Expression of *Staphylococcus aureus* Virulence Factors in Atopic Dermatitis

Si En Poh[1], Winston L.C. Koh[1], Shi Yu Derek Lim[2], Etienne C.E. Wang[2,3], Yik Weng Yew[2,4], John E.A. Common[5], Hazel H. Oon[2,5] and Hao Li[1,6,5]

Atopic dermatitis (AD) is a skin inflammatory disease in which the opportunistic pathogen *Staphylococcus aureus* is prevalent and abundant. *S. aureus* harbors several secreted virulence factors that have well-studied functions in infection models, but it is unclear whether these extracellular microbial factors are relevant in the context of AD. To address this question, we designed a culture-independent method to detect and quantify *S. aureus* virulence factors expressed at the skin sites. We utilized RNase-H–dependent multiplex PCR for preamplification of reverse-transcribed RNA extracted from tape strips of patients with AD sampled at skin sites with differing severity and assessed the expression of a panel of *S. aureus* virulence factors using qPCR. We observed an increase in viable *S. aureus* abundance on sites with increased severity of disease, and many virulence factors were expressed at the AD skin sites. Surprisingly, we did not observe any significant upregulation of the virulence factors at the lesional sites compared with those at the nonlesional control. Overall, we utilized a robust assay to directly detect and quantify viable *S. aureus* and its associated virulence factors at the site of AD skin lesions. This method can be extended to study the expression of skin microbial genes at the sites of various dermatological conditions.

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INTRODUCTION

Atopic dermatitis (AD) is a chronic skin inflammatory disease characterized by red, itchy, and dry skin (Luger et al., 2021; Paller et al., 2019). AD is a highly prevalent skin disorder in developed countries, affecting up to 60% of infants aged <1 year and up to 20% of adults (Cheok et al., 2018; Meylan et al., 2017). The pathogenesis of AD is complex and generally thought to have both underlying genetic and environmental factors. The well-characterized genetic factors include genes essential for skin barrier formation such as FLG, tight junctions, and the balance between skin proteases and protease inhibitors (Williams and Gallo, 2015). The environmental factors are less understood, with many hypothesizing an interplay of both the host microbiota and the microbes in the environment that are important in priming the immune system at a young age and in the maintenance of the epithelial barrier integrity (Paller et al., 2019).

Microbial analysis of the skin during AD has revealed clear dysbiosis of the microbial community compared with that in the healthy skin (Gong et al., 2006; Kong et al., 2012). In particular, *Staphylococcus aureus*, an opportunistic Gram-positive bacteria, largely dominates the bacterial community at lesional sites of AD, leading to a dramatic decrease in the microbial diversity (Leyden et al., 1974). The exact roles of this bacteria in vivo are not completely understood because its significance is highly context dependent—ranging from skin colonization to overt sepsis (Koziel and Potempa, 2013). Many studies using laboratory cultures and mouse infection models have revealed that disease pathogenesis is driven by several *S. aureus* virulence factors (Amagai et al. 2002; Cho et al., 2001; Falugi et al. 2013; Gonzalez et al. 2012; Jenkins et al. 2015; Kolar et al. 2013; Williams et al. 2020, 2019). These virulence factors include cell wall–associated and –secreted proteins with roles in tissue adhesion and extracellular tissue degradation, host immune modulators, bacteria toxins, and other enzymes responsible for bacterial metabolism (Lacey et al., 2016; Tam and Torres, 2019). Although these *S. aureus* virulence factors can be potentially involved in exacerbating AD, the expression of most of these proteins has not been detected on the skin sites of patients with AD. The limited number of studies investigating *S. aureus* virulence factors expression have focused on wounds (Rozemeijer et al., 2015), cutaneous abscesses (Date et al., 2014), and nasal colonization (Chaves-Moreno et al., 2016). The main difficulty when investigating the expression of microbial genes on the skin is the low microbe bioburden (Kong et al., 2017), compared with those of other sites where there is a dense microbial community (i.e., the intestinal tract). Although a

[1]*Molecular Engineering Lab, Institute of Molecular and Cell Biology (IMCB), Agency for Science, Technology and Research, Singapore, Singapore;* [2]*National Skin Centre, National Healthcare Group, Singapore, Singapore;* [3]*Skin Research Institute of Singapore, Singapore;* [4]*Lee Kong Chian School of Medicine, Nanyang Technological University, Singapore;* [5]*A*STAR Skin Research Labs (A*SRL), Agency for Science, Technology and Research, Singapore, Singapore; and* [6]*Department of Chemistry, Faculty of Science, National University of Singapore, Singapore, Singapore*

[5]*These authors contributed equally to this work.*

Correspondence: Hao Li, Department of Chemistry, Faculty of Science, National University of Singapore, 4 Science Drive 2, S9 Level 12, Singapore 117544, Singapore. E-mail: chmlihao@nus.edu.sg

Abbreviations: AD, atopic dermatitis; EASI, Eczema Area and Severity Index; rhPCR, RNase-H–dependent PCR

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previous study in patients with AD detected several toxins and superantigens with immune blotting (Moran et al., 2019), this was limited to a small set of proteins in which antibodies were readily available.

In this study, we developed a robust method to quantitatively assess the expression of multiple *S. aureus* virulence factors. We used tape strips to directly sample control and lesional skin sites of patients with AD, isolated the total RNA, and utilized RNase-H–dependent PCR (rhPCR) to preamplify the cDNA before quantitative assessment of each transcript using qPCR. Using this assay, we directly sampled a nonlesional skin site and two lesional skin sites of differing severity on patients with AD using noninvasive tape stripping to determine the expression of each virulence factor in situ. We observed that although an increased relative abundance of most virulence factors was detected at skin sites with higher disease severity, the relative expressions of these genes were largely unchanged compared with those of nonlesional sites or were even downregulated at lesional sites with high severity. Overall, we show that our technique enables robust in situ detection of viable *S. aureus* and that many of the *S. aureus* virulence factors are expressed on AD skin.

RESULTS

Assay development of RNase-H–dependent preamplification qPCR for *S. aureus* genes

We designed a targeted preamplification qPCR assay for 19 *S. aureus* genes representative of the five main categories of virulence factors: adhesins (*clfA* and *fnbpA*), exoenzymes comprising proteases (*aur, eta, scpA, splA, sspB,* and *V8*) and other secreted enzymes (*coa, geh, hysA,* and *sak*), toxins and superantigens (*hla, psmA,* and *tsst-1*), immune modulators (*cap8G, spa,* and *scin*), and metabolic enzymes (*isdA*). Two housekeeping genes *gmk* and *gyrA* were also included. The typical skin microbial load is a mere 10^3–10^4 bacteria/cm^2 (Whitman et al., 1998), and for noninvasive sampling using tape strips or swabs, this low amount of microbes does not provide sufficient RNA for robust analysis of gene expression for more than a few genes. To address this, we utilized a multiplex preamplification step employing rhPCR to amplify the cDNA after the first-strand synthesis of the mRNA. This is a method that utilizes a blocked oligonucleotide incorporated into the primer that is subsequently cleaved off by RNase-H after specific binding of the primer to the target. This ensures high fidelity hybridization of the primer and dramatically reduces the formation of primer dimers—a major side reaction in multiplex PCR that interferes with qPCR signal (Dobosy et al., 2011; Li et al., 2019). qPCR of this preamplified cDNA gene library was subsequently used to assess the relative gene expression of each *S. aureus* virulence factor. Each of the primer sequences was verified for their PCR efficiency (Supplementary Table S1), and the specificity against the common Staphylococcal skin commensal *Staphylococcus epidermidis* was determined (Figure 1a). We optimized the best rhPCR reaction mixture for the multiplex PCR to ensure low bias in the preamplification of the cDNA (Figure 1b). We next compared the blocked-cleavable primers (RNase-H–dependent primers), which are activated by RNase-H2, to conventional primers. Using a known amount of luciferase RNA (*luc*) spiked into total RNA extracted from healthy subjects' skin tape strips, we performed the preamplification PCR followed by qPCR of *luc* (Figure 1c–e). From melt curve analysis, we found that RNase-H–dependent primers significantly reduce the formation of PCR side products (Figure 1d and e), and this effect is especially obvious at low input amounts (10 and 100 *luc* copies), which is highly relevant for RNA isolated from skin tape strips or swabs. From the *luc* gene titration, we further determined that the preamplification was highly efficient for up to 100 gene copies (amplification factor = 1.87). Between 10 to 100 gene copies, *luc* can be detected but not reliably quantified (Figure 1c).

S. aureus housekeeping gene expression as a measure of viable bacteria population

To determine the expression of *S. aureus* virulence factors expressed in situ during AD, we recruited 33 patients diagnosed with moderate-to-severe AD as determined by SCORing Atopic Dermatitis (Table 1 and Supplementary Table S2). These patients were sampled at the control (nonlesional) site (if available), site(s) of low AD severity, and another site(s) with high AD severity as assessed by the local Eczema Area and Severity Index (EASI) score. Because EASI score is assigned to score a body region (Hanifin et al., 2001) and not a specific sampling site, the severity (low or high) of each lesional site was assessed by the clinician. We first determined the optimal number of tape strips for robust *S. aureus* mRNA signal using the housekeeping guanylate kinase gene *gmk* as a measure for bacterial abundance. Although bacterial abundance is usually determined using genomic DNA, we reasoned that mRNA abundance of a housekeeping gene allows for quantification of the viable *S. aureus* population. We observed that increasing the input tape strips for skin microbiome sampling increases the rate of *gmk* detection (Figure 2a). As expected, the *gmk* gene abundance also increases with increasing input. Combining four tapes provides the highest detection rate (100%), but this reduces skin site specificity and is more tedious in the clinical setting. To address this, we decided to proceed with the combined materials from two tapes (detection rate = 86%) for further studies. Using extracted RNA combined from the tape strips sampled at differing AD skin sites, we determined that the *gmk* abundance was higher at both lesional sites than at the control site and that high-severity sites have higher expression of *S. aureus gmk* (Figure 2b). This suggests that viable *S. aureus* increases with AD severity at specific sites. We further observed that the *gmk* expression (as determined by the C_T) correlated well with the site-specific EASI score ($r = -0.396$, $P = 0.009$) as well as with the objective SCORing AD for the severe sites ($r = -0.762$, $P = 0.0009$) (Figure 2c and d). Six patients with AD were resampled at the same sites on their second visit, and we observed that *S. aureus gmk* expression had reduced dramatically at all the sites (including the control sites) (Figure 2e). This corresponds to a reduction of EASI and SCORing AD at the second clinical visit (Table 1 and Supplementary Table S3), indicating that dermatitis has resolved significantly at these sites.

Detection and quantification of *S. aureus* virulent factors on AD skin sites

We next determined the gene expression of the *S. aureus* virulence factors at the sampled sites of each patient with AD.

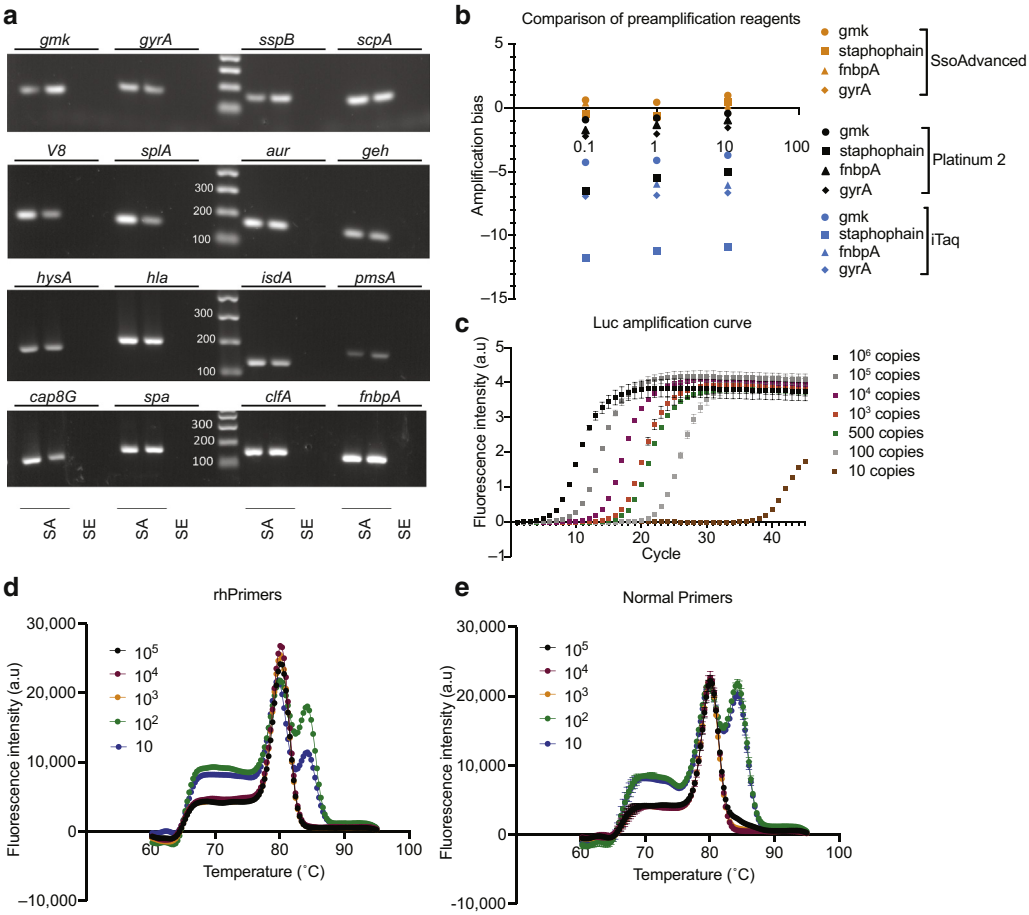

Figure 1. Optimization and validation of RNase-H–dependent preamplification qPCR assay. (**a**) Specificity of qPCR primers used against SA and SE. (**b**) Three different PCR mastermix were compared by first amplifying the reverse-transcribed RNA using the pooled primers, followed by qPCR of four **SA** genes. Calculation of amplification bias is as described in Materials and Methods. (**c**) Amplification plot showing the titration of varying amounts of *luc* added to RNA extracted from tape strips sampled from healthy individuals, which was subsequently reverse transcribed and preamplified using the pooled primers followed by qPCR of *luc*. (**d, e**) The melt curve of qPCR with varying amounts of luciferase RNA (*luc*) added to RNA as in **c**. Preamplification was performed with either (**d**) rhPrimers or (**e**) normal primers. All qPCR was done in technical duplicates or triplicates. a.u, arbitrary unit; rhPrimer, RNase-H–dependent primer; SA, *Staphyloccous aureus*; SE, *Staphylococcus epidermidis*.

From our preliminary study, we found that samples with undetectable *gmk* typically have undetectable signals for the expression of the other virulence genes. As such, qPCR of the virulence factor genes was only performed for samples where *gmk* is detected. This resulted in a total of 11 control, 16 low-severity, and 16 high-severity samples from 17 patients. From the luciferase gene titration curve, we were further able to define a limit of quantification and detection. Each gene is detectable if the melt curve from the qPCR is specific to the amplicon, whereas the expression is quantifiable only if the C_T of the signal is <25 cycles. In a pilot experiment, we observed that *pmsA*, *tsst-1*, and *eta* were not detected in any of the 24 samples collected from four patients, and these three genes were excluded from qPCR for subsequent samples. We observed that the percent detectable and quantifiable genes are the highest for the AD high-severity sites (Table 2), which is unsurprising given the higher *S. aureus* load as determined from *gmk* abundance (Figure 2). However, the exoenzymes *aur* and *hysA* and adhesin *fnbpA* were expressed at very low levels and were only quantifiable in a few samples.

For the *S. aureus* virulence factor genes that are in the quantifiable range, we observed that there is an increase in overall relative abundance at the low- and high-severity sites compared with that in the control sites (Figure 3a). This again corresponds to the increase in *S. aureus* population at the affected sites. *scin* and *spa*, two genes involved in immune modulation, were the two most highly expressed virulent factors at both the control and diseased sites (Figure 3a). We next sought to determine whether any of the virulence factors were differentially expressed during the disease. Surprisingly, we did not observe any genes that were significantly upregulated at the high-severity sites compared with those in the low-severity or the control sites. Instead, we observed that many genes, in particular, the exoenzymes *geh*, *splA*, and *V8* and the immune modulator *scin*, were downregulated at the high-severity site compared with those at the control site (Figure 3b and c). Furthermore, the normalized expression of *splA* has a negative correlation with disease severity (Figure 3d) as determined by EASI ($r = -0.4659$, $P = 0.0063$). We performed a separate analysis for patients with or without

Table 1. Patient Demographics and Clinical Characteristics

Characteristics	One Tape	Two Tapes	Four Tapes
Number recruited	11	17	5
Age (y), mean (SD)	35.4 (16.3)	30.0 (12.2)	27.2 (9.5)
Sex, n (%)			
Female	2	3	1
Male	9	14	4
Ethnicity, n (%)			
Chinese	10	13	5
Malay	1	3	0
Indian	0	1	0
Clinical severity disease scores, mean (SD)			
SCORAD visit 1	51.9 (14.1)	64.4 (14.4)	54.8 (6.9)
SCORAD visit 2[1]	NA	51.9 (25.7)	NA
EASI visit 1	13.8 (12.0)	24.4 (19.0)	16.0 (8.8)
EASI visit 2[1]	NA	19.1 (17.3)	NA

Abbreviations: EASI, Eczema Area and Severity Index; NA, not applicable; SCORAD, SCORing Atopic Dermatitis.
[1]SCORAD and EASI for visit 2 were determined from 6 of the 17 patients recruited at visit 1. Refer to Supplementary Table S3 for further details.

steroid treatment and did not observe any clear differences in both *S. aureus* abundance (as assessed by *gmk* expression) (Figure 4a and b) or virulence factor expression (Figure 4c and d). However, this analysis is limited by the low number of patients with no steroid treatment (n = 2) compared with the number of those receiving steroids (n = 12). In summary, although the abundance of each virulence factor increases at the diseased (low- and high-severity) sites, many of these *S. aureus* virulence factor genes are unchanged or downregulated compared with those of the control sites.

DISCUSSION

S. aureus is an opportunistic pathogen that has low skin colonization in healthy skin but dramatically increases in abundance at the affected skin sites of patients with AD. Although this increase in *S. aureus* abundance has been known for the last 40 years (Leyden et al., 1974), it is unclear whether the virulence factors that have been previously characterized using infection models are relevant in the context of AD pathogenesis. Because the expression of virulence factors is highly regulated (Gimza et al., 2019), their expression on the skin sites should be determined directly in situ without further microbial culturing. In this paper, we have designed a robust assay that enables the detection and quantification of a panel of *S. aureus* virulence factors using RNA isolated from samples obtained in a noninvasive manner. This enabled us to determine that the viable population of *S. aureus* increases with disease severity and that many of the well-characterized *S. aureus* virulence factors, although surprisingly downregulated at the high-severity sites, were indeed expressed at the diseased sites at a higher level than at the control sites.

The advantage of using RNA to determine *S. aureus* abundance is the ability to detect the viable population. We determined that the viable *S. aureus* population increases at the diseased site compared with that at the control sites on patients with AD. One limitation of this study is that we were unable to assess the *S. aureus* abundance and virulence factor expression of healthy individuals. Only about one third of the healthy population is colonized with *S. aureus*, and this is typically in the nares (Parlet et al., 2019). Although a previous study has shown that it is possible to directly profile the metatranscriptome of samples isolated from the nare (Chaves-Moreno et al., 2016), we reasoned that using the *S. aureus* virulence factor expression profile of the stratified, non-cornified nare surface is likely not a good control for skin sites relevant to AD. The low abundance of *S. aureus* on healthy skin sites does not allow for robust quantification using our multiplex preamplification qPCR assay. By sampling at the control and two lesional sites of varying severity, we observed that the abundance of virulence factors increased with disease severity. This confirms that *S. aureus* virulence factors were indeed expressed on AD skin sites and can potentially lead to exacerbation of the disease, especially because the skin barrier is compromised in AD. Secreted virulence factors such as proteases can potentially reach the deeper cutaneous layers and result in tissue destruction (Chua et al., 2022).

It was initially surprising that the expression of virulence factors was unchanged or even downregulated at the severe sites. Many of these genes involved in *S. aureus* virulence are controlled by the quorum-sensing *agr* regulon and are upregulated at high *S. aureus* density (Novick and Geisinger, 2008). Our data suggest that the *S. aureus* population on even the control sites of many individuals is sufficiently dense for *agr*-mediated upregulation of the virulence factors. Furthermore, the expression of *S. aureus* virulence factors is controlled by a myriad of regulatory genes (Jenul and Horswill, 2019), which in combination may downregulate virulence factor expression at high-severity sites owing to reasons yet undetermined. One possible pathway is through the transcription factor CODY, which is induced during nutrient deprivation and is a negative regulator of the *agr* regulon (Roux et al., 2014). In this manner, when a highly dense population of *S. aureus* is established in the severe lesional sites and has consumed the available resources in the nutrient-limited skin environment, expression of many secreted virulence factors controlled by *agr* decreases. Further investigations into the expression of these global transcription factors will be needed to confirm this.

In this study, we focused on detecting and quantifying the expression of *S. aureus* virulence factors where antibodies are not readily available because a previous study showed the use of a dot blot approach to detect *S. aureus* superantigens and exotoxins (Moran et al., 2019). We therefore did not include delta toxins, a key *S. aureus*–secreted toxin that was shown to directly impact AD pathogenesis (Nakamura et al., 2013). Furthermore, delta toxin was not detected at any of the nonlesional or lesional sites in the Moran et al. (2019) study, although this observation is limited by a small samples size of five patients with AD.

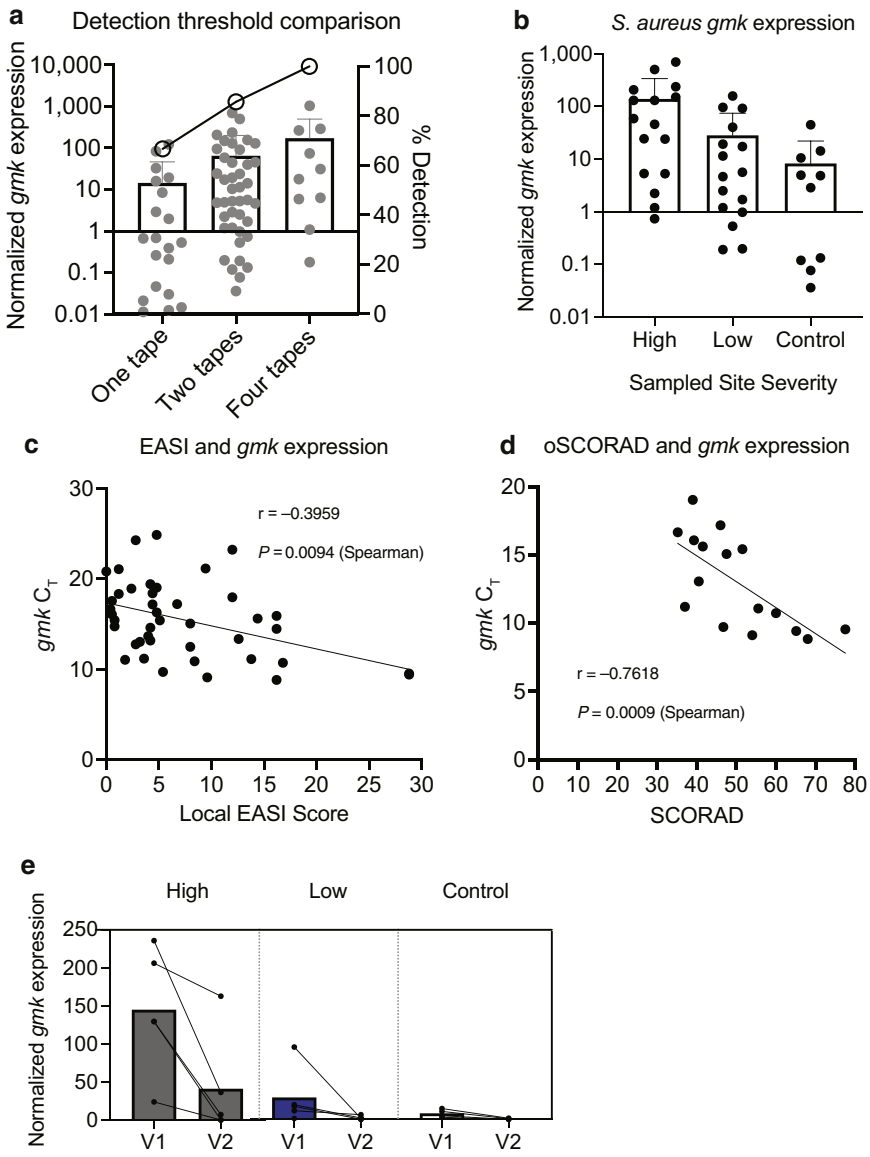

Figure 2. Using *Staphylococcus aureus* housekeeping gene expression to quantify *S. aureus* abundance on atopic dermatitis skin sites. (**a**) Comparison of detection rate and *S. aureus gmk* (normalized to internal control *luc*) when samples were extracted from one (n = 11), two (n = 17), or four (n = 5) tapes. (**b**) *S. aureus gmk* expression at control, low-severity, or high-severity sites for samples extracted from two tapes. (**c**) Spearman correlation of EASI and (**d**) oSCORAD with *gmk* expression. For the correlation, the C_T was used. (**e**) Assessment of *S. aureus gmk* (normalized to internal spike-in control *luc*) on the same sites of patients with AD at the second visit (n = 6). Error bars represent SD. EASI, Eczema Area and Severity Index; oSCORAD, objective SCORing Atopic Dermatitis.

Another limitation of this study is the inclusion of patients with topical and/or systemic corticosteroid treatment because this can affect bacteria diversity (Kong et al., 2012). However, we reasoned that because most patients with AD are under corticosteroid treatment, this is reflective of the typical AD skin environment. Although we did not observe any significant difference in the virulence factor expression between patients under corticosteroid treatment versus those without, this analysis is limited by the small cohort number and will require a follow-up study assessing the effect of corticosteroid treatment on *S. aureus* virulence factor expression.

In conclusion, we utilized a robust assay to directly detect and quantify viable *S. aureus* and its associated virulence factors at the site of AD skin lesions. This method can be further expanded to determine the expression of other skin microbial genes at low input RNA amounts and used to study other cutaneous conditions such as skin and soft tissue infections to better understand the role of microbial genes in driving these infections.

MATERIALS AND METHODS

Skin microbiome sampling of patients with AD

Skin tape strip sampling for patients with AD was approved by the National Healthcare Group domain-specific ethics review board (2018/01248), and all subjects provided written informed consent before participation. Subjects were recruited during their routine clinic appointment by the attending physician. A total of 27 male and 6 female subjects aged >16 years were included in the study. The overall disease severity of patients with AD was assessed using SCORing Atopic Dermatitis, whereas site-specific severity was assessed using EASI (local EASI). Patients on topical antibiotics for 1 week or systemic antibiotics for 2 weeks before sampling were excluded from the study. Patients were included regardless of therapy such as oral immunosuppressants and/or steroids, and no further restrictions on the intake or application of these treatments were given before sampling. All treatments for each patient are shown in Supplementary Table S1. Patients were sampled at control (nonlesional) sites and low-severity and high-severity (lesional) sites with D-SQUAME sampling discs (Clinical and Derm, Dallas, TX). For

Table 2. Percentage of Detected and Quantifiable *Staphylococcus aureus* Genes from Subjects with AD

	Percentage Detected			Percentage Quantifiable		
Gene	Control	Low	High	Control	Low	High
gyrA	100	100	100	100	100	100
aur	63.64	100	93.75	0	6.25	31.25
scpA	72.73	100	100	45.46	50	62.5
spiA	63.64	93.75	93.75	54.55	87.5	81.25
sspB	72.73	93.75	100	45.46	56.25	75
V8	63.64	100	100	63.64	100	100
coa	63.64	81.25	87.5	27.27	40	40
geh	63.64	100	93.75	54.55	93.75	93.75
hysA	9.09	31.25	56.25	9.09	18.75	18.75
sak	54.55	93.75	93.75	45.46	68.75	75
cap8G	63.64	87.5	93.75	45.46	68.75	75
scin	81.82	100	100	72.73	100	100
spa	72.73	100	100	72.73	100	100
clfA	72.73	87.5	100	54.55	62.5	75
fnbpA	72.73	81.25	87.5	9.09	31.25	31.25
hla	72.73	100	100	54.55	93.75	87.5
isdA	81.82	100	100	72.73	100	100

Abbreviation: AD, atopic dermatitis.

samplings where two or four tapes were combined for RNA extraction, tapes sampled from an area of similar disease severity (by EASI) were combined. Sampling was performed by pressing each tape strip at a specific skin site a total of 50 times. Each tape was stored at −80 °C separately in 2 ml bead-beating vials containing 0.5 mm Zirconia/Silica beads (Biospec Products, Bartlesville, OK). A total of 1 ml TRIzol reagent (Thermo Fisher Scientific, Waltham, MA) was added to each tape, and samples were frozen at −80 °C until further processing. Tapes were combined at the RNA extraction step. For patients with repeat visits, the exact same sites as the first visit were sampled. RNAs from healthy subjects were obtained from a previous study (Li et al., 2018).

RNA extraction

Tape strips in TRIzol were thawed and bead beaten twice on the Mini-Beadbeater-16 (Biospec Products) for 1 minute at room temperature, with samples cooled on ice between each bead-beating cycle. Samples were spun down at 13,000 r.p.m. for 5 minutes, and the supernatant was transferred to fresh 2 ml tubes. Total RNA extraction was performed with the Direct-zol RNA Microprep Kit (Zymo Research, Irvine, CA) as per the manufacturer's instructions, without on-column DNase treatment. Tapes of the control sites were either not combined or combined at two tapes or four tapes per column. The same was done for the lesional sites. After elution of RNA, DNase treatment was done with the TURBO DNA-free Kit (Invitrogen, Waltham, MA), as per the manufacturer's instructions.

Reverse transcription and targeted rhPCR preamplification

First-strand cDNA synthesis was performed with random hexamers and SuperScript III Reverse Transcriptase (Thermo Fisher Scientific). Luciferase control RNA (Promega, Madison, WI) was included in the reverse transcription reaction at 10^4 copies per sample as an internal control.

The cDNA was subsequently used for preamplification as per SsoAdvanced PreAmp Supermix (Bio-Rad Laboratories, Hercules, CA) instructions, with samples preamplified for 20 cycles. The GEN1 rhPCR primers were designed using PrimerBlast to contain sequences specific to *S. aureus* genes, and the 3′ blocking groups were designed according to the manufacturer's instructions (Integrated DNA Technologies, Coralville, IA). A total of 5 μM of pooled GEN1 rhPCR primer (Integrated DNA Technologies) mixture containing 44 primers were added to a final of 50 nM each. RNase H2 enzyme (Integrated DNA Technologies) was included in the preamplification mixture at 1 mU and a final concentration of 0.01% Triton X-100. After preamplification, a PCR clean-up was done with Exonuclease I (New England Biolabs, Ipswich, MA) as per the manufacturer's instructions with some modifications. A total of 1.25 μl of exonuclease I was added to 3 μl of exonuclease I digestion buffer and 0.75 μl of nuclease-free water. This 5 μl reaction mixture was added to the preamplified products, and incubation was carried out for 60 minutes at 37 °C, 15 minutes at 80 °C, and a final temperature holding at 4 °C. All samples were then diluted 10-fold with nuclease-free water before qPCR.

Assessing optimal PCR mastermix for preamplification

S. aureus SH1000 was cultured overnight, spun down, and the pellet was treated with RNAprotect (Qiagen, Hilden, Germany). TRIzol reagent was added, and the pellet was resuspended and transferred to a bead-beating tube for lysis using the same procedures as for the tape strips described earlier. Total RNA extraction was performed with the Direct-zol RNA Miniprep Kit (Zymo Research), and DNase treatment was done with the TURBO DNA-free Kit (Invitrogen) as per the manufacturer's instructions. After cDNA synthesis, preamplification was performed using the iTaq DNA polymerase (Bio-Rad Laboratories), SsoAdvanced PreAmp Supermix (Bio-Rad Laboratories), or Platinum II Taq Hot-Start DNA polymerase (Thermo Fisher Scientific) according to the manufacturer's instructions for 20 cycles. qPCR was performed for *gmk*, *gyrA*, *fnbpA*, and *sspB*. To determine the amplification bias, qPCR was performed directly using the cDNA without further preamplification for the same genes. The amplification bias is calculated by first subtracting the C_T value of the preamplified sample from that of the unamplified sample and then subtracting the theoretical C_T difference with accounting for sample dilution.

Luciferase titration assay and optimization of preamplification primer type

Serial dilutions of luciferase control RNA (Promega) were done with nuclease-free water in DNA LoBind tubes (Eppendorf, Hamburg, Germany). Luciferase RNA dilutions were spiked into pooled skin microbiome RNA samples previously extracted from a healthy subject study (Li et al., 2018). Luciferase RNA was added to a final amount of 10^6, 10^5, 10^4, 10^3, 500, 10^2, 50, and 10 copies. The mixture was used for first-strand cDNA synthesis with random hexamers and SuperScript III Reverse Transcriptase (Thermo Fisher Scientific). cDNA samples were preamplified using the SsoAdvanced PreAmp Supermix (Bio-Rad Laboratories) and treated with exonuclease I as described earlier. qPCR was done with primers against the luciferase gene (Integrated DNA Technologies). For comparison of preamplification with normal primers versus the GEN1 rhPCR primers, cDNA samples were preamplified using either a pool of 44 normal primers (Integrated DNA Technologies) or GEN1 rhPCR primers (Integrated DNA Technologies). qPCR was performed against the luciferase gene, and the melt curve was obtained for comparison of amplified products.

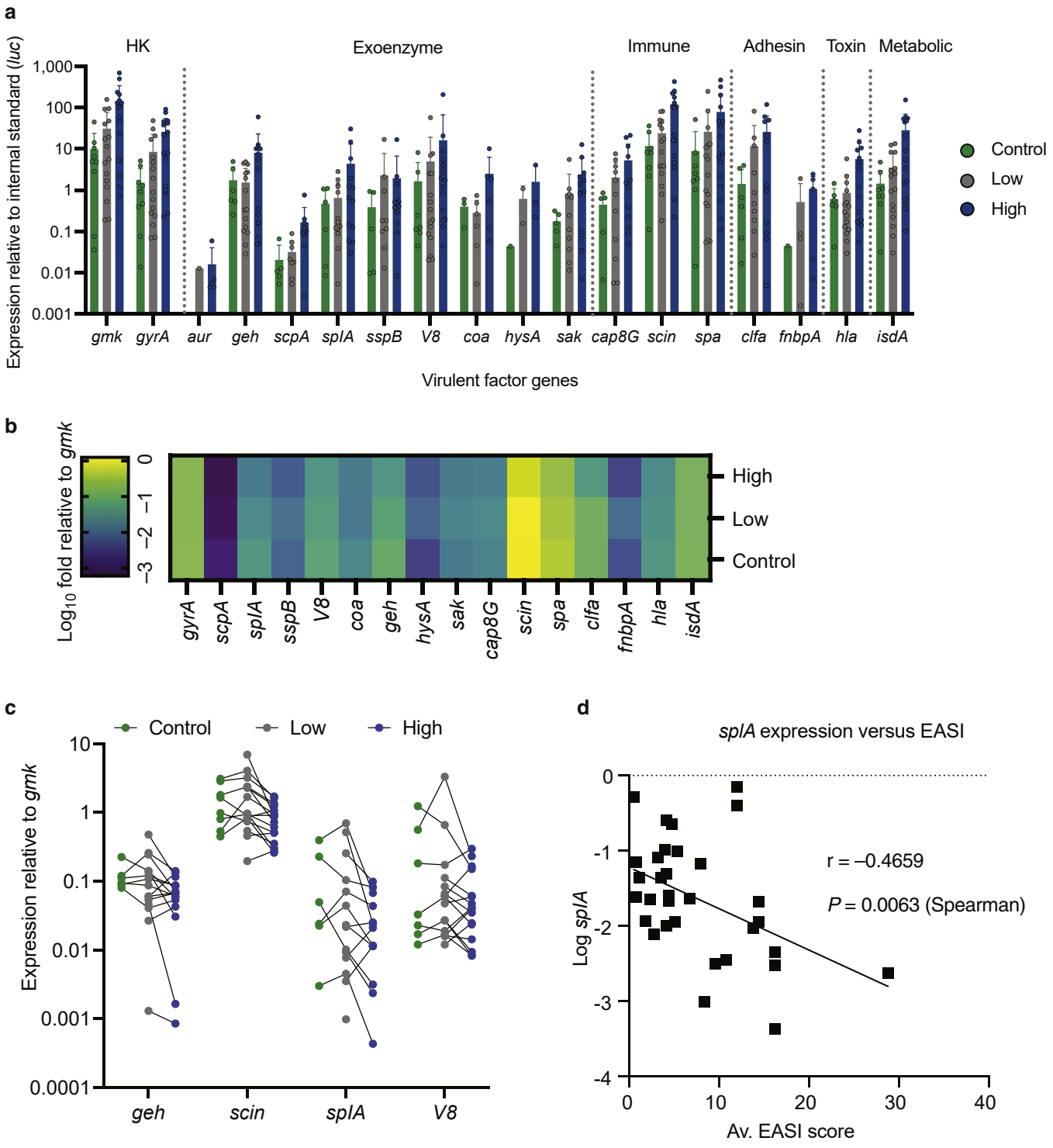

Figure 3. Expression of *Staphylococcus aureus* virulent factors on skin sites of subjects with AD. (**a**) The abundance of each virulent factor transcript at control, low-severity, or high-severity sites was assessed by the rhPCR preamplification, followed by qPCR. The expression of each gene was normalized to an internal spike-in control *luc*. (**b**) Log_{10}-normalized expression of each virulence factor relative to the housekeeping gene *gmk*. (**c**) Expression of *geh*, *scin*, *splA*, and *V8* (normalized to *gmk*) for individual patients at control, low-severity, and high-severity sites. Samples from the same subject are connected by a line. (**d**) Spearman correlation of log_{10} *splA* expression (normalized to *gmk*) with the average EASI score. The EASI score was averaged across the two sites where the samples were collected. Error bars represent SD. Av., average; EASI, Eczema Area and Severity Index; HK, Housekeeping; rhPCR, RNase-H–dependent PCR.

Quantitative PCR

Quantitative PCR was performed with LUNA Universal qPCR mastermix (New England Biolabs) as per the manufacturer's instructions on the Applied Biosystem StepOne Plus Real-Time PCR System (Thermo Fisher Scientific) for 45 cycles. *S. aureus*–specific primers (Integrated DNA Technologies) were used, designed using Primer-Blast for *S. aureus* specificity. Two technical replicates were done for each sample.

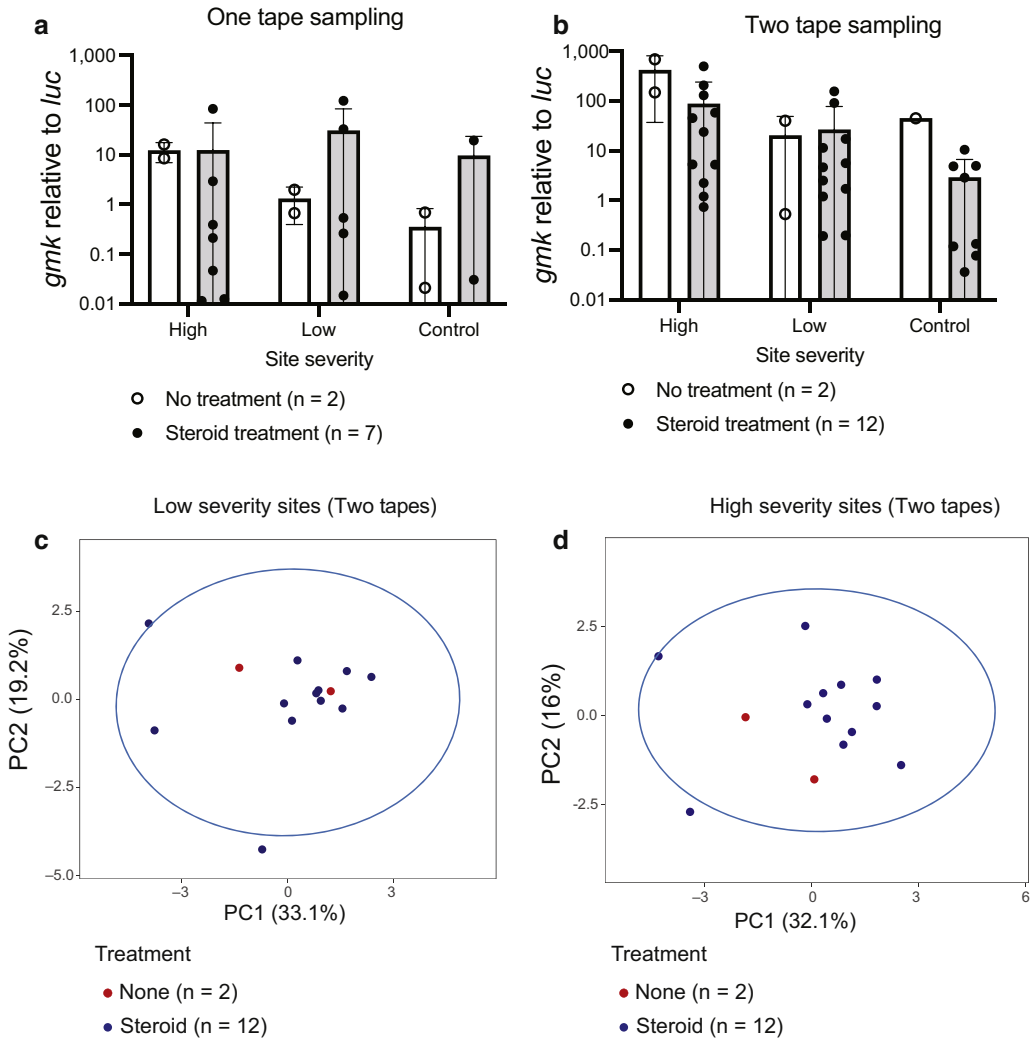

Figure 4. *Staphylococcus aureus* abundance and virulence factor expression are unaffected by steroid treatment. *S. aureus gmk* expression (normalized to internal spike-in control *luc*) from RNA obtained using (**a**) one tape or (**b**) two tapes from patients with or without steroid treatment. Statistical analysis was not performed because the no-treatment group number was too low (n = 2) compared with the steroid-treatment group (n = 12). (**c**) PCA plot for virulence factor expression for (**c**) low-severity and (**d**) high-severity sites. Error bars represent SD. PC, principal component; PCA, principal component analysis.

Data availability statement

No large datasets were generated or analyzed during this study. Minimal datasets necessary to interpret and/or replicate data in this paper are available on request to the corresponding author.

ORCIDs

Si En Poh: http://orcid.org/0000-0003-4990-4820
Winston L.C. Koh: http://orcid.org/0000-0003-4611-6490
Shi Yu Derek Lim: http://orcid.org/0000-0002-7079-2416
Etienne C.E. Wang: http://orcid.org/0000-0002-9139-1865
Yik Weng Yew: http://orcid.org/0000-0001-8915-306X
John E.A. Common: http://orcid.org/0000-0002-3280-7365
Hazel H. Oon: http://orcid.org/0000-0001-7607-3124
Hao Li: http://orcid.org/0000-0002-6652-4793

AUTHOR CONTRIBUTIONS

Conceptualization: HL, HHO, WLCK, JEAC; Data Curation: HL, WLCK; Formal Analysis: HL, SEP; Funding Acquisition: HL, HHO; Investigation: SEP, SYDL, ECEW, YWY; Methodology: WLCK, SYDL, ECEW, YWY, JEAC, HL, HHO; Project Administration: HL, HHO; Resources: HL, HHO; Software: HL; Supervision: HL, HHO; Validation: SEP, HL; Visualization: HL, SEP; Writing-Original Draft: HL, SEP, WLCK, HHO; Writing-Review and Editing: HL, HHO, ECEW, SEP, YWY, JEAC

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CONFLICT OF INTEREST

The authors state no conflict of interest.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2022.100130

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