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Biofilm production and antibiotic susceptibility of *Staphylococcus epidermidis* strains from Hidradenitis Suppurativa lesions

Running head: *S. epidermidis* in Hidradenitis Suppurativa

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Abstract

Background: An aberrant interaction between commensal skin bacteria and the host skin immune system is considered important in the pathogenesis of Hidradenitis Suppurativa (HS).

Objective: In this study we investigated the antibiotic susceptibility and biofilm-forming capabilities of *S. epidermidis* strains isolated from HS patients.

Methods: Skin biopsies were taken from active HS lesions such as inflammatory nodules and/or sinuses and non-involved skin from 26 patients and cultured under optimal microbiological conditions for 24 hours. Planktonic growth, biofilm production, antibiotic susceptibility, and biofilm eradication by clindamycin, doxycycline, rifampicin, tetracycline, were tested including a laboratory control strain of *S. epidermidis* for reference.

Results: *S. epidermidis* was cultured in 16 out of 26 HS patients (62%). In total 27 different *S. epidermidis* isolates were identified; 16 (59%) from non-involved skin and 11 (41%) from HS lesions. All bacterial strains showed planktonic growth. Twenty-four out of 27 (89%) isolates were strong biofilm producers *in vitro.* The biofilm-forming capability varied amongst the strains from non-involved skin and lesional skin. Twenty-four strains had an intermediate to resistant antibiotic susceptibility to clindamycin (89%). Rifampicin was the most effective antibiotic at inhibiting planktonic growth and at eradication of biofilm ($p<0.05$).

Conclusion: We observed a slight increase in *S. epidermidis* virulence, characterized by resistance to commonly used antibiotics, increased biofilm production, and resistance to biofilm eradication. Especially the reduced sensitivity to tetracycline and

clindamycin, two standard antibiotics in the treatment of HS is alarming. Rifampicin, also important in HS treatment, showed the greatest efficacy at eradicating the biofilm at low MIC concentrations.

Introduction

Hidradenitis Suppurativa (HS) is a chronic, debilitating skin disease characterized by recurrent abscesses, nodules, sinuses, and scarring involving the intertriginous areas of the body.[1] Although HS is not considered to be a simple infection, bacteria are thought to play a role in the pathogenesis and an aberrant interaction of commensal skin bacteria with the innate skin immune system in patients with HS has been suggested as a central element of the pathogenesis of the disease.[2-4] This implies that both immune system and the commensal flora of HS patients may have functional characteristics which influence the pathogenesis of HS.

Data on the role of the skin microbiome in the pathogenesis of other inflammatory skin diseases such as acne vulgaris, psoriasis, and atopic dermatitis are currently emerging.[5] However, only limited data on the role of skin commensals in the pathogenesis of HS are available.

Staphylococcus aureus and coagulase-negative staphylococci (CNS) are the most abundant species cultured from lesional skin of patients with HS.[6-8] Generally, *Staphylococcus epidermidis* is a non-pathogenic CNS and a part of the human skin microbiome. However, *S. epidermidis* can become pathogenic and cause severe infections especially in immunocompromised patients, and in patients with implants.[9-12]

Biofilm formation is an important functional characteristic and a crucial virulence trait of *S. epidermidis* infections.[13] Biofilms are matrix-enclosed sessile microbial communities characterized by their ability to adhere to any surface and to each other.[14] The biofilm matrix consists of a mixture of exopolysaccharides, proteins, DNA, and other macromolecules, which allows bacteria to evade the host immune system and antimicrobial exposure.[15]

The clinical course of HS shows several characteristics of a biofilm-driven disease. The chronic and recurrent course of HS, the slow wound healing process and the relative resistance towards conventional antibiotic therapy are compatible with a pathogenic role for biofilms in HS.[16] In contrast to HS-prone skin where biofilm appears to be absent [2], the presence of biofilm in chronic HS lesions has recently been described.[16, 17]

The current concept of HS treatment is immunomodulatory and anti-inflammatory therapy, and the antibiotics commonly used to treat HS possess those properties.[18]

Therefore we aimed to characterize the *in vitro* antibiotic susceptibility pattern, the *in vitro* growth and biofilm forming capabilities of *S. epidermidis* strains isolated from HS patients.

Material and methods

The study protocol has been approved by the ethical board of Region Zealand, Denmark (project number SJ-420) and the data protection agency of Denmark (REG-105-2014). Informed consent was obtained from all patients.

Skin biopsies, bacterial cultures and analyses

The *S. epidermidis* isolates were cultured from 4mm punch biopsies from active HS lesions such as inflammatory nodules and/or sinuses and non-involved skin (i.e. at least 10 centimeters away from the lesional skin) of HS patients in the Dermatological Department of Roskilde Hospital, Denmark. One laboratory control strain of *S. epidermidis*, a gift from Anders Løbner-Olesen, Department of Biology, University of Copenhagen, was used as a control. Biopsies were cultured in an initial enrichment broth by incubating at 35 °C with 5% CO_2 for three days followed by subcultures onto 5% blood and chocolate agar plates for up to 10 days and anaerobic plates, incubated under anaerobic conditions. Speciation of microorganisms was done by MALDI-TOF MS (Bruker Daltonics, Bremen, Germany). Organisms of the same species were deemed indistinguishable if they had the same colony morphology, the same basic biochemical features and an identical antibiogram.

Antimicrobial agents

The antimicrobial agents used for the disk diffusion assay were tetracycline, rifampicin and clindamycin (Neo-sensitabs ROSCO, Taastrup, Denmark). The same antibiotics, including doxycycline, were ~~also~~ used in minimum inhibitory concentration (MIC) assays. The antibiotics were prepared and stored at -20°C according to the instructions of the manufacturer.

Planktonic growth

Each bacterial isolate was inoculated in 10mL Mueller-Hinton-Broth (MHB) medium (BD diagnostics, Mississauga, Canada) and incubated overnight at 37 °C with gentle shaking. The growth of all *S. epidermidis* strains was evaluated by measuring the optical density (OD) at 600 nm (Eppendorf BioPhotometer, Eppendorf, North America). The OD_{600} nm values were measured at 0, 20, 40, 80, 120, 180, 360 and 1440 minutes. All experiments and measurements were done in duplicates.

Antimicrobial susceptibility testing – disk diffusion and minimum inhibitory concentration (MIC) assay

The antibiotic susceptibility for tetracycline, clindamycin, and rifampicin was tested using the disk diffusion method. The inhibition zone diameters were measured, recorded and categorized as Sensitive (S), Intermediate (I) or Resistant (R) according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines.

The MIC was performed to test the susceptibility to doxycycline in the strains resistant to tetracycline. Additionally, the isolates selected for biofilm eradication were also tested to determine the MIC value. For the MIC assay we used an adapted protocol from Mojsoska *et al*. [19] Bacterial suspensions in the range of 2-8x10^5 colony-forming units per mL (CFU/mL) were incubated with the antibiotics overnight. The MIC classification was based on the MIC breakpoints of the EUCAST. All these experiments were performed in triplicates.

Microtiter biofilm formation assay

The cultured bacteria were tested for their ability to produce biofilm by using the crystal violet assay. Three different types of media - MHB, LB and tryptic soy broth media with additional glucose (TSB 1% glucose) (Sigma-Aldrich, Denmark) - were used to determine the optimal growth conditions. Overnight cultures were diluted (1:100) in fresh media and 100µL was plated in a flat bottom 96-well plate. At time points 24 and 48 hours, the bacterial suspension was removed, washed two times with PBS and stained with 125µL crystal violet 0.1% for 10 minutes at room temperature. After staining the wells were washed again with PBS. Ethanol 96% was added for 10 minutes at room temperature to solubilize crystal violet. OD_{595} nm was read with a plate reader (Synergy HT BioTek Instruments, Inc, Vermont USA). The strains were classified as strong, moderate, weak and no biofilm producer.[20, 21] The experiments were performed in duplicate.

Microtiter biofilm eradication assay

A few cultured strains were assessed for their ability to eradicate biofilm. The selection of the strains was based on their resistance pattern. Diluted overnight bacterial cultures were (1:100) were cultured in flat bottom 96-well plates at 37 °C for 24 hours. After removal of the bacterial suspension, the wells were washed gently with PBS and the antibiotics were added for biofilm breakdown. The plate was further incubated as described in the biofilm growth curve section.

Data analysis

Data presented represent duplicates from at least three independent experiments. Statistical analysis was done by using GraphPad Prism version 6.0.1 (GraphPad Software, Inc., San Diego, CA, USA) using the unpaired, two-tailed Student's *t*-test. *p*-values less than 0.05 are considered statistically significant.

Results

Characteristics of patients and the S. epidermidis isolates

S. epidermidis was cultured in 16 out of 26 patients (62%). Twenty-seven different *S. epidermidis* strains were identified: 16 (59%) from non-involved sites and 11 (41%) from lesional sites (Table 1). Eight of 16 patients (50%) did not receive any antibiotic treatment one month prior to the biopsies. Three patients (19%) were on oral tetracycline and one 1 patient (6%) was using topical clindamycin. Information about antibiotic treatment at the time of biopsy is missing for 4 patients (25%). In 6 out of 16 patients (38%), more than one strain was cultured from a non-involved or lesional biopsy. On average, more strains were cultured in the group on antibiotics (average of 0.8 strains) compared to the group not on antibiotics (average of 0.7 strains). However, in the group which did not use antibiotics, more strains were cultured in non-involved skin (average of 1 strain) in comparison to lesional skin (average of 0.3 strain). This difference was not found in the patients with antibiotic use in the past (average of 0.75 strains in both non-involved and lesional HS skin) (data not shown).

Planktonic growth

After 24 hours the strains reached an OD value between 2 and 5 on a scale from 0 to 5 (Fig. 1A), with the laboratory control strain showing the highest OD measurement after 24 hours.

Antimicrobial susceptibility

Twenty-six out of 27 strains were sensitive to rifampicin (96%). Twenty out of 27 strains (74%) were sensitive for tetracycline.

Seven out of 27 strains (26%) were classified as resistant (5 strains) or intermediate (2 strains) sensitive to tetracycline. The tetracycline-resistant strains were also resistant to doxycycline. Two strains from three patients with a known medical history of recent use of tetracycline use were classified as resistant. Three out of 27 strains were sensitive for clindamycin (11%), whereas resistance (9 strains) and intermediate sensitivity (15 strains) was observed in 24 strains (24/27; 89%). A strain isolated from a patient with known previous topical use of clindamycin was resistant to that antibiotic (Table 1). Proportional difference in susceptibility patterns between *S. epidermidis* strains from non-involved and lesional skin was demonstrated for all antibiotics, and in particular for clindamycin (Table 2).

Biofilm production

Twenty-four out of 27 clinical isolates (89%) could be classified as strong biofilm producers. The laboratory control strain was also categorized as a strong biofilm producer. Two strains, from two different patients, both from lesional HS sites, were classified as non-biofilm-forming strains (Table 1, Fig. 1B). One strain, isolated from non-involved skin, was classified as a moderate biofilm-producer (Table 1, Fig. 1B).

Biofilm eradication assay

Based on the biofilm growth curves and the MIC values, we selected bacterial isolates from two patients for the biofilm eradication assay, patient number 8 (strains 91, 92 and 93) and patient number 10 (strain number 96, 97 and 98). Patient 8 (unknown antibiotic use in past) and patient 10 (tetracycline antibiotic use in past) were selected because multiple strains were cultured from their skin biopsies and because strains from patient 8 showed a significantly different susceptibility pattern in the MIC compared to patient 10. The laboratory control strain served as a reference.

Rifampicin and clindamycin eradicated the biofilm in a significant manner in all tested HS strains ($p<0.05$, Fig. 2B,C). Tetracycline also eradicated the biofilm significantly in almost all HS strains. Notably, tetracycline induced a significant increase in biofilm in one strain (patient 10, strain 96, $p<0.05$, Fig. 2C). Doxycycline eradicated the biofilm significantly (patient 10, strain 96, $p<0.05$, Fig. 2C). In two strains (strain 92 and 96) doxycycline was not eradicating the biofilm significantly (Fig. 2B,C). Only tetracycline failed to eradicate biofilm in the laboratory control *S. epidermidis* strain (Fig. 2A).

Discussion

The skin is colonized by a broad spectrum of microorganisms. Skin commensals mostly do not harm, live in symbiosis and in general are even beneficial to the host. An important host factor regulating the composition and balance of the skin commensals is the skin innate and adaptive immune response to microorganisms.[22] Several recent reports indicate that skin commensals may be involved in the pathogenesis of HS. Microbiological studies in HS patients have shown primarily skin or intestinal (especially in the anogenital area), anaerobic commensals in lesional HS tissue.[7, 23, 24] Data suggests a shift in microbiota from pre-clinical paucity to an abundance in chronic lesions.[2, 17] An aberrant interaction between the commensal skin bacteria and the skin immune system has been suggested to play a role in HS. A current hypothesis is that in genetically susceptible individuals follicular plugging and early inflammation is triggered by an abnormal immune response to intrafollicular skin commensals. [25] We investigated *S. epidermidis* because it is a natural constituent of the human skin microbiome, the follicular infundibulum and because it has been isolated from HS lesions.[26-28]

Our finding that more than one *S. epidermidis* strain was cultured from non-involved and/or lesional skin in 6 HS patients indicates a polyclonal *S. epidermidis* population within the patient.[29] In healthy volunteers it has also been shown that an individual can carry many *S. epidermidis* strains with differing antibiotic resistance patterns, capacities to form biofilm and overall gene distribution.[30]

By growing the strains in a planktonic phase, we were able to analyze the strains in more detail, defining growth rates and antibiotic resistance patterns. Growth rates influence the virulence of bacteria as well as their antibiotic resistance.[31, 32] We included a laboratory control strain which grew to a higher optical density indicative of more exuberant growth than the HS strains, and did not observe significant differences in growth rates between the strains isolated for lesional or non-lesional skin.

Antibiotic susceptibility is another functional characteristic of bacteria. We tested the susceptibility towards the most commonly used antibiotic treatments of HS. The finding that ninety-six percent of cultured *S. epidermidis* strains were sensitive to rifampicin is largely consistent with other studies that performed susceptibility testing on *S. epidermidis* strains.[33-35] However, none of these studies were on *S. epidermidis* strains from HS lesions. Nevertheless, it is an important and encouraging finding because rifampicin is an important antibiotic in different guidelines for the treatment of HS. Recent studies demonstrated acquired resistance for antibiotics by *S. epidermidis*.[10] The observed resistance for rifampicin illustrates the potential of *S. epidermidis* to transform from a commensal into a pathogen.

The strains with resistance to tetracycline were also cross resistant to doxycycline. This resistance percentage is interesting since tetracyclines form a first line systemic antibiotic treatment for patients with HS and/or acne. One study investigated the resistance pattern of 129 isolates from HS lesions. Unfortunately, the authors did not specify the resistance pattern per bacterial species. From the 129 isolates, 42 were resistant to tetracycline (33%).[36]

Clindamycin appeared as the weakest antibiotic with almost 90% of the strains showing an intermediate sensitivity to resistance. Resistance to clindamycin was significantly higher in our study, compared to Cavanagh *et al*. (33% vs 8%) who investigated the antimicrobial susceptibility of *S. epidermidis* strains from healthy individuals.[35] High antimicrobial resistance to clindamycin has been observed in bacterial isolates obtained from HS patients. An overall resistance rate of 71 out of 129 isolates (71%) was found.[36] In daily clinical practice, the combination therapy of rifampicin with clindamycin is often prescribed for the treatment of HS. Clindamycin is known to enhance the bactericidal properties of rifampicin *in vitr*o.[37] Additionally*, in vivo* experiments have demonstrated synergistic bactericidal effects of rifampicin and clindamycin on *S. aureus* strains.[38]

Regardless, only one isolated strain was cross resistant towards both clindamycin and rifampicin. This strain was isolated from a patient that had no medical history of previous or ongoing rifampicin use, but had reported use of clindamycin lotion in the past, which could have induced the resistance to clindamycin.[39]

Treatment of HS is mainly based on immunomodulatory and anti-inflammatory therapy, and not on the bacteriocidal of bacteriostatic effect of antibiotics.[18] Therefore our findings regarding the antibacterial effect on our strains is of less importance in the treatment of HS, though it has profound importance when considering the escalating problems tied to antimicrobial resistance.

Taking into consideration that biofilm formation is regarded as *S. epidermidis* most notable virulence trait, particularly in terms of medical treatment, the propensity to form biofilm could be considered an important virulence feature. Most of the *S. epidermidis* strains from HS patients were classified as strong biofilm producers *in vitro*. However, two strains, classified as non-biofilm producers, were isolated from lesional HS skin. Therefore, based on this study it was not possible to relate biofilm formation to either lesional or non-involved skin or HS pathogenesis.

Rifampicin showed the highest biofilm eradication activity against strains from HS patients and also the laboratory control strain. Gomes *et al*. performed biofilm eradication by testing 5 *S. epidermidis* strains against 8 antibiotics. Rifampicin appeared the most potent antibiotic used [40]. Similar results were obtained in another study.[41] Although different techniques were used in these two studies, they are in accordance with our results, emphasizing rifampicin's potency against *S*. *epidermidis* biofilms.

Even though the tested HS strains had an intermediate to resistant response towards clindamycin when growing as individual bacteria, clindamycin eradicated the biofilm in all tested HS strains. A changed phenotype of bacteria that are embedded in the biofilm could have led to tolerance towards clindamycin. For instance, tolerance mechanisms in biofilms could involve reduced bacterial growth, the presence of persistent cells and mechanisms that control antibiotic-induced oxidative stress.[42]

Interestingly, enhancement of biofilm formation was seen in only one strain. Previous studies have shown that antibiotics can increase the synthesis of the extracellular

polymeric substance and biofilm formation by upregulating transcription genes responsible for different virulence factors in biofilm production [43].

One of the strengths of this study is that optimized conditions for culturing and biofilm testing of *S. epidermidis* were carefully chosen and tested for all the performed experiments. Secondly, we used a *S. epidermidis* laboratory control strain to increase the validity of our experimental set-up.

A limitation of this study is that we did not include biopsies of healthy controls without a (family) history of HS. By including healthy individuals we could have made several comparisons with strains from non-involved skin from HS patients. Secondly, we tested a selection of the isolated strains for biofilm eradication, and we missed information about previous antibiotic use and medical history of a few included HS patients.

Summarizing, we observed a slight increase in *S. epidermidis* virulence, characterized by resistance to commonly used antibiotics, increased biofilm production, and resistance to biofilm eradication. Especially the reduced sensitivity to tetracycline and clindamycin, two key antibiotics used in supportive treatment of HS and acne is alarming. Future larger case-control studies are needed focusing on functional characteristics, including the response of the skin immune system to *S. epidermidis* strains.

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Conflicts of interests

None of the authors has any conflict of interest to declare.

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Legends for figures

Figure 1. Growth properties of the isolated *S. epidermidis* strains. (**a**) Planktonic growth. After 24 hours most isolates showed acceptable growth (OD value between range of 2-4 on a scale from 0-5). The red line shows the control strain. Only one strain, number 93, follows the growth of the laboratory control strain. Isolate 74 and 75 (both from one patient) grew the slowest. (**b**) Biofilm production. Almost all *S. epidermidis* strains are strong producers of biofilm *in vitro.* The variation between the amount of biofilm is visible in both non-involved skin and lesional isolates. After 24 hours the biofilm production decreased in all strains (not shown).

Figure 2. Eradication of bacterial biomass in 24 hours preformed biofilms exposed to optimal doses of rifampicin, tetracycline, clindamycin and doxycycline for 24 hours. * $p < 0.05$ (Graph Pad version 6.0.1, unpaired student's t-test). (**a**) The laboratory control strain. Tetracycline is not able to eradicate the biofilm. All other tested antibiotics eradicated the biofilm. (**b**) Patient 8, strains number 91, 92 and 93. Rifampicin, clindamycin and tetracycline were able to eradicate the biofilm in all strains. Doxycycline did not eradicate the biofilm in strain 92. (**c**) Patient 10, strain number 96, 97 and 98.

Rifampicin, clindamycin and tetracycline eradicated the biofilm. In strain 96, doxycycline did not eradicate the biofilm and tetracycline showed an increase of biofilm formation.

Table 1. Antimicrobial susceptibility and biofilm production pattern for all strains.

			Disk diffusion			MIC		
Patient	**Strain**	**N/L**	**Rif**	**Tet**	**Clinda**	**Doxy**	**Antibiotic use**	**Biofilm production**
1	71	N	R	S	R		Clindamycin lotion	Strong
2	72	L	S	S	I		Tetracycline	Strong
3	73	L	S	R	R	R	Tetracycline	Strong
4	74	N	S	S	I		None	Strong
	75	N	S	S	S			Strong
5	76	N	S	R	R	R	None	Strong
6	77	N	S	S	I		None	Strong
	78	N	S	S	I			Strong
7	79	N	S	S	R		None	Strong
8	91	N	S	S	I		Unknown	Strong
	92	N	S	I	I	I		Strong
	93	L	S	S	I			Strong
	94	L	S	I	S	S		Strong
9	95	N	S	S	R		None	Moderate

10	96	N	S	R	R	R	Tetracycline	Strong
	97	N	S	R	R	R		Strong
	98	L	S	R	R	R		Strong
11	99	L	S	S	R	R	None	Strong
12	100	N	S	S	I		None	Strong
	101	L	S	S	I			None
13	107	L	S	S	I		Unknown	Strong
	108	N	S	S	S			Strong
	109	L	S	S	I			Strong
14	110	N	S	S	I		Unknown	Strong
	111	N	S	S	I			Strong
15	112	L	S	S	I		Unknown	None
16	119	L	S	S	I		None	Strong
Control	56		S	R	I	R		Strong

MIC, minimum inhibitory concentration; N, non-involved skin; L, lesional skin; Rif, rifampicin; Tet, tetracycline; Clinda, clindamycin; Doxy, doxycycline; R, resistant; S, sensitive; I, intermediate; Control, laboratory control strain.

Table 2. Proportional difference in the antibiotic susceptibility to rifampicin, tetracycline, and clindamycin between non-involved (n=16) and lesional (n=11) skin in HS patients.

	Rifampicin		**Tetracycline**		**Clindamycin**	
	Non-involved	*Lesional*	*Non-involved*	*Lesional*	*Non-involved*	*Lesional*
Sensitive	15 (94%)	11 (100%)	12 (75%)	8 (73%)	2 (13%)	1 (9%)
Resistant	1 (6%)	0 (0%)	3 (19%)	2 (18%)	6 (38%)	3 (27%)
Intermediate	0 (0%)	0 (0%)	1 (6%)	1 (9%)	8 (50%)	7 (64%)