Skin microbiota sampling in atopic dermatitis: to swab or scrub?

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ABSTRACT

Background

Collecting high quality samples for skin microbial analysis is challenging due to the low number of microorganisms on the skin. Studies comparing different available techniques for skin sampling are scarce.

Objective

This study compares the detection of total bacterial DNA, *Staphylococcus (S.) aureus* DNA, microbial diversity and fungi by two different methods, namely dry flocked swabbing and scrubbing.

Methods

As part of an ongoing study in atopic dermatitis, we collected 39 swab and 39 scrub samples from 16 patients. *S. aureus* specific and total bacterial DNA were measured with quantitative (q)PCR. To identify bacteria and fungi we sequenced the 16S rRNA gene and the fungal internal transcribed spacer region 1.

Results

The qPCR showed a higher absolute amount of total bacterial DNA in the scrubs ($p<0.001$). Sequencing of 16S rRNA identified 323 and 318 different genera in the swabs scrubs, respectively. The majority was identified equally well with the two techniques and biodiversity was not significantly different. Interestingly, we found fungal DNA more often in the scrubs than in the swabs (36% versus 9%).

Conclusion

Scrubs result in a higher collection of bacterial and especially fungal DNA. Therefore, they are preferable for studying low-biomass skin areas or fungi.

INTRODUCTION

Changes in the cutaneous microbial ecosystem are associated with skin diseases. For example, atopic dermatitis (AD) is characterized by overgrowth of *Staphylococcus (S.) aureus*.[1] Advances in sequencing technologies and metagenomics have enabled more precise studies of the microbial composition of the skin, including the interactions between microbes.[2] Together with these advances, questions arose about the optimal approaches to study the skin microbiota.[3] Recent studies focused on primer selection and the quality control of sequencing runs.[3-6] In addition, consistency in the sampled skin site was shown to be important, as bacterial communities appear to be body-site specific.[7] However, collecting a valid sample of the skin still remains a challenge due to the low number of microorganisms on skin compared to other body sites.[8] There are different techniques to profile the skin microbiota, with skin punch biopsy, swabs, tape-stripping and scrubs being the most widely used. Punch biopsy gives a complete microbial profile of all the skin layers, but its use is limited by the invasiveness.[9] Superficial swabs are practical and the smooth surfaced cotton swabs (premoistened) are often used for skin sampling.[7] However, flocked (brush like) swabs could increase the detection of bacteria compared to smooth swabs. [10,11] The scrub was pioneered for culture-based studies and its use in microbiome studies has only been investigated in a single pilot study with three healthy volunteers.[7,12,13] Compared to the swab, this technique might profile microbes at a deeper level of the skin, where bacterial composition and behaviour could be different.[14-16] Each technique has its own specific sampling depth and usability characteristics, leading to possible variation in the type and density of the collected micro-organisms. The choice of sampling method depends on the research question, sampling site, the feasibility of the method and the amount of total DNA required for further analyses. Selecting the right technique is crucial. However, studies that compare sampling methods in relation to different skin microbial outcomes are scarce.

As part of an ongoing interventional study to understand the role of microbes in AD and changes in skin microbial populations after treatment, we compared total bacterial DNA, *S. aureus* DNA, microbial diversity and the presence of fungi between dry nylon-flocked swabs and scrubs.

METHODS

Study design and patients

The aim of the study was to investigate skin microbial collection methods for use in a future interventional study in patients with AD.[17,18] Patients were recruited between June 2014 and January 2015 in the Erasmus Medical Center and the Havenziekenhuis,

Rotterdam. Inclusion criteria were diagnosis of AD, age above 18 years and the ability to read the relevant patient information and provide informed consent. Criteria for exclusion were the use of systemic antibiotics, corticosteroids or immunosuppressive medication 6 months before the study and the use of topical antibiotics or steroids 7 days before the study. AD was diagnosed according to the UK Working Party's diagnostic criteria.[19] Severity of AD was scored by the patient using the self-administrated eczema area and severity index (SA-EASI).[20] Additional information about medication use and the presence of atopic diseases was retrieved via medical history. Swab- and scrub samples were collected from skin lesions in patients that met the criteria for inclusion (visit 1). A second, third and fourth swab- and scrub sample were taken one, two and three weeks after visit 1 (visit 2, 3, and 4), only in those patients that were positive for *S. aureus* at visit 1 (figure S1). The medical ethical committee of the Erasmus Medical Center approved the study and written informed consent was obtained from all participants.

Skin microbiome samples – swab and scrub

Microbial skin samples were collected from a skin lesion, preferably located at the antecubital fold. In case of absence of antecubital lesions, the most predominant skin lesion was chosen. Two types of samples were obtained, a swab and a scrub sample. These two samples were collected simultaneously from non-overlapping areas of the same skin lesion. First, a sterile dry nylon-flocked swab (Copan480CE) was rubbed along the antecubital crease for 30 seconds and put back in the tube containing liquid Amies Medium. The liquid medium was additionally extracted into an eppendorf. Second, a modified scrub method was used, based on the method of Williamson and Kligman.[12,21] A sterile PVC sampling ring with an internal diameter of 4 cm was placed just below the antecubital crease and 0.75 mL of sterile wash fluid (0,85% NaCl, 0.1% bacteriological peptone, 0.1% Tween 80) was added. After scrubbing the surface of the skin within the ring with a sterile swab for 1 min, the fluid was collected in a 2 ml eppendorf. Samples were stored at -80^{o}C until further processing for DNA based analysis. All samples were collected by a medical doctor wearing sterile gloves.

The patients were informed to avoid bathing or showering in the 12 hours before sampling and could not use personal antibacterial hygiene products from 48 hours before sampling.

DNA isolation and qPCR

For cutaneous DNA isolation approximately 150 uL material was directly transferred to a DNA isolation plate and 0.5 mL phenol pH8.0 (Phenol solution, catalogue P4557, Sigma-Aldrich, St Louis, MO) was added. The samples were mechanically disrupted by bead beating twice for 3 minutes with a 96-well plate Beadbeater (Biospec Products, Bartlesville). Following bead beating, samples were centrifuged at 1880 rcf (4000rpm)

for 10 minutes to separate the aqueous and phenolic phases. The aqueous phase was transferred to a new 96-well plate and DNA was purified with the AGOWA mag Mini DNA Isolation Kit (AGOWA, LGC genomics, Berlin, Germany) in accordance with the manufacturer's recommendations. After elution, the total bacterial load in each sample was assessed by quantitative (q)PCR using an universal bacterial primer-probe set.[22] The total load of *S. aureus* DNA was assessed by qPCR using the following primers : 16S-S. aur-F1 (5'-GCG AAG AAC CTT ACC AAA TCT TG-3') and 16S-S.aur-R1 (5'-TGC ACC ACC TGT CAC TTT GTC-3'), and the 16S-S.aur MGB Taqman® probe (5'-CAT CCT TTG ACA ACT CT-3') with a FAM label.

16S rRNA sequencing

Analysis of the microbiome composition was performed by massively parallel sequencing of the archaeal and bacterial V4 hypervariable region of the 16s rRNA gene on an Illumina MiSeq sequencer (Illumina, San Diego, CA). To prevent over-amplification, the barcoded DNA fragments spanning the V4 hypervariable region were amplified from a standardized level of template DNA (1 ng). Amplicons generated using adapter primers F515 and 806R (using 30 PCR cycles), were bidirectionally sequenced as described previously.[24] Pre-processing, analysis of unique sequences and classifications were performed using modules implemented in the Mothur software platform.[25] Standardized mock communities were included to check for technical performance of all experimental steps. Sequences were grouped with the OTU clustering module in MOTHUR with a 0% cut-off leading to separate clusters for each unique sequence. To assign taxonomic information to the different unique sequences, they were blasted in the Ribosomal Database Platform (RDP).[26] Negative control samples of the lysis buffer contained substantial amounts of *Schlegelella*, and *Enhydrobacter*, both described as contaminant genera before.[23] Therefore, these genera were excluded for further analysis. Shannon diversity index was determined to describe the microbial diversity for the swabs and the scrub samples.

Sequencing of Fungal ITS amplicons

Barcoded amplicons of fungal internal transcribed spacer regions (ITS) were generated using a two step PCR approach. Fungal ITS-1 regions were first amplified with the following primers: nex-ITS-BITS-F: TCGTCGGCAGCGTCACCTGCGGARGGATCA and nex-ITS-B58S3-R GTCTCGTGGGCTCGGGAGATCCRTTGYTRAAAGTT (adapted from Bokulich & Mills[27]). Each reaction contained 5ul undiluted stock DNA, 1 unit Phusion Hot start II (F-549L) enzyme, 1x High Fidelity buffer, 200nM deoxynucleotide triphosphates (Thermo Scientific) and nuclease free PCR grade water to a 25 µl final reaction volume. PCR reactions consisted of an initial denaturation step of 98 °C, for 5 min and 30 amplification cycles (98 °C for 10 sec, annealing 48 °C for 30 sec and elongation 72 °C for 30 sec and

final extension step (72 °C for 5 min) followed by cool down (10 min at 4 °C). A negative control (blank) was included for each set of 24 PCR reactions. Reactions were cleaned by solid-phase reversible immobilization (SPRI) using AMPure XP SPRI beads (Beckman Coulter, Inc.). Dual barcodes (8 bp) and Illumina Sequencing adapters were attached using the Nextera XT Index Kit (Illumina, San Diego, CA) according to the manufacturer's protocols. Barcoded amplicons were quantified using the Caliper LabChip GX II system (Perkin Elmer, Hopkinton, USA), normalized to the same concentrations, pooled, and gel purified using the Qiaquick spin kit (Qiagen) and AMPure XP SPRI beads. Pooled amplicons were 250-bp paired-end sequenced using the MiSeq (Illumina). Raw Illumina fastq files were demultiplexed, quality filtered, and analyzed using modules implemented in the Mothur software platform.[25] Unique sequences were taxonomically classified by the RDP-II Naïve Bayesian Classifier [26] using a 60% confidence threshold against the Mothur formated UNITE Database (Version No. 7).[25] The mock samples confirmed good technical performance.

Statistical analysis

Patient and sample characteristics are presented with median and interquartile range (IQR) for continuous data and counts and percentages for categorical data. Total bacterial DNA, *S. aureus* specific DNA and Shannon diversity were compared between baseline swabs and baseline scrubs, using a non-parametric Wilcoxon signed rank test for related samples. A linear mixed effects model was used to account for the repeated measurements (different time points) for each patient.[28] This mixed effects model determined if there was a significant difference between scrubs and swabs regarding uptake of *S. aureus* specific DNA, total bacterial DNA and Shannon diversity, adjusted for the use of antibiotics (during the study), use of antibacterial products (before/during the study) and the time variable. *P*-values were corrected for multiple testing (3 tests) and therefore a *P*-value of ≤0.0167 was considered significant. To display the microbial composition per sample, as measured using 16S rRNA, the different genera were expressed using the mean relative abundance per sample. SPSS version 21 was used for descriptive data analysis. Microsoft Excel 2010 was used to generate graphs and figures.

RESULTS

Patient characteristics

For this study, 21 patients with AD were screened for participation (visit 1). Of these, 17 patients were included for analysis as four patients did not meet the diagnostic criteria for AD. Ten of these 17 patients were positive for *S. aureus* at the screening. To mimic the longitudinal design of the intervention study and to take into account the

microbial variation over time, a follow-up including three measurements was performed in these ten patients (visits 2 to 4; one week between each visit; figure S1). The mean self-reported eczema severity at baseline, measured with the SA-EASI [20], was 23.2 (IQR 17.48; scale 0-96). Patient characteristics are shown in table 1.

Table 1. Patient characteristics (n=17)

Male : female	6 : 11
Age	
Median (IQR)	38 (26)
Atopy (n (%))	
Asthma	9 (52.9)
Rhinitis	13 (76.5)
Food allergy	9 (52.9)
Age of onset (n (%))	
Early childhood	14 (82.4)
Adult	3 (17.6)
SA-EASI baseline (range 0-96)†	
Median (IQR)	23.2 (17.48)
Use of medication at start of the study (n(%))	
Emollient	17 (100)
Topical calcineurin inhibitor	2 (11.8)
Topical steroids	14 (82.4)
Topical OTC antibacterial (shower) product	1 (5.9)
Use of medication during the study (other than emollient) (n(%))	
Topical calcineurin inhibitor	2 (11.8)
Topical steroids	5 (29.4)
Topical OTC antibacterial (shower) product	10 (58.9)
Systemic antibiotics	1 (5.9)

† n=15 due to missing data, OTC = over the counter

Sample characteristics

The screening of 17 patients and follow-up of ten patients yielded a total of 47 swab- and 42 scrub samples. For an optimal comparison between swab and scrub samples, we included only swab-scrub pairs that were taken at the same skin site at the same time. Thirty-nine swab-scrub pairs could be included, of which 37 were taken from the antecubital fold, one from the knee and one from the upper arm. Of the 39 paired swab-scrub samples, 38 and 35 (obtained from 16 patients) could be included for qPCR and sequencing analysis, respectively. One swab and four scrub samples did not contain sufficient bacterial DNA to generate a PCR amplicon for qPCR or sequencing respectively (figure S1).

Total bacterial DNA and *S. aureus* DNA – swab vs. scrub (qPCR)

Comparison of the scrubs and the swabs that were collected at baseline in 13 patients, showed that the absolute amount of both total bacterial DNA and *S. aureus* DNA was higher in the scrub samples, indicating a better resolution/sensitivity of the scrub measurement. However, when the variation at different time points and use of antiobiotics/antibacterial products (during the study) was taking into account (Methods section; 38 samples), only the total amount of bacterial DNA found with qPCR was significantly higher in the scrubs (log1.23fg/ul; p<0.001; table 2a-b).

Table 2a. Baseline scrubs versus baseline swabs

	Swab (median (IQR))	**Scrub** (median (IQR))	**P- value**
total bacterial DNA (fg/ul)	14888 (6355-26192)	144828 (79881-589027)	0.001*
***S. aureus* DNA** (fg/ul)	83 (0-453)	547 (107-9518)	0.008*
Shannon-diversity	2.93 (2.37-4.29)	2.31 (1.50-3.82)	0.019

The analysis includes 13 baseline samples from 13 individuals. All p values are calculated using Wilcoxon signed rank test for related samples. * significant P-value (<0.0167)
NOTE: The table presents absolute amounts. The relative amount of S. aureus compared to the complete microbiome cannot be calculated due to primer specific differences between the specific S. aureus primer and the universal primer for total bacterial DNA.

Table 2b. Mixed effects model scrub versus swab

	Coefficient (95% CI)	**P- value**
Log total bacterial DNA (fg/ul)	1.23 (0.58-1.88)	< 0.001*
Log *S. aureus* DNA (fg/ul)	0.94 (-0.50-2.38)	0.195
Shannon-diversity	0.73 (-1.88-0.43)	0.210

The analysis includes 38 samples from 16 individuals. * significant P-value (<0.0167)

Microbial diversity – swab vs. scrub (16S rRNA sequencing)

In the 35 swabs that could be analysed with 16S rRNA sequencing, a total of 441044 reads could be assigned to 321 genera. These belonged to 20 different phyla of which Firmicutes, Proteobacteria, Actinobacteria and Bacteriodetes were most abundant. The five most predominant genera found at baseline (visit 1), available from 13 patients, were *Staphylococcus (60%)*, *Corynebacterium (6%), Streptococcus (5%), Micrococcus (2%)* and *Paracoccus(2%).* In the 35 scrub samples, 533004 high quality reads belonging to 316 different unique sequences and 15 different phyla were obtained. *Staphylococcus (76%), Corynebacterium (7%), Micrococcus (2%), Streptococcus (2%)* and *Acinetobacter (1%)* were the five most predominant genera. Figure 1 shows the mean relative abundances of the 5 predominant genera per sample per technique. Of the 356 detected genera, 281 were identified by both sampling methods (78.9%). These represented over 99% of the total reads, showing that dominant genera are detected equally well by the

Figure 1. Relative abundance of 5 most abundant genera in (a) swabs and in (b) scrubs

NOTE: Numbers 1 to 16 refer to the 16 different patients. v1-v4 refer to the 4 different visits, all with one week in between. Individual nr. 13 started oral AB during the study.

swab and the scrub method (figure 2). The Shannon diversity index indicated that bacterial diversity did not significantly differ between the swab and the scrub samples (table 2a-b; p=0.210).

Fungi

Fungal DNA was found in four of the 47 collected swabs (8.5%). These four swabs belonged to four different individuals. In the scrub samples, fungal DNA was present in 15 of the 42 samples (35.7%), belonging to 8 individuals. More different unique sequences were identified in the scrubs (285) than in the swabs (130). The fungal generum that was most abundant in both the swabs and scrubs was *Malassezia* (table S1).

Figure 2. Venn diagram illustrating the observed overlap of (a) the identified reads (sequences) and (b) the identified genera, using 36 swabs and scrubs

DISCUSSION

This study compares the use of dry flocked swabs and scrubs to measure the skin microbiota in patients with AD, adjusting for a time variable and the use of antibacterial products (during the study) in a mixed model. A higher absolute amount of both bacterial and fungal DNA was recovered from the scrub samples, compared to the swabs. The potential of the scrub method to increase bacterial discovery rates was described earlier based on culture methods.[29] Secondly, dry swabs and scrubs were found to detect the dominant genera equally well at a given time (figure 2). This confirms that dry swabs can be used for microbiome analysis of the skin. Finding the same bacterial species using a swab and a scrub method, suggests that the deeper level (reached by the scrub) harbours no different bacteria from the superficial layer. It also raises the question whether the scrub actually reaches much deeper than the swab. What technique should be used for microbiome experiments depends on the type of study.

The increased resolution of the scrub makes it the preferred method, especially for characterizing fungi, rare microorganisms and for low biomass areas of the skin. However, dry flocked swabs are more user-friendly, can be applied on all skin sites and identify the dominant sequences of the microbiome equally as well as scrubs. Therefore,

the swabs can be considered for large scale studies and for body sites that are difficult to sample with the scrub.

The four most abundant genera that we found on the skin with 16S rRNA sequencing were *Staphylococcus* (Firmicutes), *Corynebacteria* (Actinobacteria), *Streptococcus* (Firmicutes) and *Micrococcus* (Actinobacteria), representing common bacterial phyla of the normal skin microbiota.[30] A clear dominance of staphylococci was found, as expected in patients with AD.[31] We found a low abundance of fungi, with fungal DNA in only 36% of the scrub samples and in less than 10% of the swab samples. This indicates the importance of using adequate collection methods when sampling the skin for fungi. The skin fungal microbiota in healthy controls mainly consists of the genus *Malassezia*.[32] Patients with AD are thought to have a higher abundance and diversity of *Malassezia* species compared to controls, suggesting a contribution of fungal species to AD.[33] In our AD patients, *Malassezia* was the most abundant genus on the skin. However, we were not able to draw any conclusions about the relationships between fungi on the skin and AD.

Limitations of this study include the use of the 515F/806R primer for amplification of the V4 variable region, a standard primer at the time we performed our analysis. The 806R primer is now known for its poor coverage of Propionibacteria. Recent studies were published that recommend amplification of the V1-V3 region for the skin or a the use of a modified V4 primer.[4,5] In our samples Propionibacterium were low abundant. However, this could also be inherent to eczema lesions where staphylococci overgrow. We do not expect that the choice of sequence region influences our conclusions about the swab-scrub comparison. Our study included negative controls (lysis buffer), but contamination from other sources e.g. the air or the swab solution (prepared sterile) cannot be excluded. We used a cohort of AD patients in this study and the results could be specific for this group. The use of antibacterial products during the study could have influenced the bacterial composition on the skin. However, this effect is expected to be equally present in the swabs as in the scrubs. Finally, the scrub method itself has some practical limitations as the collection of scrub samples is restricted to areas of the body where the cup can be placed. Despite these limitations, this study provides insight into the specific characteristics of different skin microbiota collection methods which is important for designing future skin microbiome studies.

In conclusion, scrubs result in increased yields of bacterial and especially fungal DNA. This increases the resolution of the measurement and makes the scrub preferable when working with fungi or low- biomass skin areas. However, dry flocked swabs identify the dominant genera of the bacterial microbiota equally as well as scrubs. Therefore, swabs can be considered for large population studies that examine higher-biomass areas of the skin.

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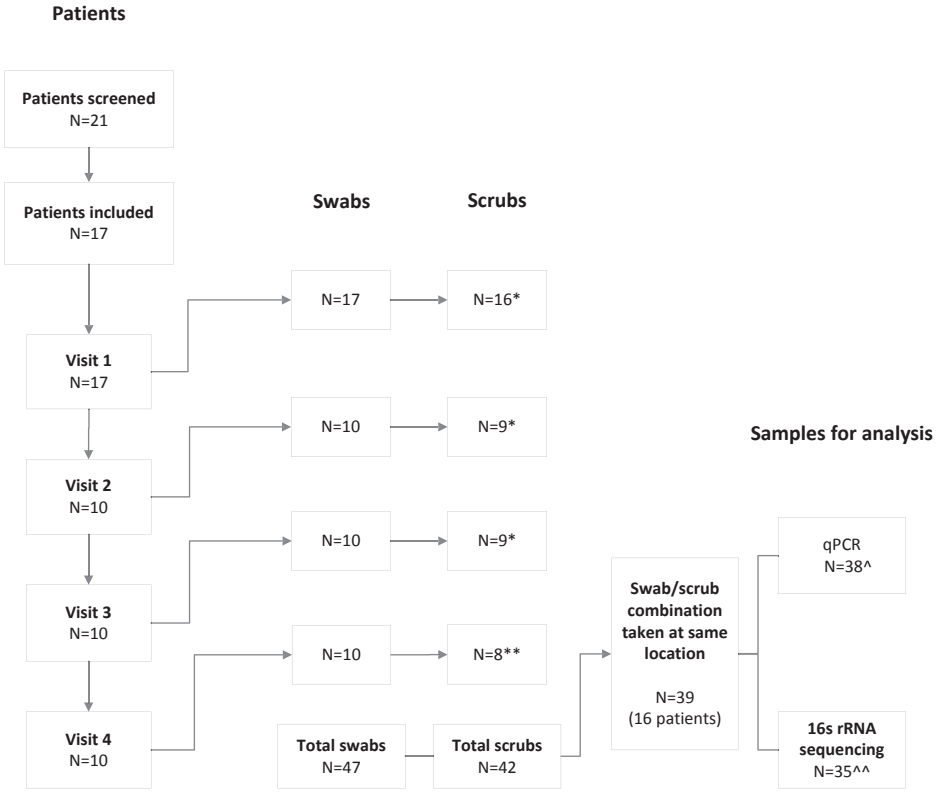
SUPPLEMENTARY MATERIAL

Figure S1. Flowchart of the included patients and collected samples



* 1 missing (eczema on the volar hand making scrub not feasible)
** 2 missings (eczema on the volar hand + logistic problem)

^ 1 missing samples (failure to collect DNA)
^^ 4 missing samples (bacterial DNA yield to low to generate a PCR amplicon)

Table S1. Presence of fungal genera in the swab and the scrub samples

	Swab **Number of sequences per sample (n=4)** Mean (range)	**Scrub** **Number of sequences per sample (n=15)** Mean (range)
Total fungal DNA	31173 (22780-40086)	37663 (20511-85031)
Malassezia	7554 (2172-13555)	13620 (112-43939)
Unclassified	6263 (982-14500)	1365 (0-4519)
unclassified_Malasseziales	5477 (3655-7118)	3623 (118-8994)
Candida	4036 (0-10823)	626 (0-4056)
Unclassified	2830 (1308-3490)	2566 (150-8160)
Cladosporium	821 (43-1825)	1289 (0-4099)
Cryptococcus	317 (0-919)	664 (0-3954)
Sporobolomyces	127 (31-267)	5305 (0-74381)