulated *IL36G* expression 4.2-fold ($P < .05$), 2.9-fold, 7.8-fold ($P < .001$), and 6.2-fold ($P < .01$), respectively, relative to PBS-treated controls. It is interesting to note that when these BV-associated bacteria were combined into a polymicrobial cocktail and then used to infect 3-D aggregates, we observed a significant ($P < .0001$) induction of *IL36G* (12.1-fold). In addition, we tested expression of other proinflammatory genes, such as *IL8*, *CCL20* (*MIP3A*),

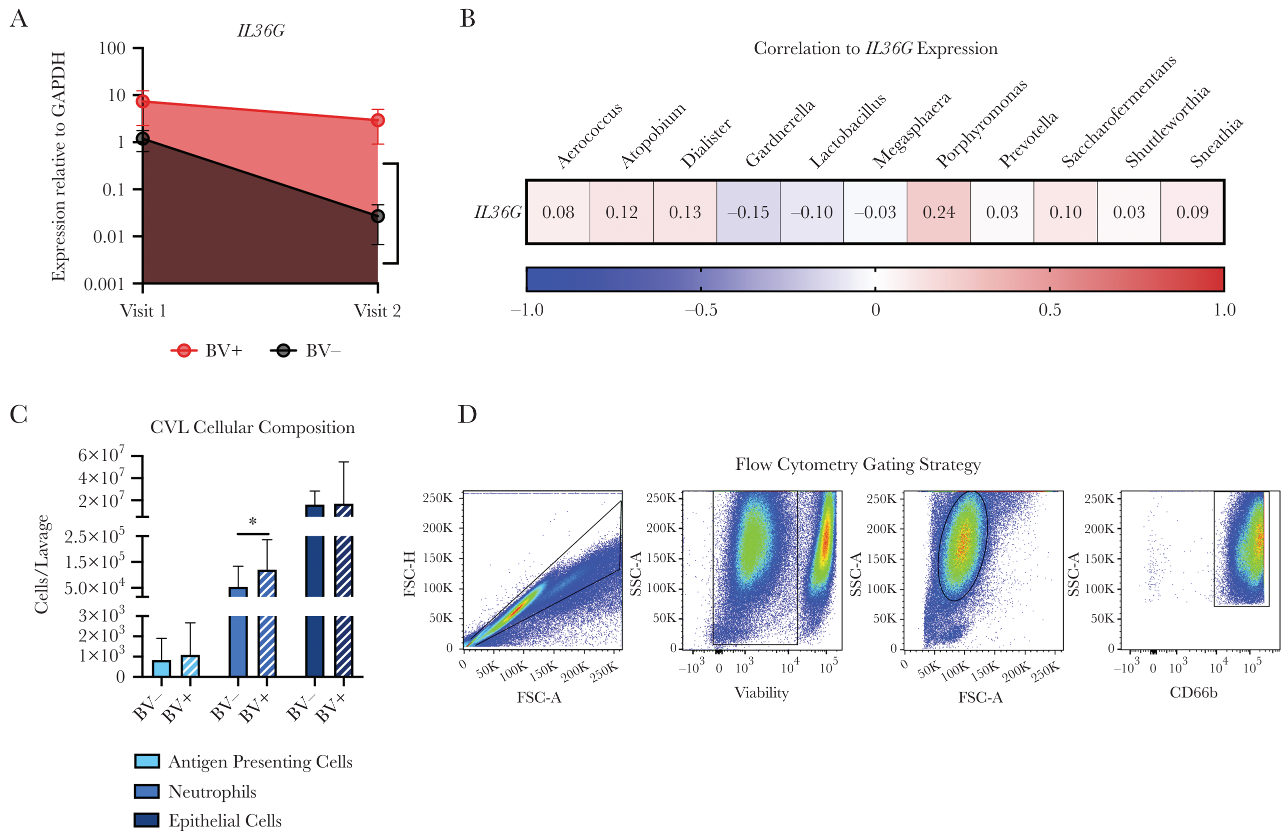

Figure 1. Women with bacterial vaginosis (BV) exhibit elevated *IL36G* levels in epithelial cells that corresponds with increased neutrophil counts in cervicovaginal lavages (CVLs). (A) *IL36G* expression was assessed by quantitative polymerase chain reaction in epithelial cells isolated from CVLs. Participant samples were grouped as BV negative (BV⁻) and BV positive (BV⁺) and then separated by office visit for analysis. Expression levels were normalized to *GAPDH* and data depict mean ± standard deviation (SD). (B) Correlation of BV-associated bacteria with an average relative abundance greater than 1% to *IL36G* expression in cervicovaginal epithelial cell pellets. *IL36G* gene expression was $\log_2$ transformed before analysis. Correlation coefficients were calculated using Spearman's rank coefficient analysis. (C) Counts of antigen-presenting cells, neutrophils, and epithelial cells in CVLs were assessed by flow cytometry. (D) Representative gating strategy for flow cytometry analysis of CD66b⁺ neutrophils in CVLs. (A and B) Data depict mean count ± SD. Statistical analyses were performed by area under the curve followed by unpaired, 2-tailed, Student *t* test with Welch's correction (A) or unpaired, 2-tailed, Student *t* test with Welch's correction (B). *, $P < .05$.

and *TNF* (Supplementary Figure 1). Hierarchical clustering of these targets together with *IL36G* revealed that *P bivia* and *G vaginalis* did not induce robust proinflammatory responses and cluster with *L crispatus* and PBS controls (Figure 2C), not *A vaginae*, *S amnii*, or the polymicrobial cocktail, which exhibited proinflammatory profiles.

DISCUSSION

Since their discovery in 1999, the IL-36 cytokines have been investigated in the context of chronic inflammatory diseases and infectious diseases in the lungs and skin [12, 14]. More recently, we and others have shown that IL-36γ is expressed in the FRT, upregulated in cervical cancer, and induced in response to an array of microbial products, pathobionts, and HSV-2 [4–8]. Despite the wide prevalence of BV, there remains an unmet need to better understand the immunologic microenvironment during this non-*Lactobacillus*-dominant state and the contribution of various BV-associated bacterial species to disease. In this study, we sought to evaluate the contribution of IL-36γ in the epithelial cell-driven host response to BV using clinical samples and a physiologically relevant human 3-D in vitro model.

In our clinical samples, we measured significantly elevated *IL36G* expression levels in epithelial cells isolated from women with BV. In contrast, we found that women with *Lactobacillus*-dominated VMB exhibited lower levels of *IL36G*, suggesting a role for IL-36γ in BV. These findings are in line with recent reports that showed that IL-36 cytokine levels are increased in women with highly diverse VMBs [9] and that elevated levels of IL-1α and IL-1β were predictive of non-*Lactobacillus*-dominance in South African women [15]. Although specific BV-associated genera alone did not correlate with *IL36G* expression in clinical samples, utilizing our human 3-D in vitro model system we demonstrate that specific BV-associated bacteria induce *IL36G* in a species-specific manner. In contrast, we found that the vaginal health-associated *L crispatus* did not induce *IL36G*. Furthermore, when BV-associated bacteria were combined in a polymicrobial cocktail, we measured a significant 12-fold increase in *IL36G* expression relative PBS controls.

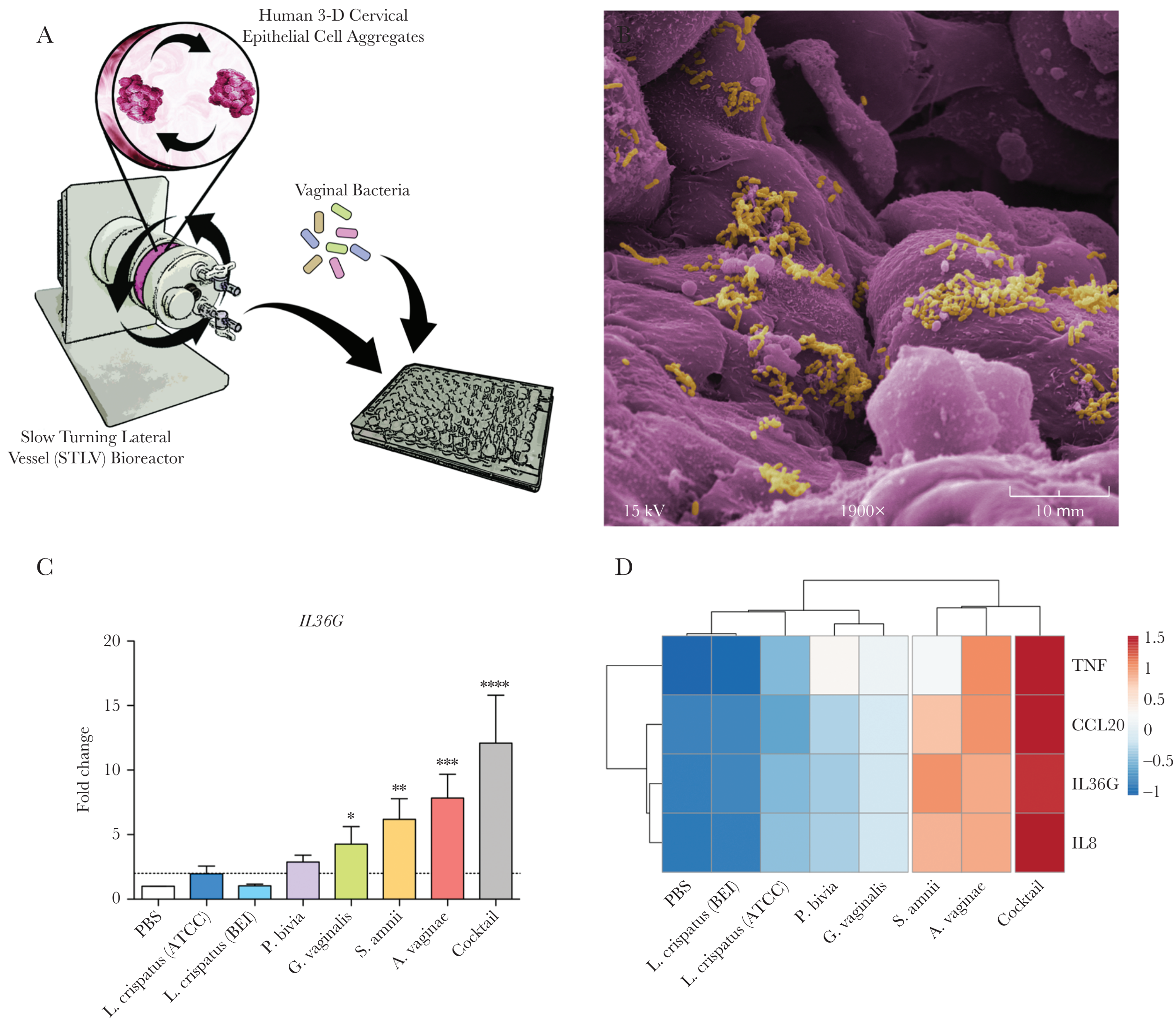

Figure 2. Bacterial vaginosis (BV)-associated bacteria induce *IL36G* in human 3-dimensional (3-D) cervical epithelial cells in a species-specific manner. (A) Schematic of slow-turning lateral vessel (STLV) bioreactor used to generate human 3-D cervical epithelial cells. (B) Pseudocolored scanning electron micrograph depicting polymicrobial cocktail infection of human 3-D cervical epithelial cell aggregate. (C) Human 3-D cervical epithelial cells were infected with individual vaginal health-associated *Lactobacillus crispatus* (designated ATCC and BEI isolates), BV-associated bacteria (*Gardnerella vaginalis*, *Prevotella bivia*, *Sneathia amnii*, and *Atopobium vaginae*) at a multiplicity of infection (MOI) of 10, and a polymicrobial cocktail (a mixture of *G vaginalis*, *P bivia*, *S amnii*, and *A vaginae* each at an MOI of 2.5 for a total MOI of 10) for 24 hours. *IL36G* expression in human 3-D cervical epithelial cells was assessed by quantitative polymerase chain reaction using ΔΔCt method. Gene expression was normalized to *GAPDH* and is expressed as fold change relative to phosphate-buffered saline-treated control. Data depict mean ± standard deviation and represent results from 3 to 4 independent experiments. Statistical analysis performed on $\log_2$-transformed data using one-way analysis of variance with Bonferroni's multiple comparisons test. *, $P < .05$; **, $P < .01$; ***, $P < .001$; ****, $P < .0001$. (D) hierarchical clustering analysis of proinflammatory gene expression in human 3-D cervical epithelial aggregates after infection with vaginal bacteria. Bacteria were grouped within clusters determined by hierarchical clustering analysis. Gene expression was normalized to *GAPDH*. Clustering of the heat map was based on Euclidean distance between rows and columns and average linkage clustering.

This highlights the complex, polymicrobial nature of BV and could potentially explain the lack of correlation between individual genera and *IL36G* expression in our clinical samples. The absence or low relative abundance of a given genera may be compensated by the presence of another, and complex interactions or synergy between specific bacteria could further exacerbate the inflammatory response. We previously demonstrated that IL-36γ signaling in the FRT stimulates proinflammatory signaling pathways and promotes neutrophil infiltration infection and could be essential in response to bacterial infection, similar to genital HSV-2 infection [4]. Although BV is not characterized as a neutrophilic disease [13], we measured a significant ($P < .05$) 2.2-fold increase in neutrophils in CVLs collected from BV^+ study participants that corresponded with elevated *IL36G* expression. The functional impact of IL-36γ and IL-36γ-mediated neutrophil infiltration in the context of BV warrants further investigation. However, the data presented in this report provide further evidence that IL-36γ functions as a

key regulator of mucosal inflammation, neutrophil trafficking, and immunity in the lower FRT.

This well controlled clinical data set provided important insights into IL-36γ, but these findings should be interpreted in the context of several limitations. First, this analysis included a limited number of study participants (n = 23) that precluded statistical significance on several analyses. Second, the participants were predominately African American women, and results may not represent or be generalizable to all women in the United States. Finally, although women were advised to abstain from sexual intercourse for at least 24 hours before study visits, this does not rule out the risk of measuring elevated immune mediators due to recent intercourse. Larger clinical studies in the future may provide additional support for IL-36γ as a potential marker for FRT inflammation and further define the function of IL-36γ in the response to BV and other clinically relevant STIs, including human immunodeficiency virus.

CONCLUSIONS

In this study, we demonstrate that upregulated *IL36G* expression in epithelial cells isolated from BV^+ women corresponded with increased neutrophil counts in CVLs, highlighting the potential impact of increased *IL36G* expression in women with BV. Using a well characterized human 3-D cervical epithelial cell model, we show that specific BV-associated bacteria (*A vaginae* and *S amnii*), and a polymicrobial cocktail, induce *IL36G* and other proinflammatory genes. It is interesting to note that the polymicrobial cocktail composed of multiple bacterial species induced *IL36G* greater than any individual species, highlighting the potential collaboration between bacteria that can impact levels of genital inflammation. Collectively, we demonstrate that BV-associated bacteria induce *IL36G* in vivo and in vitro. Taken into consideration with our previous findings on the function of IL-36γ in genital HSV-2 infection, this study provides new compelling evidence indicating that IL-36γ may function broadly as an important mediator of host defense in the FRT.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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Disclaimer. The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the US Centers for Disease Control and Prevention or the Department of Health and Human Services.

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