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DISSERTATION

Analysis of Medical Biofilms with Emphasis on Indwelling Device-Related Infections Using Fluorescence *In Situ* Hybridization (FISH)

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1. Abstract

Auf Deutsch

Hintergrund: Katheterassoziierte Blutstrominfektionen und Sepsis durch bakterielle Biofilme sind eine Hauptursache für Morbidität und Mortalität bei Patienten auf Intensivstationen und bei Krebspatienten mit Langzeitanwendung von Zentralvenenkathetern. Bakterielle Biofilme sind Aggregate von Bakterien, die in einer Schicht aus extrazellulären Polymer-Substanzen eingebettet sind, wodurch ihre Bekämpfung mit Standard-Antibiotika-Schemata erschwert wird. Die mikrobiologische Kulturmethode der koloniebildenden Einheiten (KBE) wird routinemäßig zur Untersuchung von katheterassoziierten bakteriellen Infektionen eingesetzt. Die Kultur von Katheterspitzen auf Agarplatten liefert jedoch keine Informationen über den Ort und die Aktivität von lebensfähigen, aber nicht kultivierbaren Zellen (VBNC), so dass eine komplementäre Nachweismethode empfehlenswert ist.

Zielsetzung: Ziel dieser Arbeit war die Erstellung eines ‚Proof of Concept' für die Evaluation der 16S-rRNA-gerichteten Fluoreszenz-*in-situ*-Hybridisierung (FISH) in Kombination mit digitaler Bildanalyse zur Visualisierung, Lokalisierung und Quantifizierung der Wirkung zweier Antibiotika (Daptomycin und Vancomycin) auf *Staphylococcus epidermidis*-Biofilme auf Zentralvenenkathetern. Darüber hinaus wurden die durch FISH gewonnenen Ergebnisse mit denen der KBE Standarddiagnosemethode verglichen.

Methodik: *S. epidermidis*-Biofilme wurden unter Verwendung eines *in vitro*-Modells einer katheterassoziierten Infektion gezüchtet und nachfolgend einer Antibiotikabehandlung unterzogen. Anschließend wurde die Biofilmfläche und der Anteil aktiver Zellen unter Verwendung von FISH in Kombination mit Mikroskopie und digitaler Bildanalyse untersucht. Die FISH-Daten wurden mit den Daten der KBE, der Standarddiagnosemethode für katheterassoziierte Infektionen, verglichen. Die Anti-Biofilm-Aktivität von Daptomycin und Vancomycin wurde mit einer Kontrollgruppe Phosphat-gepufferter Kochsalzlösung verglichen.

Ergebnisse: *S. epidermidis*-Biofilme wurden *in vitro* auf Polyurethankathetern hergestellt und FISH erfolgreich angewendet, um die Wirkung der Antibiotika auf Biofilme zu messen. Sowohl Daptomycin als auch Vancomycin konnten die Biofilm-Parameter

Fläche und FISH-positiver Anteil signifikant reduzieren. Daptomycin zeigte im Vergleich zu Vancomycin eine stärkere Anti-Biofilm-Aktivität.

Schlussfolgerungen: Die durchgeführte Forschungsarbeit ist die erste Studie ihrer Art, bei der FISH als Analysemethode für die Anti-Biofilm-Aktivität von Antibiotika auf *S. epidermidis*-Biofilmen auf Kathetern verwendet wurde. Die Ergebnisse dieser ‚Proof of Concept' Studie unterstützen nachdrücklich die Verwendung von FISH als zusätzliches Analyseinstrument, um wichtige Informationen über den Ort und die Aktivität von Bakterienzellen in Biofilmen zu erhalten, die nicht durch KBE-Daten bereitgestellt werden können. Die Erkenntnisse dieser FISH-Studie sind ein wertvoller Beitrag zur rechtzeitigen und erfolgreichen Behandlung von bakteriellen Biofilminfektionen auf Kathetern.

In English

Background: Catheter-related blood stream infections and sepsis due to bacterial biofilms are a major cause of morbidity and mortality in intensive care unit patients and cancer patients with long-term use of central venous catheters (CVCs). Bacterial biofilms are complex aggregates of bacteria embedded within a layer of extracellular polymeric substances making them harder to combat using standard antibiotic regimens. Microbiological culture method of colony forming units (CFUs) is routinely used towards the examination of catheter-associated bacterial infections. However, culture of catheter-tips on agar plates fails to provide information on the location and activity of viable but non-culturable cells (VBNCs), warranting the need for a complimentary diagnostic method.

Objectives: The aim of this proof of concept study was to evaluate the use of 16S rRNA directed fluorescence *in situ* hybridization (FISH) in combination with digital image analysis to visualize, localize and quantify the effect of the two antibiotics daptomycin and vancomycin on *Staphylococcus epidermidis* biofilms on central venous catheters. The FISH results were compared to those obtained using the standard culture method of counting CFUs.

Methods: *S. epidermidis* biofilms were grown using an *in vitro* model of CVC infection followed by antibiotic treatment. Subsequently, biofilm area and the fraction of

metabolically active cells was investigated using FISH and microscopy in combination with digital image analysis. FISH data was compared to the standard method of diagnosis of catheter infections namely CFU data. Anti-biofilm activity of medically important antimicrobial compounds daptomycin and vancomycin was compared to phosphate buffered saline controls.

Results: The *in vitro* model produced *S. epidermidis* biofilms on polyurethane catheters and FISH was applied successfully to measure the effect of the antibiotics on the biofilms by digital image analysis. Both daptomycin and vancomycin were able to significantly reduce the area and the FISH-positive fraction of the biofilms. Daptomycin showed a more pronounced anti-biofilm activity in comparison to vancomycin.

Conclusions: This is the first study of its kind to employ FISH in combination with digital image analysis to measure anti-biofilm activity on catheters colonized by *S. epidermidis* biofilms. The results of this proof of concept study strongly support the use of FISH as an important complementary tool to obtain crucial information on the location and activity of bacterial cells within biofilms, not provided by CFU data. The insights provided by FISH are a valuable contribution towards the timely and successful management of bacterial-biofilm infections on catheters.

2. Introduction

Biofilm-associated infections account for an estimated 80 % of all bacterial infections in humans and therefore pose a major challenge to the medical field (Fleming and Rumbaugh, 2017). Indwelling medical devices such as prosthetic joints, ventricular assist devices and other biomedical implants are prone to colonization by bacterial biofilms leading to severe infections (Harris et al., 2017; Margaryan et al., 2020; Toba et al., 2011). Three out of four patients in intensive care units (ICUs) require insertion of intravascular catheters (Timsit et al., 2020). Central-venous catheters (CVCs) are essential for the care and management of critically ill patients in ICUs as well as cancer patients. CVCs can become colonized by bacterial biofilms causing bacteremia and sepsis ultimately leading to higher mortality in patients with long-term catheter use and increased financial burden on the health care system (Blot et al., 2005; Maki et al., 2006). The International Nosocomial Infection Control Consortium (INICC) summarized data from 43 countries

during the period of 2007-2012 and reported a risk of 6.8 catheter related bloodstream infections (CRBSIs) per 1000 central line-days in ICUs (Rosenthal et al., 2014). Apart from the economic aspects, biofilm formation in CVCs has severe consequences for patient health including device failure followed by removal or replacement of the catheter. Detachment of biofilm aggregates from infected medical devices may lead to colonization of new sites as well as systemic infections (Rafii, 2015).

Staphylococcus epidermidis is a Gram-positive, coagulase-negative staphylococcus and is a commensal skin bacterium, constituting a part of the normal flora on skin and mucous membranes in humans and has a protective role in host immunity (Nakatsuji et al., 2018). Nevertheless, it is the most common opportunistic pathogen implicated in life-threatening biofilm infections in patients with indwelling medical devices such as CVCs (Heilmann et al., 2019; Paharik and Horswill, 2016). *S. epidermidis* is responsible for nosocomial CRBSIs following invasive procedures such as insertion of CVCs worldwide (Büttner et al., 2015). Biofilm-formation is the major virulence factor of *S. epidermidis* (Nuryastuti and Krom, 2017; Otto, 2014), especially due to the absence of toxins and other virulence factors (Van Mellaert et al., 2012). The biofilm-forming capability of *S. epidermidis* enables it to evade the host immune response leading to bacteremia (Kleinschmidt et al., 2015). A study comparing two *S. epidermidis* strains, a biofilm-producing and a non-biofilm-producing strain concluded that the biomass of the biofilm attached to the device surface was greater for the biofilm-producer than for the non-biofilm-producer (Fazly Bazzaz et al., 2014). In addition, the same study showed that the biomass of the biofilm increased with prolonged incubation time from 24 hours to 72 hours. Latest publications such as (Büttner et al., 2020) continue to elucidate novel mechanisms by which *S. epidermidis* adheres to implant surfaces causing hard to treat biofilm infections.

Bacterial biofilms are distinct from planktonic i.e. free-floating, single bacterial cells in that the bacterial cells within a biofilm have the same genotype as planktonic cells but a different phenotype with an altered growth rate and transcription in order to switch to a biofilm mode of existence (Branda et al., 2004; Flemming et al., 2016). Biofilm formation on surfaces occurs in a five-step process: state of reversible attachment, sessile state, growth including microcolony formation, maturation and finally dispersal (Magana et al., 2018). Biofilm architecture involves bacterial cells embedded in mushroom-shaped structures separated by water channels and varying oxygen permeability at different

depths of the biofilm giving rise to distinct environments within the same biofilm. The biofilm lifestyle bestows certain advantages upon the individual bacterial cells within the biofilm: access to nutrients, protection from antimicrobials by transfer of antibiotic resistance genes via plasmids and a clear division of labor simulating functions similar to multicellular organisms (Jefferson, 2004; Shapiro, 1998). It has been observed that mutations leading to antibiotic resistance have been observed more frequently in biofilm-forming strains than in planktonic cultures (Nesse and Simm, 2018). Bacterial cells grown as a biofilm are up to thousand-fold more resistant to antibiotic treatment than the planktonically-grown cells of the same organism (Chadha, 2014; Sharma et al., 2019).

Bacterial biofilms consist of a complex community of bacterial cells attached to a living or inert surface while being embedded within a matrix of extracellular polymeric substances (EPS). The EPS matrix, a hydrated 3D network of polysaccharides held together by hydrogen bonds, serves to perform various functions aiding in the biofilm-lifestyle, ranging from adhesion to surfaces, mechanical stability, nutrient uptake and waste product removal (Flemming and Wingender, 2010; Muhammad et al., 2020). Embedded in the EPS matrix, biofilm cells are protected from the host immune system as well as from the action of antibiotics (Khan et al., 2020).

Another unique feature of biofilms is that the bacterial cells communicate with each other by way of chemical signals in the form of homoserine lactones via a cell to cell communication process based on population density known as quorum sensing (Mangwani et al., 2016; Miller and Bassler, 2001). This exchange of chemical molecules enables the cells to get a constant overview of the composition and metabolic status of their neighbors within the biofilm. A recent study demonstrated that a molecule regulated by quorum sensing promotes biofilm formation and antimicrobial tolerance via the production of reactive oxygen species leading to disruption of membrane integrity followed by autolysis (Hazan et al., 2016). Quorum sensing plays a major role in all stages throughout the biofilm lifecycle including formation, toxin production, detachment and establishment of new colonizers as well as initiation of pathogenicity (Köhler et al., 2010). Other resistance mechanisms in biofilms include starvation-induced stringent response (Lebeaux et al., 2014) and extracellular DNA (eDNA) with the ability to chelate to antimicrobials (Penesyan et al., 2015).

A recent review outlined mechanisms of resistance of Gram-positive bacteria to current antibiotic regimens as follows: reduced permeability, induction of efflux pumps transportation of antimicrobials out of the cell, gene mutations eliminating target binding sites, acquired exogenous DNA and plasmid-associated enzymes that either alter or degrade the antibiotic (Jubeh et al., 2020). *S. epidermidis* in particular, resists antibiotic action due to biofilm formation and the surrounding EPS matrix acts as a permeability barrier against penetration of the antimicrobial agent. Another review listed additional factors specific to biofilms such as nutrient and oxygen gradients, altered microenvironment, matrix polysaccharides, stringent response, oxidative stress response, biofilm-specific gene products, horizontal gene transfer of resistance genes by conjugation and persister cells that contribute to biofilm resistance and tolerance (Hall and Mah, 2017; Sharma et al., 2019). These mechanisms work either individually or in synchrony depending on the antimicrobial agent, the species of biofilm and the specific growth conditions and contribute to the recalcitrance of biofilms to antimicrobial therapy thereby making eradication of biofilm infections challenging (Mah and O'Toole, 2001; Stewart, 2015).

Persister cells with the ability to survive antibiotic treatment may cause recurrence of infection in patients with long-term indwelling medical implants (Balaban et al., 2019; Spoering and Lewis, 2001). Balaban et al. (2019) defined persisters as survivor cells representing a sub-population that display antibiotic persistence and have the ability to regrow once the antibiotic is removed. In addition, the authors state that the presence of antibiotic persister cells in biofilm infections is responsible for the lack of clearance of bacteria by antibiotic treatment. This review distinguishes between persistence which is characterized by biphasic killing curve and resistance which is based on higher minimal inhibitory concentrations (MICs). However, the authors emphasize that bimodality may also be due to resistant mutants and therefore the true hallmark of persister cells is their regrowth following prior exposure to an antibiotic.

Biofilm-associated perseverance in the face of antibiotics in *Pseudomonas aeruginosa,* widely considered the model organism for biofilms studies, is attributed to two distinct mechanisms: first, antibiotic tolerance owing to a combination of physical, physiological and genetic factors; and second, antibiotic-resistance owing to mutations occurring after repeated antibiotic exposure (Ciofu and Tolker-Nielsen, 2019). Strategies combatting

both aspects of biofilm recalcitrance are needed in order to make progress towards prevention and treatment of biofilm infections in indwelling medical devices. Recent approaches towards biofilm eradication in CVCs include antibiotic lock technique, compounds targeting the EPS matrix and anti-persister agents (Gominet et al., 2017). New strategies such as bundling of metal-coated devices including CVCs have shown promising results towards improving outcomes for severely ill patients in ICUs (Zampieri et al., 2020).

The choice of strain *S. epidermidis* PIA 8400 for inoculating the *in vitro* catheter model was dictated by its demonstrated ability to form stable, reproducible biofilms. Polysaccharide intercellular adhesin (PIA) is a glycan of beta-1,6-linked 2-acetamido-2-deoxy-D-glucopyranosyl residues and is an essential factor for the virulence of *S. epidermidis* biofilms (Rohde et al., 2010). The production of PIA, also referred to as poly N-acetylglucosamine (PNAG), is mediated by the *icaADBC* operon in staphylococcal biofilms (Lin et al., 2015) (Arciola et al., 2015). Production of PIA plays an important role in adhesion to surfaces and antibiotic tolerance, it has previously been shown that PIA-positive *S. epidermidis* biofilms have a higher tolerance to antibiotics than PIA-negative biofilms (Costa et al., 2009). A study found that biofilms isolated from high-shear environments, for example the catheter lumen in patients, are more likely to produce PIA-dependent biofilms than those from low-shear biofilm infections (Schaeffer et al., 2016). Another recent study showed that PIA synthesis and regulation of the *icaADBC* locus in *S. epidermidis* biofilms is mediated by two transcriptional repressors namely IcaR and TcaR (Hoang et al., 2019). Many studies have investigated biofilm formation and elimination. Nevertheless, there is a lack of standardization and consensus amongst the various methodologies used (Azeredo et al., 2017; Roy et al., 2018). Previous studies examining the efficacy of antimicrobial agents on biofilms have used colony forming units (CFUs) as a measure of anti-biofilm efficacy (Jahanbakhsh et al., 2018; Kirker et al., 2015; Poonacha et al., 2017; Raad et al., 2003; Sherertz et al., 2006; Wiederhold et al., 2005). However, CFU data gives an incomplete picture and fails to acknowledge the role of persister cells in recurring biofilm infections (Lewis, 2007). The results of a retrospective cohort study of CVC tip cultures indicate a steady decline in the use of this practice for diagnosing CRBSIs in hospitals, instead emphasis is laid on the presence of positive blood cultures (Lai et al., 2019).

Vancomycin is a glycopeptide antibiotic and shows bactericidal activity by inhibiting the final stage of cell wall synthesis in Gram-positive bacteria. For empirical therapy of CRBSIs caused by methicillin resistant *Staphylococcus* spp. treatment with vancomycin has been the standard therapy option. There has been an alarming trend towards higher MICs due to emerging resistance to vancomycin in blood stream infections caused by methicillin-resistant coagulase-negative staphylococci (CoNS) (Asadpour and Ghazanfari, 2019; Lubin et al., 2011). In cases of MICs of vancomycin exceeding 2 µg/ml in staphylococcal biofilm infections, drug therapy with daptomycin has been recommended (da Costa et al., 2020; LaPlante and Mermel, 2009).

Daptomycin is a cyclic lipopeptide antibiotic that works by disrupting the cell membrane leading to the shutdown of nucleic acid and protein synthesis in Gram-positive bacteria. Daptomycin is the recommended treatment option for *S. aureus* infections of blood stream and heart valves (Fowler et al., 2006). Since its introduction to the market in 2006, daptomycin has been the subject of several studies comparing its efficacy to other available antibiotics (Chaftari et al., 2010; Weiss et al., 2009). Daptomycin is the last-line of anti-staphylococcal antibiotics available currently (Jiang et al., 2019).

The choice of the two antibiotics to test the herein established *in vitro* biofilm model incorporates vancomycin representing the first-line of therapy and daptomycin as an alternative medical intervention strategy in cases of staphylococcal catheter infections. A study comparing the effects of antibiotics on cell viability within CoNS biofilms found that daptomycin showed good penetration and potent bactericidal activity while vancomycin showed poor penetration and no bactericidal activity on the biofilms (Ozturk et al., 2016). A retrospective study of patients with CRBSI due to infected long-term CVCs where previous therapy with vancomycin had failed, reported successful eradication of the CoNS infection with daptomycin lock therapy (Tatarelli et al., 2015). Another study reported the administration of daptomycin following treatment failure of first-line glycopeptides in cases of Gram-positive bacterial infections of prosthetic devices and came to the conclusion that daptomycin was an effective treatment option and was well-tolerated by patients (Seaton et al., 2013). Despite increasing reports of emerging daptomycin-resistance, a recent publication conducted a meta-analysis of the prevalence of antibiotic resistance in 25 countries and reported that the incidence of daptomycin-resistant CoNS strains is relatively low (0.3 %) worldwide (Shariati et al., 2020).

Fluorescence *in situ* hybridization (FISH) is a culture-independent, molecular technique that allows visualization and identification of bacteria at a single cell level (Aistleitner et al., 2018; Eichinger et al., 2019; Gescher et al., 2008; Moter and Göbel, 2000; Wecke et al., 2000). The positive FISH signal correlates to ribosomal activity within the cell and is therefore an indirect indicator of the metabolic state of the bacterial cells. Moreover, the FISH technique enables the study of spatial distribution of cells within biofilms (Bisht and Wakeman, 2019). FISH technique is based on the binding of fluorescence-labelled oligonucleotide probes to bacterial ribosomes enabling the rapid and accurate identification of pathogens by microscopy (Frickmann et al., 2017).

There exists a knowledge gap regarding the precise effect of antibiotics on individual cells within mature *S. epidermidis* biofilms on catheters. The main objective of this proof of concept study was to test if FISH is an appropriate technique to measure the efficacy of antibiotics on *in vitro* grown *S. epidermidis* biofilms, simulating infections in patients with long-term placement of central venous catheters.

To this end, the present study was designed with the following four objectives: the first goal was to establish an *in vitro* model as a proof of concept simulating catheter infection using the polysaccharide intercellular adhesin (PIA)-positive *S. epidermidis* strain 8400 on polyurethane catheters followed by antibiotic treatment. The second goal involved a novel approach combining FISH and digital image analysis to visualize, precisely locate and quantify the activity of antibiotics on *S. epidermidis* biofilms formed using the *in vitro* model of catheter infection. In order to verify the feasibility of this new approach, the antibiotics vancomycin and daptomycin, which represent the standard medical treatment and an alternative therapy option, respectively, were chosen to be tested preliminarily. Using a fluorescence microscope, images of vancomycin- and daptomycin-treated catheters as well as phosphate buffered saline (PBS) controls were to be obtained. The digital image analysis program *daime* was to be employed to analyse the FISH images. The following two variables of antibiotic efficacy were to be measured using *daime*: total biofilm area (μm^2) and fraction of FISH-positive biofilm cells (%) followed by comparison between the test groups of vancomycin, daptomycin and PBS-controls. The third goal was to compare the FISH results obtained using this novel approach to the standard method used in routine diagnostics namely CFU data. The location and distribution of the FISH-positive cells within the biofilms for the PBS controls and antibiotic treated catheters

were to be compared. This was to be achieved by evaluating the statistical significance between test groups using the FISH method and compare these to CFU analysis. The statistical output was then to be interpreted to draw conclusions regarding the effect of antibiotic therapy in comparison to PBS controls on the biofilms using the two compared methods.

Striving towards understanding biofilm infections, how they develop and how best to prevent and treat them, is a topic of major interest for several disciplines. This study is a multi-disciplinary approach leaning on several scientific areas including microbiology, molecular biology, biofluid mechanics and microbial ecology. The resulting publication (Sutrave et al., 2019) of this study in the multidisciplinary journal PLOS ONE continues to ensure that these findings reach a wide audience including clinicians, healthcare workers and scientists across many subject areas.

3. Materials and Methods

3.1. *In Vitro* Model for Biofilm Formation on Catheters

Biofilms of the *Staphylococcus epidermidis* strain 8400 producing the polysaccharide intercellular adhesin (PIA) were grown in 50 ml-scale biofilm reactors. The PIA strain has been well characterized and proven to form reproducible biofilms with PIA-production being the first factor identified to be involved in biofilm formation in *S. epidermidis* (Mack et al., 1994). PIA-producing homologues of *S. epidermidis* have been shown to be better equipped at evading the human innate immune response than their counterparts which are lacking this adhesin in their EPS matrix (Vuong et al., 2004).

A 10 % tryptic soy broth (TSB) was inoculated with the clinical isolate *S. epidermidis* strain 8400, an optical density (600 nm) of 0.3 of the resulting suspension was achieved and the suspension was incubated with the catheters at 37 °C for 7 days. The medium was replaced with fresh 100 % TSB medium supplemented with 25 % glucose every 24 hours. The catheters remained immersed in the medium throughout the experiment while being continuously agitated. During this time, a biofilm formed in the lumen and on the outside of the polyurethane catheters.

3.2. Antimicrobial Treatment

The concentrations of the two antibiotics used were 160 µg/ml for daptomycin (Novartis Pharma AG, Basel, Switzerland) and 100 µg/ml for vancomycin (Sigma-Aldrich Chemie GmbH, Munich, Germany) based on peak plasma concentrations in humans (Benvenuto et al., 2006; Pai et al., 2017). The TSB medium was replaced with Mueller-Hinton medium supplemented with calcium along with daptomycin or vancomycin at the above concentrations, and pumped (50 µl/min) through the catheters into the bioreactor, owing to the calcium-dependent mode of action of daptomycin (John et al., 2011). The control catheters were treated accordingly with medium supplemented with calcium and phosphate buffered saline (PBS). The catheters remained for 24 hours at 37 °C in the antibiotic and control solutions while being continuously stirred ensuring a homogeneous mixture of antibiotics throughout the liquid medium. The catheters were then harvested and cut into two halves of 1 cm each: one half of each catheter was used for CFU analysis and the other half for FISH analysis.

3.3. CFU Measurement

The 1 cm section of the catheter set aside for CFU analysis was transferred to 1 ml PBS (pH 7.4) and vortexed for 1 minute in order to homogenize the biofilm. Serial dilution was carried out, 100 µl aliquots were plated on Muller-Hinton agar and the plates were incubated for 48 hours at 37 °C. The resulting colonies were then counted and final counts were calculated taking the dilution factor into account.

3.4. Fixation and Embedding

The second half (1 cm section) of each catheter was prepared for FISH by carefully placing the catheter in 3.7 % (w/v) paraformaldehyde in PBS containing 50 % (v/v) ethanol at 4 °C overnight and washed in 6 % buffered sucrose solution for 24 hours at 4 °C. The catheter sections were then dehydrated in 50 %, 70 %, 96 % and 100 % (v/v) ethanol in progressive steps of 2 minutes and a final step of 30 minutes. Each 1 cm catheter section was cut into four pieces; the pieces were embedded upright in cold polymerizing resin Technovit 8100 (Kulzer, Wehrheim, Germany) as published previously (Moter et al., 1998). With the aid of a microtome, 2 µm thick sections of the catheter pieces were cut into 8 cross-sections with a total of 32 cross-sections per catheter. The sections were

placed on glass slides pre-treated with a coating agent poly-L-lysine (Thermo Fisher Scientific Inc., Waltham, MA) for fixation and then air-dried.

3.5. Oligonucleotide Probes and FISH

Sample preparation involved an enzymatic degradation step with lysozyme for 10 minutes followed by lysostaphin for 5 minutes carried out at 30 °C as previously published (Gescher et al., 2008). Internal controls for every experiment included slides of ethanol-fixed bacterial smears of *Escherichia coli*, *Staphylococcus aureus* and *Streptococcus pyogenes* and subjected to enzymatic degradation following the same procedure as for the catheter sections.

As published previously, a hybridization buffer mix containing the nucleic acid stain 4′,6-diamidino-2-phenylindole (DAPI) and the oligonucleotide probes EUB338 (pan-bacterial probe), STAPHY specific for *Staphylococcus* spp., and non-EUB338 was applied to the sections followed by washing and mounting (Amann et al., 1990; Wallner et al., 1993). EUB338 and STAPHY were labelled at the 5' end with the fluorescent indocarbocyanine dye Cy3; non-EUB338 was labelled with Cy5. Non-specific probe binding was ruled out by ensuring there was no fluorescence signal for non-EUB338 with the Cy5 filter.

3.6. Epifluorescence Microscopy

Epifluorescence microscopy images were obtained with the help of AxioCam MRm (Zeiss) using the AxioVision 4.6 software as previously published (Schillinger et al., 2012). The experiments were carried out in triplicate, making 47 catheters in total: control (n = 24), vancomycin (n = 11) and daptomycin (n = 12). One vancomycin-treated catheter was deemed unusable during processing. Two FISH images were taken per cross-section using the epifluorescence microscope. If no biofilm was seen, an area value of zero was assigned for that particular image. For each catheter 100x magnification images of 32 cross-sections at different planes were taken, each with two images of the outer surface of the catheter, resulting in a total of 64 images per catheter. The images thus obtained were statistically evaluated: control (n = 1536), vancomycin (n = 704) and daptomycin (n = 768).

3.7. Digital Image Analysis

For the purpose of this study, quantification of biofilm area and percentage of FISH-positive cells was achieved using the Adobe After Effects 5.5 software and the program *daime: digital image analysis in microbial ecology* (Daims et al., 2006). The DAPI and Cy3 grayscale images were transformed into binary images using the luminance threshold setting option of After Effects and exported as previously published (Schillinger et al., 2012). The images were then segmented using *daime* and artefacts were removed where necessary. The *daime* program was employed to calculate the biofilm areas of both the Cy3 and the DAPI channels with the DAPI area set as mask for the Cy3 layer. The total biofilm area per catheter (DAPI) and the percentage of the FISH-positive fraction (Cy3) were thus calculated.

3.8. Statistical Analysis

The data obtained were analyzed using the statistical package SPSS V.19 (IBM, USA). Significance was assumed at $p \leq 0.05$ for all statistical tests. The Kolmogorov-Smirnov test was performed to assess deviations from a normal distribution. Group differences were assessed by paired Student's t-test in the case of normally distributed data; alternatively, the Mann-Whitney U test was used in combination with Levene's test to prove the equality of variances.

4. Results

4.1. *In Vitro* Catheter Biofilm Model

After 7 days of growth, the *in vitro* model produced thick *S. epidermidis* PIA biofilms on the surface of the catheters visible to the naked eye (Figure 1). There was variation in the amount of biofilm on the polyurethane catheters, with a thin layer of biofilm cells in the lumen of the catheter and more developed biofilms showing characteristic mushroom-shaped structures on the outer surface of the catheters. The *in vitro* biofilm model was robust and enabled uncomplicated medium exchange and handling of the biofilms during antibiotic treatment. The set-up remained free of contamination throughout the duration of the experiments.

Figure 1: Image showing macroscopic *S. epidermidis* biofilms on the surface of polyurethane catheters in an *in vitro* model of catheter infection

4.2. FISH and Digital Image Analysis

The FISH technique in conjunction with digital image analysis with *daime* provided promising results leading to the visualization, localization and quantification of the FISH-positive cells following antimicrobial application. FISH was applied successfully to calculate the area and the percentage of FISH-positive cells after antibiotic treatment. The PBS treated control biofilms showed a consistent distribution of the FISH-positive cells going from the periphery to the deeper areas of the biofilms; the FISH-positive cells were clustered towards the outer edges (younger cells) of the biofilm with fewer FISH-positive cells scattered within the inner layers (older cells). Control biofilms and those treated with vancomycin showed a similar distribution of FISH-positive cells with a higher density of these cells observed towards the outer edges of the biofilm. Biofilms treated with daptomycin showed single FISH-positive cells spread throughout the biofilm.

For the first biofilm parameter i.e. area, both antibiotics significantly reduced the total biofilm area in comparison to the controls ($p \leq 0.05$). Daptomycin showed a greater reduction in total biofilm area than vancomycin, however, this result was not significant at

the p ≤ 0.05 level. For the second biofilm parameter i.e. percentage of the FISH-positive fraction, both antibiotics reduced the fraction of FISH-positive cells significantly in comparison to the controls (p ≤ 0.05). The percentages of FISH-positive fractions after treatment with PBS (control) and antibiotics were 56 % for the control, 28 % for vancomycin and 12 % for daptomycin. These inter-group differences were all statistically significant at the p ≤ 0.05 level.

4.3. FISH vs. CFU Data

The calculated Log_{10} CFU/ml values were 12.15 ± 0.82 for control-biofilms, 4.91 ± 0.28 for vancomycin-treated biofilms and 2.49 ± 0.57 for daptomycin-treated biofilms (Figure 2). The inter-group differences between controls, vancomycin and daptomycin were all statistically significant at the p ≤ 0.01 level. Neither vancomycin nor daptomycin showed 100 % bactericidal activity. The CFU results correlate to the FISH data, where remaining FISH-positive cells were observed following antibiotic treatment for both antibiotics.

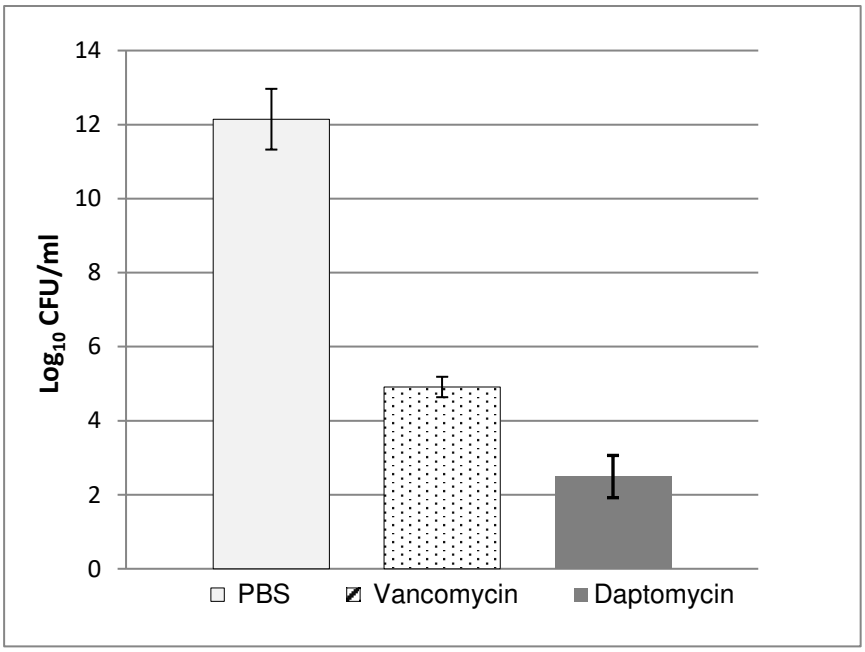

Figure 2: Log_{10} CFU/ml values for catheters treated with PBS, vancomycin and daptomycin

5. Discussion

Not all bacterial biofilms are harmful to humans, for example, biofilms help to maintain the normal microbiota of skin and gastrointestinal tract by providing protection against harmful microorganisms and aiding in digestion. However, certain bacterial biofilms have been implicated in various ailments and chronic diseases (Costerton et al., 1999) such

as periodontitis (Schlafer et al., 2010), cystic fibrosis (Pawar et al., 2015; Pustelny et al., 2015), otitis media in children (Vermee et al., 2019), bacterial endocarditis (Tascini et al., 2013); and chronic foot ulcers in diabetic patients (Kim et al., 2020). While chronic wounds represent a classic example of mixed infection where multiple species of aerobic and anaerobic bacteria exist within a single biofilm (James et al., 2008; Murphy et al., 2020), the majority of indwelling device-related infections involve mono-species biofilms. With increasing lifespans and improved access to medical interventions globally, the need for indwelling medical devices such as CVCs will continue to rise in the long-term.

The present study was able to achieve its three main objectives successfully and provided the following insights: firstly, the feasibility of an *in vitro* model for growing PIA-positive *S. epidermidis* biofilms on catheters followed by antibiotic treatment was shown. Secondly, the FISH technique was able to pinpoint the location of metabolically active biofilm cells after antibiotic therapy. The FISH method showed distinct differences in the distribution of FISH-positive cells amongst control biofilm and those treated with vancomycin and daptomycin. Following antibiotic treatment there was a reduction in biofilm area. Lastly, the CFU data was directly correlated to the FISH method. Both methods showed daptomycin had higher potency than vancomycin against biofilm cells. *S. epidermidi*s PIA 8400 provided an ideal basis for the establishment of this proof of concept model and aided in obtaining statistically sound data. However, further validation of the system would involve testing a broad range of *S. epidermidis* strains as well as other relevant staphylococcal species. While *P. aeruginosa* biofilms have been most widely used as prototypes for studying resistance and tolerance mechanisms, so far the number of studies focusing on other biofilm-building pathogenic bacteria is limited.

The *S. epidermidis* PIA 8400 strain used to inoculate the *in vitro* catheter model proved to be an ideal biofilm-builder forming dense biofilms visible to the naked eye on the outer surface of the catheters. A recent study used the same *S. epidermidis* strain to establish an *in vitro* model for biofilms on porcine heart valves simulating infective endocarditis in patients (Lauten et al., 2020). Similar to the PBS-treated control biofilms, a majority of the FISH-positive cells in the biofilms treated with vancomycin were clustered on the edges of the biofilms. In contrast, the FISH-positive cells in those biofilms treated with daptomycin were fewer and scattered throughout the biofilms. The digital image analysis program *daime* was implemented successfully to measure the anti-biofilm efficacy for the

two antibiotics tested by enabling the calculation of the biofilm area and the FISH-positive fraction of the biofilm cells. A recent study introduced a multicolour FISH technique also using the *daime* program to enable the simultaneous detection of several microorganisms in mixed cultures (Lukumbuzya et al., 2019).

FISH provided insights into the distinct differences in the architecture and location of the remaining FISH-positive cells between the two antibiotics, likely owing to their disparate modes of antimicrobial activity. The antimicrobial action of the glycopeptide vancomycin involves inhibition of cell wall synthesis by preventing transpeptidation of peptidoglycan subunits whereas the lipopeptide daptomycin works by disrupting the cell membrane by depolarization and shutting down synthesis of nucleic acids and proteins. These differences in the modes of action of the two antibiotics may account for the differences in the distribution of the remaining FISH-positive cells following antibiotic treatment. A possible explanation for the higher efficacy of daptomycin in eradicating biofilm cells may be its ability to act on slow-growing or non-growing cells within the biofilm. Daptomycin together with calcium and anionic phospholipid phosphatidylglycerol (PG) forms complexes or micelles that play a crucial role in cell membrane insertion followed by oligomerization, translocation across the cell membrane and subsequent membrane disruption due to lipid extraction and ion leakage (Miller et al., 2016).

The two antibiotics tested here have previously been examined in other *in vitro* studies where FISH was employed to investigate the anti-biofilm activity of daptomycin- and vancomycin-loaded microparticles in staphylococcal biofilms (Bettencourt et al., 2015; Santos Ferreira et al., 2018). Similar to the present study, both these studies also found that daptomycin showed a greater anti-biofilm effect when compared to vancomycin. In the present study, statistical analysis showed that while both antibiotics reduced the percentage of FISH-positive cells in comparison to PBS controls, daptomycin was significantly more efficient than vancomycin for this parameter. However, for the other parameter of total biofilm mass, the difference in the effect of the two antibiotics was not statistically significant at $p \leq 0.05$ level. Administration of high-dose daptomycin (>6 mg/kg/day) may be a therapy option in order eradicate the remaining FISH-positive cells within the biofilm after antibiotic treatment. Owing to its broad range of activity and ease of administration, doses of daptomycin up to 12mg/kg/day have been shown to be safe and effective in treating severe infections (Hamed et al., 2016).

Previous studies have used fluorescent labelling to measure the rate of penetration of antibiotics through dense staphylococcal biofilms and reported that vancomycin showed partial permeation of the biofilms (Jefferson et al., 2005) while daptomycin penetrated thick biofilms within minutes (Stewart et al., 2009). In keeping with these studies, FISH-positive cells were observed on the edges of the vancomycin-treated biofilms, in contrast to those scattered singly within the deeper layers of the daptomycin-treated biofilms. Both FISH analysis and CFU results were in agreement and showed that neither vancomycin nor daptomycin achieved complete eradication of the biofilm cells following antibiotic treatment. The remaining metabolically active, FISH-positive cells following daptomycin treatment that were observed scattered singly within the biofilms may indicate persister cells that are refractory to antibiotic therapy and thereby contribute to biofilm recalcitrance. The cells towards the core of the biofilm are in hypoxic zones that are characterized by nutrient and oxygen starvation, lower rate of metabolism and down-regulation of biosynthesis functions as compared to the cells at the exterior layers of the biofilms (Dincer et al., 2020; Stewart et al., 2016). Persister cells are reservoirs of cells surviving antibiotic treatment and have the ability to regrow and cause infection relapse. In order to verify if the remaining FISH-positive cells after daptomycin treatment are indeed persisters, the survival of these cells and their regrowth under the same conditions needs to be tested. In accordance with Balaban et al. (2019), the new population of cells arising from these persisters after removal of antibiotic would then show renewed susceptibility to the drug.

By reducing the time to obtain results, FISH has vast potential as a valuable alternative molecular technique to conventional microbial culture as it provides information on the spatial distribution of metabolically active cells within biofilms (Cattò and Cappitelli, 2019; Frickmann et al., 2017). FISH results correlated to CFU data showing the same trend of daptomycin being more effective than vancomycin at reducing the percentage of biofilm cells. When compared to PBS controls, daptomycin demonstrated higher efficacy than vancomycin in reducing the total biofilm area as well as the percentage of FISH-positive cells of the *in vitro S. epidermidis* biofilms. However, neither antibiotic achieved 100 % eradication and FISH-positive cells were observed after antibiotic treatment. This result supports the standard clinical practice of removing the infected catheter to prevent recurrence of infection.

In agreement with the 3R principles: Replacement, Reduction and Refinement in the use of laboratory animals for research, the present *in vitro* model of catheter infection is the first step in the establishment of the system before moving towards *in vivo* studies for testing antimicrobial efficacy (Törnqvist et al., 2014). As a further step in validating the results of this *in vitro* study, an *in vivo* rat model of CVC infection (unpublished data) by using the same following variables: catheter specifications, the PIA-positive *S. epidermidis* 8400 strain and the two antibiotics vancomycin and daptomycin has been established. The optimized rat model is an improvement over previously described models (Andes et al., 2004; Rupp et al., 1999) due to its unique subcutaneous catheter fixation. The yet to be published results of this *in vivo* study show a significantly higher reduction of FISH-positive cells in biofilms that were treated with daptomycin as compared to vancomycin and PBS controls. Hence, the results of the *in vivo* model are in line with the results of the *in vitro* study described here. However, major limitations of the *in vivo* study are the high standard deviations of the FISH results. In order to achieve lowering standard deviations and thereby improve the statistical analysis a large number of animals per test group would need to be analyzed.

Several promising new antibiofilm strategies against staphylococcal biofilms have been proposed in recent years including polymeric coatings with the ability to selectively release antibiotics in response to adhering bacteria (Albright et al., 2017), PIA/PNAG as potential vaccine candidate (Arciola et al., 2015) inhibition of quorum sensing known as quorum quenching (QQ) (Rampioni et al., 2014) and 3D bioprinting of biofilm constructs to study antibiotic tolerance (Ning et al., 2019). Another approach involves repurposing known drugs to be used in new therapeutic areas. A recent study highlighted the use of the repurposed drug eltrombopag (thrombopoietin receptor agonist) in combination with vancomycin as a novel treatment avenue showing promising antimicrobial activity against *S. epidermidis* biofilms and their persister cells (Zhu et al., 2021). Detailed studies involving combinations of these treatment strategies are needed to gain further insights into their effect on biofilms.

Bacteria have been able to develop resistance against every antibiotic agent, in some cases even prior to their availability in the market, however, this development of resistance to an available drug does not necessarily indicate failure of the drug (Kupferschmidt, 2016). Nevertheless, it is important to be vigilant in prescribing and using

antibiotics. Keeping in mind the high disease burden on patient health as well as the economic repercussions resulting from the rise of antimicrobial resistance, there is an urgent need for education amongst health care professionals and the general public regarding the repercussions of improper use of antibiotics. The antibiotic stewardship programs introduced in health care facilities (May et al., 2020; Trivedi et al., 2020) are a step in the right direction as they provide much needed guidelines for healthcare professionals working towards improving outcomes for patients while promoting accountability in the use of antimicrobials.

The present study is the first of its kind to spatially visualize and quantify the activity of two antibiotics in an *in vitro* model developed using *S. epidermidis* biofilms grown on catheters simulating life-threatening infections in patients with the need for long-term placement of central venous catheters. These results have clinical implications towards the ongoing health crisis of multidrug resistance facing humanity. While this simple *in vitro* model of catheter infection provides new insights into the effect of antibiotics on biofilms on a small scale, the major challenge remains in adapting the lessons learned here for enabling translation into real-world conditions for patients with indwelling-device infections. Given the complexity of biofilm-associated infections, the interplay of diverse mechanisms involved in antibiotic recalcitrance and the predicted rise in the use of indwelling medical devices in the future, continued research efforts from a multidisciplinary perspective are necessary to tackle the challenges of preventing and treating biofilm infections in the clinical setting.

6. Limitations

Owing to the proof of concept nature of the study investigated here, the scope of the experimental design has certain inherent limitations. Since the goal of the experiments was to test the feasibility of establishing an *in vitro* biofilm model and to measure the effect of antibiotics using a novel methodology of 16S rRNA directed FISH probes in combination with a digital image analysis program, as a starting point a single established biofilm-building *S. epidermidis* strain (PIA 8400) was tested along with two antibiotics: (1) the standard antibiotic regimen vancomycin and (2) daptomycin with known anti-biofilm potential. Under these preliminary test conditions, the results demonstrated the

practicability of the use of FISH in combination with digital image analysis for efficacy testing of two antibiotics in an established *in vitro* biofilm model of catheter infection.

It would be worthwhile to test several other *S. epidermidis* strains as well as other bacterial species causing catheter infections using the *in vitro* biofilm system described here in order to evaluate the genetic variability of these strains. A recent publication constructed a phylogenetic tree of 415 *S. epidermidis* isolates and identified 61 genes associated with known pathogenicity traits including biofilm formation (Méric et al., 2018). Given the vast range of pathogenic *S. epidermidis* isolates, a medically-relevant, well-established, biofilm-building strain PIA 8400 was used as the basis for this study.

Additionally, the evaluation of other antibiotics currently used in clinical practice would give a more representative picture of the effect of antimicrobials on catheter biofilms. The present model can be used for efficacy testing of other antibiofilm compounds such as rifampicin and β-lactam antibiotics. However, it is important to note that rifampicin when used alone has been shown to cause rapid development of resistance and therefore it has been suggested that rifampicin be used only in combination with other antibiotics (El Haj et al., 2018; Zimmerli and Sendi, 2018). In this class of antibiotics, resistance has been known to occur due to inactivation of the antibiotic agent by the activity of β-lactamases. According to a recent publication (Ciofu and Tolker-Nielsen, 2019), β-lactams are ineffective against *P. aeruginosa* biofilms. Owing to their mode of action targeting synthesis of peptidoglycans, β-lactams are effective against actively dividing cells and not the slow-growing cells typically found biofilms. Slow growth of bacteria within biofilms is the primary mechanism of tolerance to β-lactam antibiotics. Apart from strategies such as new antibiotic combinations (Jagadale et al., 2019; Jahanbakhsh et al., 2020) and antimicrobial peptides such as β-lactam inhibitors that lower resistance to β-lactam antibiotics (Ferrer-Espada et al., 2020), another approach involves employing antibiotic adjuvants, which are non-toxic compounds that enhance the action of β-lactams antibiotics (Gillard et al., 2018; Idowu et al., 2020).

In order to simulate catheter infection in patients, biofilms were cultivated for 7 days where typical biofilm architecture showing mature multi-layered biofilms was observed by microscopy on the surface of the catheters. Reducing the incubation time for biofilm growth to few hours instead of several days as in the present study would greatly speed up the experimental time and expedite results. However, this would also affect the

maturity of the biofilms and consequently the FISH analysis, especially in light of a study showing that older, more developed biofilms are less susceptible to antibiotics than younger ones (Stewart, 2015). Other biofilm disinfection studies have been carried out using much shorter culture times between 24–48 hours (Díaz-Ruíz et al., 2018; Ravn et al., 2018; Santos Ferreira et al., 2018). Since the purpose of the model developed here was to simulate infections in patients with long-term placement of catheters, the incubation time of 7 days was selected to obtain optimal, mature biofilms showing multi-layered architecture. Furthermore, longer incubation times may potentially contribute to an increase in the number persister cells within biofilms thereby closely simulating conditions in chronic biofilm infections.

FISH is not a stand-alone technique owing to its long turnaround time per experiment, need for specific laboratory equipment, user-expertise in epifluorescence microscopy and the employment of digital image analysis software. However, the major advantage of FISH is the unequivocal identification, spatial distribution of the metabolically active cells and their quantification within the biofilm following antibiotic treatment. In spite of the limitations detailed here, the established *in vitro* catheter biofilm model in conjunction with FISH and digital image analysis has the potential for investigating further avenues of research including additional bacterial strains and a wider palette of promising antibiofilm agents and combination therapies in the future. In addition, the large number of FISH images ($n > 3000$) analyzed here are time-consuming and labour-intensive aspects of this study. Lowering the number of images obtained by microscopy would reduce the time spent in generating and analyzing FISH images, however, it would also shrink the dataset and have an influence on the statistical analysis by increasing the standard deviations.

Another drawback of the *in vitro* model presented here is the lack of host environmental factors including the host immune response. The *in vivo* model of catheter infection in rats (to be published) aims to overcome this limitation by providing the missing information on the behaviour of biofilms within the host. Nevertheless, this *in vitro* study is the first step in examining the effect of antibiotics on biofilms ultimately giving insights into biofilm infections in catheterized patients.

7. Conclusions

The proof of concept study described here was successful in achieving all three of its goals: firstly, the establishment of an *in vitro* model for growing mature *S. epidermidis* biofilms on catheters by simulating the patient situation. Secondly, FISH was demonstrated to be a valuable tool for measuring anti-biofilm activity *in vitro.* FISH offered the following valuable information: spatial resolution, quantification of biofilm area and the FISH-positive fraction as well as localization of the remaining FISH-positive cells following antibiotic therapy. Thirdly, the comparative efficacy of vancomycin and daptomycin against PIA-positive *S. epidermidis* biofilms formed within the *in vitro* system was directly correlated to the routine culture method of CFU counts.

This study is the first of its kind to use a novel approach of combining FISH with digital image analysis to gain insights into antibiotic activity in biofilms. The results of this approach provide a vital contribution towards understanding and treating biofilm infections in central venous catheters. FISH is an important complementary technique to standard culture results as it fills in the knowledge gaps on the location and ribosomal activity of cells within *S. epidermidis* biofilms on catheters following administration of the antibiotic regimens. The *in vitro* model established here can henceforth be used to test other biofilm-building bacterial strains implicated in colonization of CVC catheters in patients with the need for long-term catherization as in the case of cancer patients and those in ICUs. Further investigations using FISH and digital image analysis are needed to study antibiotic recalcitrance mechanisms, thereby improving clinical outcomes for patients.

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9. Statutory Declaration

"I, Smita Sutrave, by personally signing this document in lieu of an oath, hereby affirm that I prepared the submitted dissertation on the topic **Analysis of Medical Biofilms with Emphasis on Indwelling Device-Related Infections Using Fluorescence *In Situ* Hybridization (FISH)** independently and without the support of third parties, and that I used no other sources and aids than those stated.

All parts which are based on the publications or presentations of other authors, either in letter or in spirit, are specified as such in accordance with the citing guidelines. The sections on methodology (in particular regarding practical work, laboratory regulations, statistical processing) and results (in particular regarding figures, charts and tables) are exclusively my responsibility.

Furthermore, I declare that I have correctly marked all of the data, the analyses, and the conclusions generated from data obtained in collaboration with other persons, and that I have correctly marked my own contribution and the contributions of other persons (cf. declaration of contribution). I have correctly marked all texts or parts of texts that were generated in collaboration with other persons.

My contributions to any publications to this dissertation correspond to those stated in the below joint declaration made together with the supervisor. All publications created within the scope of the dissertation comply with the guidelines of the ICMJE (International Committee of Medical Journal Editors; www.icmje.org) on authorship. In addition, I declare that I shall comply with the regulations of Charité – Universitätsmedizin Berlin on ensuring good scientific practice.

I declare that I have not yet submitted this dissertation in identical or similar form to another Faculty.

The significance of this statutory declaration and the consequences of a false statutory declaration under criminal law (Sections 156, 161 of the German Criminal Code) are known to me."

Date Signature

Declaration of Own Contributions

I, Smita Sutrave, contributed the following to the below listed publication:

Sutrave, S., Kikhney, J., Schmidt, J., Petrich, A., Wiessner, A., Kursawe, L., Gebhardt, M., Kertzscher, U., Gabel, G., Goubergrits, L., Affeld, K., Moter, A., 2019. Effect of daptomycin and vancomycin on *Staphylococcus epidermidis* biofilms: An *in vitro* assessment using fluorescence *in situ* hybridization. PLOS ONE 14, e0221786. https://doi.org/10.1371/journal.pone.0221786

Contribution (in detail):

Investigation: application of the FISH technique to catheter sections embedded in cold polymerizing resin along with controls, epifluorescence microscopy and documentation of catheters showing biofilm using 10x objective FISH images (n = 3008), microscopy of positive and negative controls, microscopy of representative biofilm areas using for 100x objective for daptomycin- and vancomycin-treated biofilms as well as PBS control biofilms, naming and storage of the TIFF images generated.

Methodology: digital image analysis and generation of biofilm parameters: area and FISH positive fraction data using *daime* software, preparation of Excel spread sheets with raw data on total biofilm area and FISH positive fraction.

Formal Analysis: statistical analysis via numerous consultations and exchanges with the statistician (co-author listed in the publication), statistically significant differences between test groups, generation of bar graphs with standard deviations and p-values and interpretation of the statistical output for the manuscript.

Writing – Original Draft: review of literature, management of references using citation manager, detailed description of materials, methods and equipment used along with manufacturers' details, description of the results obtained, representation of results in the form bar graphs, figure legends, image presentation including labelling and scale bars, discussion of current results in comparison to other similar studies, limitations of current study and further avenues of research in the context of device-related biofilm studies.

Writing – Review and Editing: incorporation of valid suggestions of co-authors, formulating a response to reviewers, manuscript editing based on reviewer comments, recombining bar graphs and images into representative composite figures.

Date Signature

10. Extract from the Journal Summary List

ISI Web of Knowledge[SM]

Journal Data Filtered By: **Selected JCR Year: 2017** Selected Editions: SCIE,SSCI
Selected Categories: **"MULTIDISCIPLINARY SCIENCES"** Selected Category Scheme: WoS
Gesamtanzahl: 64 Journale

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
1	NATURE	710,766	41.577	1.355810
2	SCIENCE	645,132	41.058	1.127160
3	Nature Communications	178,348	12.353	0.926560
4	Science Advances	10,194	11.511	0.057080
5	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA	637,268	9.504	1.108220
6	National Science Review	952	9.408	0.004340
7	GigaScience	1,694	7.267	0.011030
8	Scientific Data	1,567	5.305	0.008550
9	Journal of Advanced Research	1,843	4.327	0.003820
10	Annals of the New York Academy of Sciences	46,160	4.277	0.033270
11	Science Bulletin	1,952	4.136	0.005900
12	Scientific Reports	192,841	4.122	0.718960
13	Journal of the Royal Society Interface	11,357	3.355	0.030960
14	Research Synthesis Methods	1,374	3.218	0.006030
15	PLoS One	582,877	2.766	1.862350
16	PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY A-MATHEMATICAL PHYSICAL AND ENGINEERING SCIENCES	17,807	2.746	0.028220
17	Royal Society Open Science	2,145	2.504	0.009260
18	PROCEEDINGS OF THE ROYAL SOCIETY A-MATHEMATICAL PHYSICAL AND ENGINEERING SCIENCES	17,157	2.410	0.018270
19	PeerJ	7,377	2.118	0.031600
20	NPJ Microgravity	94	2.000	0.000350
21	SCIENCE AND ENGINEERING ETHICS	1,496	1.859	0.002520
22	COMPLEXITY	1,369	1.829	0.002380
23	Science of Nature	324	1.789	0.001260

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
24	PROCEEDINGS OF THE JAPAN ACADEMY SERIES B-PHYSICAL AND BIOLOGICAL SCIENCES	1,355	1.771	0.001950
25	Proceedings of the Romanian Academy Series A-Mathematics Physics Technical Sciences Information Science	375	1.752	0.000940
26	FRACTALS-COMPLEX GEOMETRY PATTERNS AND SCALING IN NATURE AND SOCIETY	1,077	1.629	0.000870
27	SCIENTIFIC AMERICAN	6,410	1.579	0.003880
28	INTERNATIONAL JOURNAL OF BIFURCATION AND CHAOS	6,094	1.501	0.007220
29	Symmetry-Basel	777	1.256	0.001630
30	SOUTH AFRICAN JOURNAL OF SCIENCE	2,332	1.191	0.001950
31	Jove-Journal of Visualized Experiments	10,616	1.184	0.034680
32	JOURNAL OF THE INDIAN INSTITUTE OF SCIENCE	375	1.151	0.000640
33	JOURNAL OF THE ROYAL SOCIETY OF NEW ZEALAND	682	1.147	0.000380
34	Mathematical Modelling of Natural Phenomena	627	1.101	0.002110
35	SCIENCE PROGRESS	458	1.098	0.000410
36	ARABIAN JOURNAL FOR SCIENCE AND ENGINEERING	2,678	1.092	0.005250
37	ISSUES IN SCIENCE AND TECHNOLOGY	400	1.030	0.000860
38	RENDICONTI LINCEI-SCIENZE FISICHE E NATURALI	549	0.986	0.001220
39	ANAIS DA ACADEMIA BRASILEIRA DE CIENCIAS	2,362	0.956	0.003040
40	Frontiers in Life Science	125	0.907	0.000310
41	CURRENT SCIENCE	10,146	0.883	0.007220
42	Proceedings of the Estonian Academy of Sciences	534	0.843	0.000540
43	ADVANCES IN COMPLEX SYSTEMS	614	0.769	0.000760
44	DISCRETE DYNAMICS IN NATURE AND SOCIETY	1,726	0.757	0.003940
44	Iranian Journal of Science and Technology Transaction A-Science	313	0.757	0.000470

Selected JCR Year: 2017; Selected Categories: "MULTIDISCIPLINARY SCIENCES"

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
46	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES INDIA SECTION A-PHYSICAL SCIENCES	226	0.754	0.000550
47	Kuwait Journal of Science	118	0.693	0.000170
48	Sains Malaysiana	1,216	0.565	0.001510
49	SCIENTIST	247	0.537	0.000520
50	AMERICAN SCIENTIST	2,360	0.525	0.001360
51	NATIONAL ACADEMY SCIENCE LETTERS-INDIA	390	0.519	0.000780
52	DEFENCE SCIENCE JOURNAL	837	0.510	0.000610
53	ENDEAVOUR	479	0.500	0.000460
53	TRANSACTIONS OF THE ROYAL SOCIETY OF SOUTH AUSTRALIA	414	0.500	0.000110
55	HERALD OF THE RUSSIAN ACADEMY OF SCIENCES	314	0.472	0.000330
56	Maejo International Journal of Science and Technology	183	0.469	0.000270
57	SCIENCEASIA	536	0.447	0.000590
58	Chiang Mai Journal of Science	455	0.409	0.000570
59	NEW SCIENTIST	917	0.386	0.001120
60	INTERDISCIPLINARY SCIENCE REVIEWS	238	0.311	0.000080
61	JOURNAL OF THE NATIONAL SCIENCE FOUNDATION OF SRI LANKA	213	0.305	0.000240
62	COMPTES RENDUS DE L ACADEMIE BULGARE DES SCIENCES	570	0.270	0.000410
63	ACTA SCIENTIARUM-TECHNOLOGY	246	0.231	0.000400
64	R&D MAGAZINE	19	0.109	0.000000

11. Printout of Selected Publication

Sutrave, S., Kikhney, J., Schmidt, J., Petrich, A., Wiessner, A., Kursawe, L., Gebhardt, M., Kertzscher, U., Gabel, G., Goubergrits, L., Affeld, K., Moter, A., 2019. Effect of daptomycin and vancomycin on *Staphylococcus epidermidis* biofilms: An *in vitro* assessment using fluorescence *in situ* hybridization. PLOS ONE 14, e0221786. https://doi.org/10.1371/journal.pone.0221786

PLOS ONE

RESEARCH ARTICLE

Effect of daptomycin and vancomycin on *Staphylococcus epidermidis* biofilms: An *in vitro* assessment using fluorescence *in situ* hybridization

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Abstract

Colonization of in-dwelling catheters by microbial biofilms is a major concern in patient health eventually leading to catheter-related blood stream infections. Biofilms are less susceptible to standard antibiotic therapies that are effective against planktonic bacteria. Standard procedure for the detection of microorganisms on the catheter tip is culture. However, viable but non-culturable cells (VBNCs) may be missed. The aim of this study was to evaluate the use of fluorescence *in situ* hybridization (FISH) as an indicator to visualize and quantify the effect of the antibiotics daptomycin and vancomycin on biofilms *in situ*. We established an *in vitro* catheter biofilm model of *Staphylococcus epidermidis* biofilms on polyurethane catheters. Biofilm activity was measured by FISH and correlated to colony forming units (CFU) data. Digital image analysis was used for quantification of total biofilm mass and the area of the FISH positive biofilm cells. FISH showed a pronounced effect of both antibiotics on the biofilms, with daptomycin having a significantly stronger effect in terms of both reduction of biofilm mass and number of FISH-positive cells. This supports the anti-biofilm capacity of daptomycin. Interestingly, neither antibiotic was able to eradicate all of the FISH-positive cells. In summary, FISH succeeded in visualization, quantification, and localization of antibiotic activity on biofilms. This technique adds a new tool to the arsenal of test systems for anti-biofilm compounds. FISH is a valuable complementary technique to CFU since it can be highly standardized and provides information on biofilm architecture and quantity and localization of survivor cells.

Introduction

In-dwelling medical devices such as central-venous catheters can become colonized by biofilms, leading to severe bacteraemia and sepsis. Biofilm-associated infections are tolerant to

standard antibiotic regimens making treatment difficult [1]. Catheter-related blood stream infections have been shown to increase the financial burden for intensive care unit patients and the health care system in addition to causing higher patient mortality [2]. Long-term use of central venous catheters is associated with a risk of bloodstream infection and sepsis (2.7 cases per 1000 catheter-days) [3].

Vancomycin is the standard antibiotic agent for empirical therapy in cases of catheter-related blood stream infections due to methicillin-resistant *Staphylococcus* spp.; nonetheless, for vancomycin minimal inhibitory concentrations (MICs) >2 μg/mL alternative treatment with daptomycin is recommended [4]. Emergence of resistance to vancomycin in blood stream infection cases of methicillin-resistant coagulase negative staphylococci caused by higher MICs has previously been reported [5, 6].

Newer antibiotics such as daptomycin are in use for the strains that are difficult to eradicate. Since 2006 daptomycin has been in use against bacteraemia and right-sided endocarditis caused by *S. aureus* [7]. Several studies have compared the efficacy of daptomycin to that of other antibiotics and concluded that daptomycin has potent activity in the treatment of staphylococcal biofilm-related infections [8, 9].

According to the manufacturer, daptomycin has a distinct mode of action in Gram-positive bacteria. In a calcium-dependent binding, daptomycin causes depolarization of the cell membrane followed by a shutdown of cellular processes such as nucleic acid and protein synthesis. The patent for daptomycin expired in 2016 and generic forms are now available for use.

Previously, biofilm disinfection studies examining the effect of antimicrobials on biofilms have used disintegration of the biofilm and count of colony forming units (CFU) as a measure of antimicrobial efficacy [10]. CFU analysis gives an incomplete picture of cell viability in biofilms following antibiotic treatment. The development of persister cells, which are not detected by CFU measurements, causes recurrent infection, making antibiotic treatment even more challenging [11].

16S rRNA directed fluorescence *in situ* hybridization (FISH) is a culture-independent, tool for visualization and identification of bacteria. The intensity of the FISH signal directly correlates to the ribosomal content within the cells and is therefore an indirect indicator of activity. The objective of this study was to combine digital image analysis and FISH to visualize, precisely locate and quantify the activity of antibiotics on *Staphylococcus epidermidis* biofilms formed in vitro.

Materials and methods

In vitro model for biofilm formation on catheters

The bacterial test strain chosen for this study is the polysaccharide intercellular adhesin (PIA) positive *S. epidermidis* strain 8400 isolated in 1992 from a clinical sample [12]. This strain is well characterized and has been shown to build mature and reproducible biofilms. Olson et al. showed that PIA-dependent *S. epidermidis* biofilms have a higher tolerance to antibiotics than their PIA-independent counterparts [13]. Biofilms were grown in 50 ml-scale biofilm reactors (see S1 Fig). 10% tryptic soy broth (TSB) was inoculated with the clinical isolate *S. epidermidis* strain 8400, an optical density (600nm) of 0.3 of the resulting suspension was achieved and the suspension was incubated with the catheters at 37˚C for 7 days. The medium was replaced with fresh 100% TSB medium supplemented with 25% glucose every 24 hours. The catheters remained immersed in the medium throughout the experiment while being continuously agitated by a magnetic stirrer. During this time a biofilm formed in the lumen and on the outside of the polyurethane catheters.

Antimicrobial treatment

The concentrations of daptomycin (Novartis Pharma AG, Basel, Switzerland) of 160 μg/mL and vancomycin (Sigma-Aldrich Chemie GmbH, Munich, Germany) 100 μg/mL were chosen according to the described peak plasma concentrations in humans [14, 15]. The TSB medium was replaced with Mueller-Hinton medium supplemented with calcium along with daptomycin or vancomycin at the above concentrations, and pumped (50 μl/min) through the catheters into the bioreactor. Calcium was added to the medium due to the calcium-dependent mode of action of daptomycin. The control reactor was treated accordingly with medium supplemented with calcium and phosphate buffered saline (PBS). The catheters remained for 24 h at 37˚C in the antibiotic/control solution which was being stirred continuously to ensure a homogeneous mixture of antibiotics in the medium.

The catheters were harvested and cut into two halves. One half (1 cm) of each catheter was used for CFU analysis; the other half was used for FISH analysis.

CFU measurement

The section of the catheter to be used for CFU analysis was transferred to 1 ml PBS (pH 7.4) and vortexed for 1 minute to homogenize the biofilm. Serial dilution was carried out, 100 μl aliquots were plated on Muller-Hinton Agar and the plates were incubated for 48 h at 37˚C. Colonies were then counted and final counts were calculated taking the dilution factor into account.

Sample preparation and FISH

One half of each catheter was prepared for FISH by fixation and embedding as described [16]. Each catheter was cut into four pieces; the pieces were then embedded upright in cold polymerizing resin Technovit 8100 (Kulzer, Wehrheim, Germany). Using a microtome, 2μm thick sections of the catheter pieces were cut into 8 cross-sections with a total of 32 cross-sections per catheter (see S2 Fig).

Enzymatic pretreatment using lysozyme and lysostaphin was conducted as described [17]. Control slides with cultures of *Escherichia coli*, *S. aureus* and *Streptococcus pyogenes* were included in every experiment.

The hybridization buffer containing the nucleic acid stain 4′,6-diamidino-2-phenylindole (DAPI) and the oligonucleotide probes EUB338 (specific for most bacterial species), STAPHY specific for *Staphylococcus* spp., and non-EUB338 to rule out unspecific probe binding was applied to the sections [17–19]. EUB338 and STAPHY were labelled at the 5' end with the fluorescent indocarbocyanine dye Cy3; non-EUB338 was labelled with Cy5. Hybridization, washing and mounting was carried out as described [17].

Epifluorescence microscopy and digital image analysis

Epifluorescence microscopy was carried out as previously published [20]. Images were obtained with the help of AxioCam MRm (Zeiss) using the AxioVision 4.6 software.

A total of 47 catheters was investigated: control (n = 24), vancomycin (n = 11) and daptomycin (n = 12). For each catheter 100x magnification images of 32 cross-sections at different planes were taken, each with two images of the outer surface of the catheter, resulting in a total of 64 images per catheter. Thus, the total number of images statistically evaluated was as follows: control (n = 1536), vancomycin (n = 704) and daptomycin (n = 768).

Quantification of biofilm area and percentage of FISH-positive cells was achieved using the Adobe After Effects 5.5 software and the image analysis program *daime* [21]. The DAPI and

Cy3 grayscale images were transformed into binary images using the luminance threshold setting option of "after effects" and exported as previously published [20]. The images were then segmented using *daime* and artefacts were removed where necessary. The *daime* program was employed to calculate the biofilm areas of both the Cy3 and the DAPI channels with the DAPI area set as mask for the Cy3 layer. The total biofilm area per catheter (DAPI) and the percentage of the FISH-positive fraction (Cy3) were thus calculated.

Statistical analysis

The data was analysed using the statistical package SPSS V.19 (IBM, USA). Significance was assumed at $p \leq 0.05$ for all tests. A Kolmogorov-Smirnov test was performed to assess deviations from a normal distribution. Group differences were assessed by paired Student's t-test in the case of normally distributed data; otherwise the Mann-Whitney U test was used in combination with Levene's test to prove the equality of variances.

Results

In vitro catheter biofilm model

The *in vitro* catheter biofilm model successfully produced thick biofilms on the outer surface of the catheters. The biofilm formation within the catheter lumen was less pronounced, ranging widely from a few cells to dense biofilms. The *S. epidermidis* PIA 8400 strain chosen for the present study showed variation in the amount of biofilm obtained, in spite of the same stringent experimental conditions being maintained.

FISH and digital image analysis

The EUB338-Cy3 and STAPHY-Cy3 FISH probes hybridized successfully to the biofilm samples showing bright orange fluorescence. No unspecific probe binding was detected with the non-EUB338-Cy5 probe. DAPI stained nucleic acids were observed emitting blue fluorescence. Characteristic biofilm was observed in the biofilms obtained from the *in vitro* model. A distinct pattern of distribution of the FISH-positive cells was seen; the FISH-positive cells were densely clustered towards the periphery of the biofilm, with a few single cells found scattered deeper within the biofilm.

A major reduction in the total area of biofilm on catheters treated with daptomycin and vancomycin as compared to the controls was seen as well. Daptomycin showed a greater reduction in total biofilm area than vancomycin, establishing a trend mirrored in the reduction of the FISH-positive fraction (Fig 1). However, due to a high standard deviation this difference was not statistically significant at $p \leq 0.05$.

The percentage of FISH-positive cells was significantly reduced within the biofilm area evaluated. Both antibiotics reduced total biofilm area; notably, daptomycin showed a greater reduction in FISH-positive cells than vancomycin compared to the control. Following treatment with PBS (control) and antibiotics, the percentage of FISH-positive cells was found to be 56% for the control, but reduced to 28% after vancomycin and to 12% after daptomycin treatment, respectively (Fig 2).

The control biofilms, treated with PBS, showed a distinct pattern of distribution of the FISH-positive cells within the biofilms (Fig 3). The outer periphery of the biofilm showed a bright FISH-positive signal along with scattered cells found towards the centre of the biofilm mass and no FISH-positive cells on the areas of the biofilm adjacent to the catheter.

Biofilms treated with vancomycin exhibited the same distribution of FISH-positive cells as seen in the case of the controls, there being a higher density of these cells towards the outer

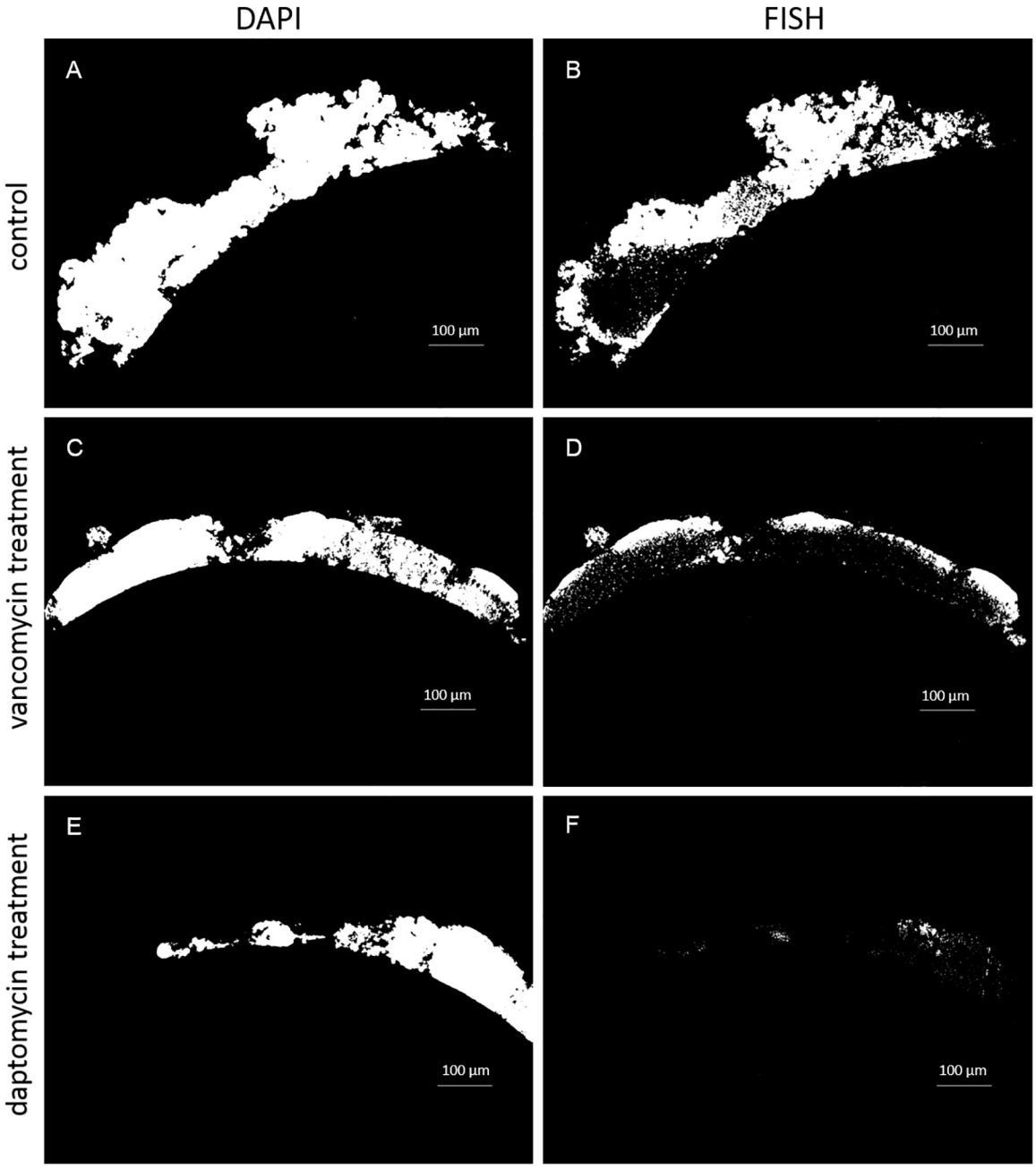

Fig 1. Digital image analysis using *daime* shows the reduction of total biofilm area and FISH-positive fraction, respectively, for *in vitro S. epidermidis*. A and B show DAPI and FISH masks, respectively, of the control biofilm treated with PBS. C and D show DAPI and FISH masks for biofilm on a catheter treated with vancomycin, and E and F show DAPI and FISH masks for biofilm on a catheter treated with daptomycin, respectively. The reduction of FISH positive areas after treatment with vancomycin (D) and even more with daptomycin (F) is clearly visible.

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edges of the biofilm (Fig 4A–4C). Daptomycin treated biofilms showed single FISH-positive cells spread throughout the entire mass of the biofilm (Fig 4D–4F).

FISH vs. CFU data

Log_{10} CFU/mL values for control catheters and catheters treated with vancomycin and daptomycin were 12.15±0.82, 4.91±0.28 and 2.49±0.57, respectively. Statistical differences between

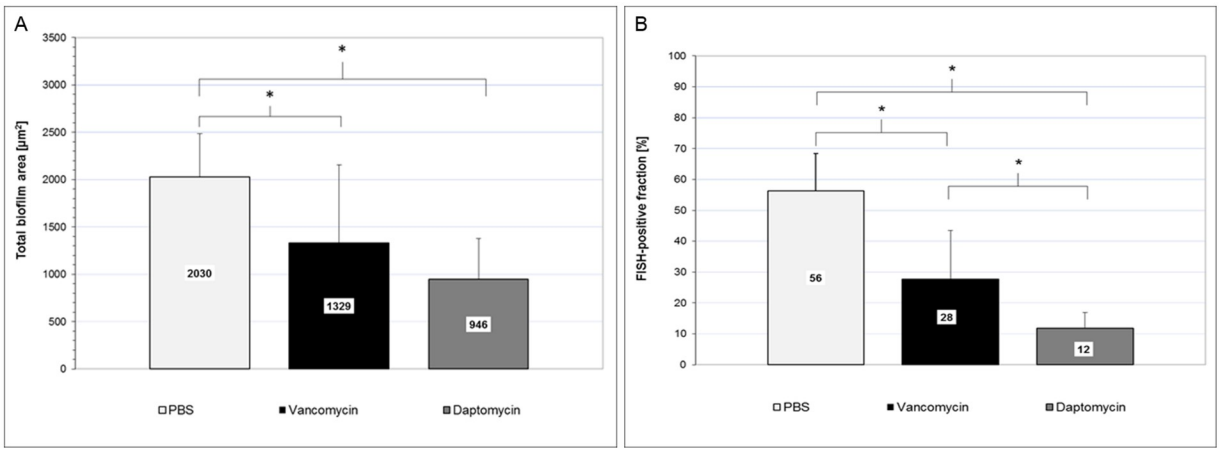

Fig 2. Reduction of the total biofilm mass (A) and FISH-positive fraction (B) in biofilms under antibiotic treatment. Total biofilm area [µm^2] represented by the DAPI mask in *daime* and fraction of FISH-positive cells in [%] as compared to the total biofilm area, for *in vitro* grown biofilms of *S. epidermidis* treated with PBS, vancomycin and daptomycin, respectively. Data was found to be normally distributed; therefore, mean values and Student's t-test were used to denote group differences. *significant difference between test groups at $p \leq 0.05$.

https://doi.org/10.1371/journal.pone.0221786.g002

any test groups (control, vancomycin and daptomycin) were highly significant ($p \leq 0.01$). Vancomycin and daptomycin met and exceeded the 3-log reduction in CFU/mL criteria to be considered bactericidal [22]. As evidenced by the cells that are FISH-positive after antibiotic treatment, neither antibiotic achieved a 100 percent kill in the *in vitro* experimental setup described here. The CFU data, as expected, showed the same general pattern of reduction in cell count following the application of antibiotics as FISH, but without the spatial aspect of distribution of cells within the biofilm gained via FISH.

Discussion

The validity of FISH in establishing the improved efficacy of antibiotic-loaded microparticles in staphylococcal biofilms has recently been shown [23]. The present study demonstrates, for the first time, the use of FISH for visualization, quantification, and localization of the rRNA containing cells within a catheter-related biofilm following antibiotic treatment. Daptomycin reduced the total biofilm area as well as the percentage of FISH-positive cells of the *in vitro S. epidermidis* biofilms; vancomycin showed this reduction to a lesser extent. FISH demonstrated distinct differences in the distribution of FISH-positive cells between the two antibiotics, perhaps due to their disparate modes of action. Our results prove FISH to be an invaluable addition to the current arsenal of tools available for the evaluation of antibiotic action on biofilms.

As expected, the FISH data correlate to the CFU results. It is important to point out the advantages of FISH over CFU towards a better understanding of biofilms under antibiotic therapy. Firstly, FISH provides spatial resolution aiding in visual input and localization within the biofilm. Secondly, FISH enables quantification of the biofilm in terms of area and percentage of FISH-positive cells. Lastly, FISH helps us examine and interpret the means by which the surviving cells lead to clinical relapse as well as provide a feeding ground for new and incoming bacterial cells.

Various *in vitro* models of biofilm formation for testing antimicrobial activity have been published so far [13, 24, 25]. In the current stage of research on catheter-related infections, there is a lack of standardized models for testing *in vitro* biofilms on catheter surfaces [26]. The *in vitro* model developed in this study produced thick biofilms on the catheters, but the

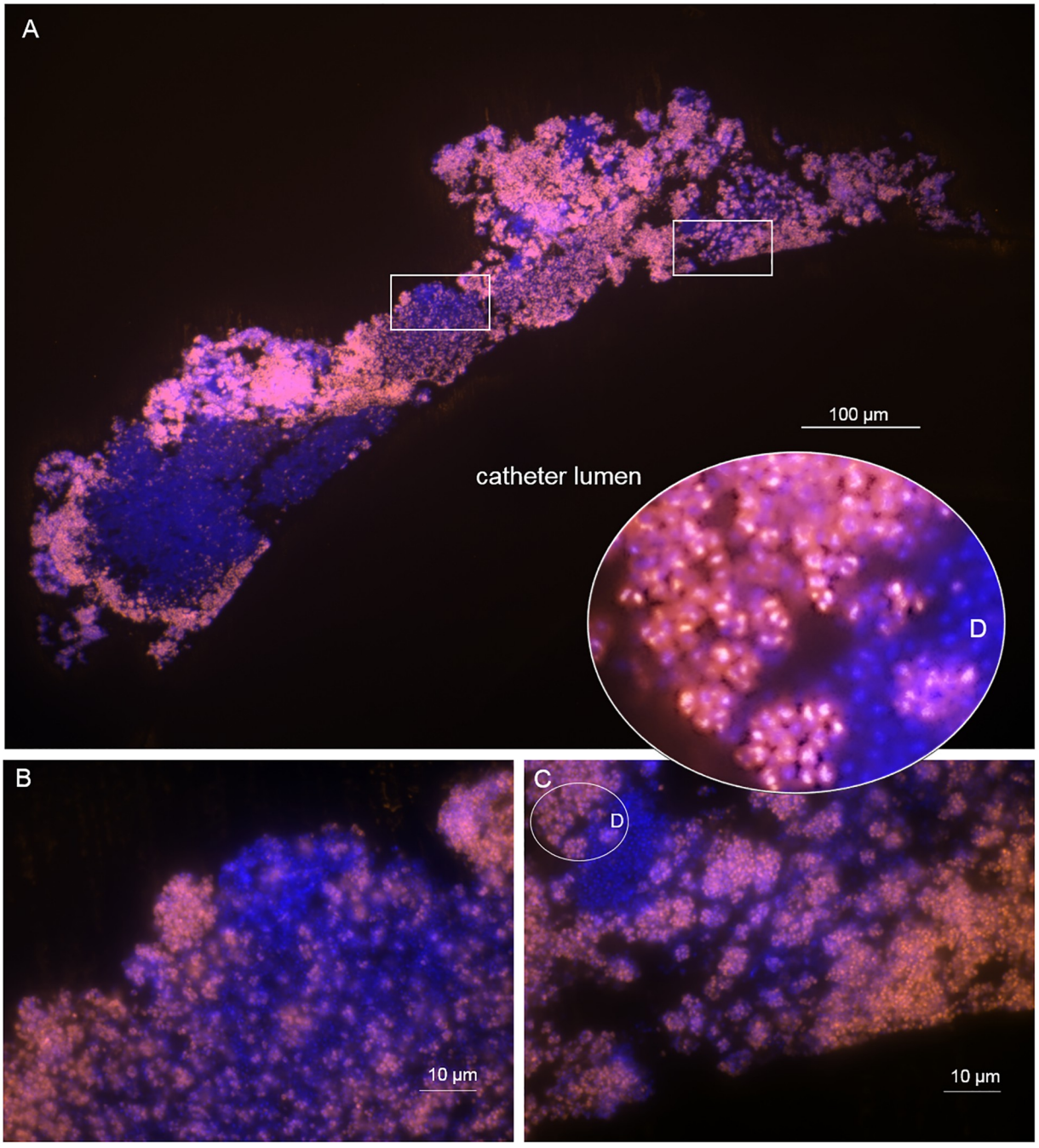

Fig 3. High proportion of FISH-positive cells in biofilms on a control catheter. *In vitro S. epidermidis* biofilm on control catheter treated with PBS. The blue layer represents the nucleic acid stain DAPI. The orange layer represents the FISH-positive cells (Cy3). B, C are magnifications of the insets from A showing FISH-positive cocci. At higher magnification of inset D, single bacterial cells are visible with differential FISH signal intensity. Of interest, note the bright fluorescence in particular in double-cocci that seem to divide, whereas the blue cocci resemble cells with low ribosome content.

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biofilm growth was not uniform between catheters within each biofilm reactor. Furthermore, the long turnaround time per experiment limits the use of the model as a prototype for *in vitro* biofilm studies on catheters. A shorter incubation time for biofilm growths such as 6–10 hours could reduce the standard deviation and speed up the analysis. In this study we aimed at rich

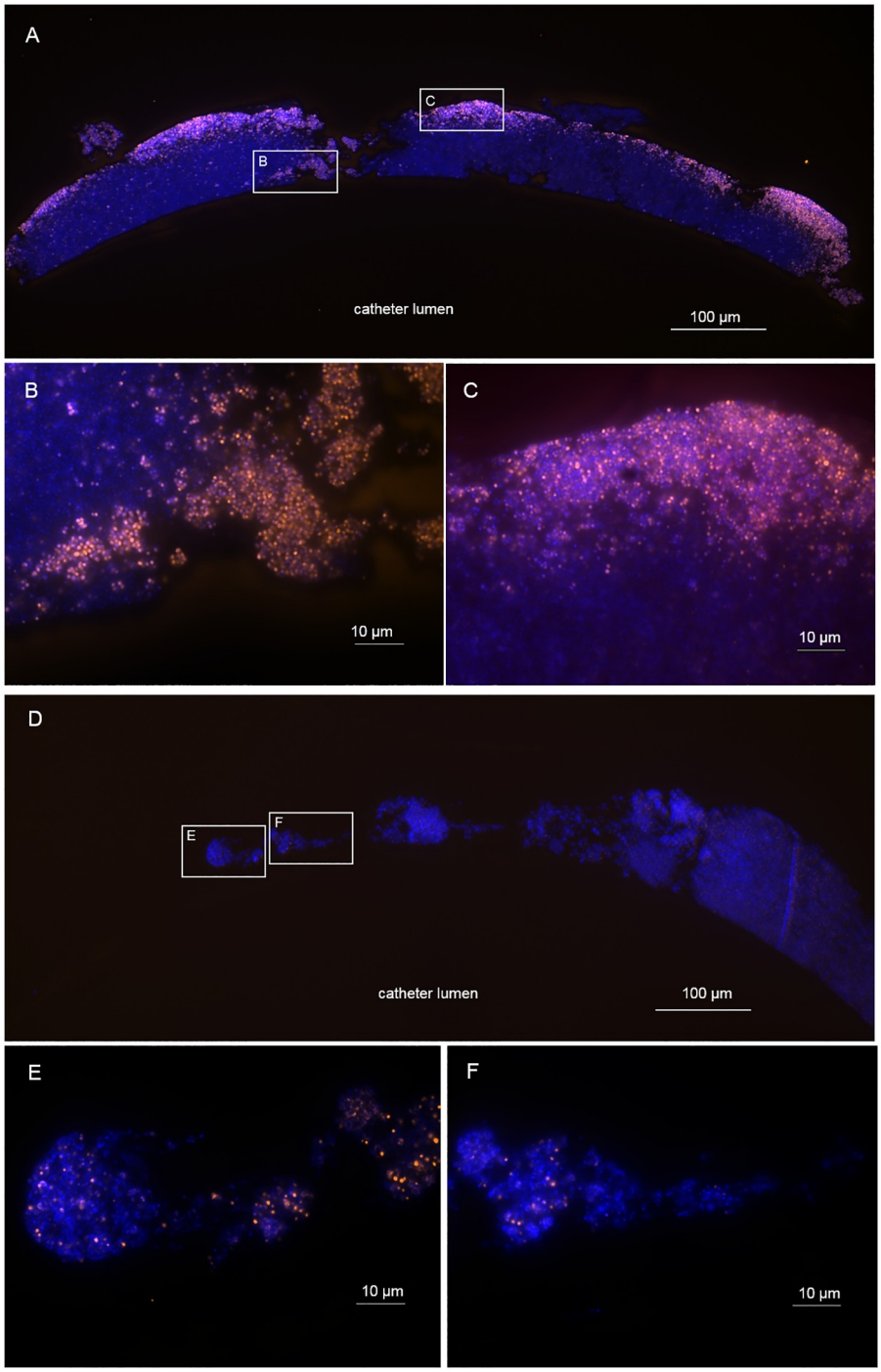

Fig 4. FISH shows differential pattern of remaining FISH-positive bacteria in biofilms and reduction of the FISH-positive fraction upon antibiotic treatment. *In vitro S. epidermidis* biofilm on catheter treated with vancomycin (4A-C) or daptomycin (4D-F), respectively. The blue layer represents the nucleic acid stain DAPI. The orange layer represents the FISH positive, ribosome-rich cells (Cy3). B, C are magnifications of the insets from A showing FISH-positive cocci in the outer layers of the vancomycin treated biofilm. Upon daptomycin treatment (4D-F), only single cells remain FISH-positive in all parts of the biofilm. 4E and F are magnifications of the insets from 4D showing single FISH-positive cocci remaining under daptomycin treatment.

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biofilms to demonstrate the feasibility of analysis of biofilm architecture to be able to compare the anti-biofilm activity of two antibiotics. Furthermore, as a clinical situation we aimed at being correspondent to port systems.

An *in vitro* model of *S. epidermidis* device-related infections for antibiotic testing has been shown to be a valid first step before moving to an in vivo model [27]. The standard protocol in dealing with catheter-related infections is the removal of the catheter, although this is not always feasible with a port system. The results of our study are in full agreement with this approach, as eradication of all biofilm cells is difficult to achieve.

Consistent with previous studies, limited activity of vancomycin against the *in vitro S. epidermidis* biofilms was observed in the present study [28]. Owing to the evidence of development of resistance to vancomycin reported [29], daptomycin is recommended as an antimicrobial alternative for the treatment of biofilm associated infections in catheterized patients [4].

Mascio et al. showed that daptomycin at higher concentrations (100 μg/mL) has bactericidal activity against stationary-phase *S. aureus* cells and concluded that this unique ability of daptomycin has a direct application on biofilm-associated cells [30].

Our results showing very promising therapeutic activity of high dose daptomycin (160 μg/mL) against *S. epidermidis* are in agreement with a recent study using daptomycin-lock therapy in a rabbit catheter model [31].

Analysis of the location of FISH-positive cells in the biofilms on antibiotic-treated catheters showed that daptomycin penetrated throughout the depth of the biofilm. In keeping with the findings of Stewart et al. the results obtained clearly indicate penetrance of the antibiotic is not a limiting factor in the treatment of *S. epidermidis* biofilms with daptomycin [32].

Very large-scale studies would be needed in order to prove the efficacy of antibiofilm agents and would involve a considerable length of time before the beneficial effects begin to reach the patient. Therefore, it is imperative to employ *in vitro* testing of antimicrobials. FISH is a relevant method to test not only anti-biofilm agents but also infected catheters from patients.

Supporting information

S1 Fig. Schematic illustration of the *in vitro* biofilm model for growing *S. epidermidis* biofilms. Each biofilm reactor consisted of four polyurethane catheters (Instech Solomon 3 Fr BPU-T30, length 3 cm, outer diameter 0.91 mm, inner diameter 0.58 mm) and an air filter (pore size 0,22 μm, Carl Roth GmbH, Germany), all catheters were connected via tubes to a peristaltic pump (Ismatec® Reglo Digital) for pumping of antibiotic and control solutions. The numbers shown the diagram represent: (1) biofilm reactor with bacterial suspension, (2) air filter, (3) polyurethane catheter, (4) reservoir with medium with or without antibiotic solution and (5) peristaltic pump.
(TIF)

S2 Fig. Illustration showing the processing of the catheter samples in methacrylate. Each catheter is cut into four equal sections. These are upright embedded into methacrylate resin. The methacrylate block is sectioned into a total of 32 cross sections per catheter.
(TIF)

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Writing – review & editing: S. Sutrave, J. Kikhney, U. Kertzscher, G. Gabel, L. Goubergrits, K. Affeld, A. Moter.

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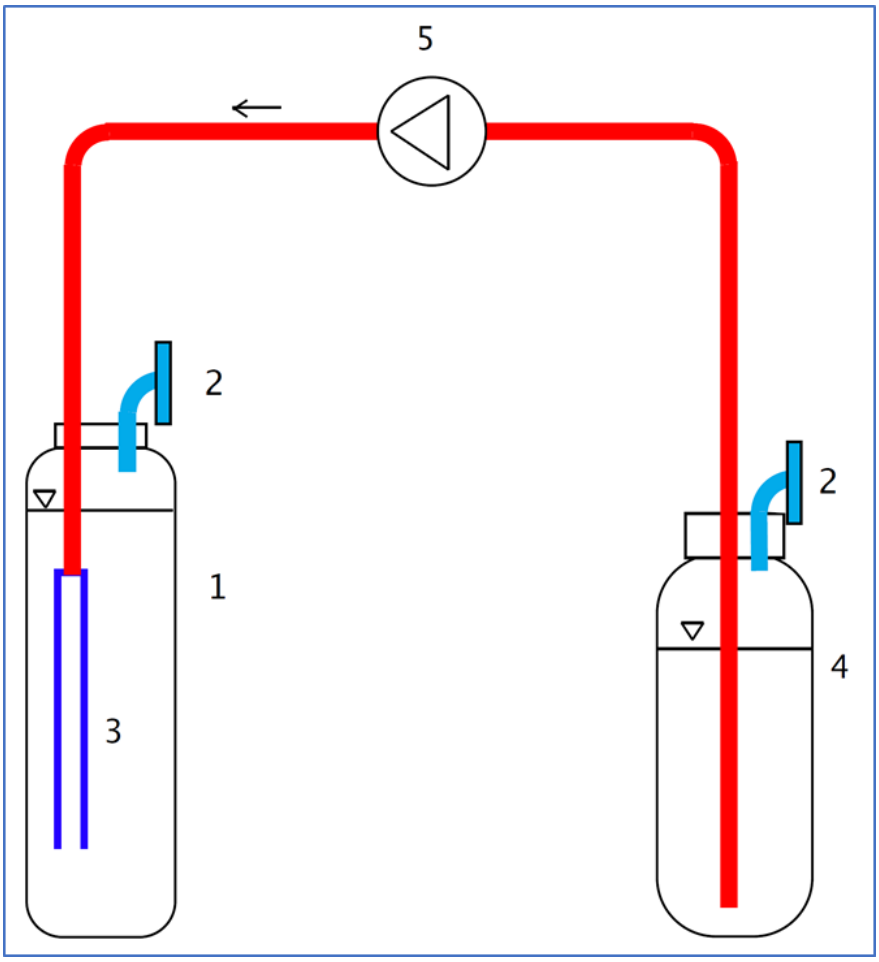
Supplement 1



S 1 Fig. Schematic illustration of the *in vitro* biofilm model for growing *S. epidermidis* biofilms. Each biofilm reactor consisted of four polyurethane catheters (Instech Solomon 3 Fr BPU-T30, length 3 cm, outer diameter 0.91 mm, inner diameter 0.58 mm) and an air filter (pore size 0,22 µm, Carl Roth GmbH, Germany), all catheters were connected via tubes to a peristaltic pump (Ismatec® Reglo Digital) for pumping of antibiotic and control solutions. The numbers shown the diagram represent: (1) biofilm reactor with bacterial suspension, (2) air filter, (3) polyurethane catheter, (4) reservoir with medium with or without antibiotic solution and (5) peristaltic pump.

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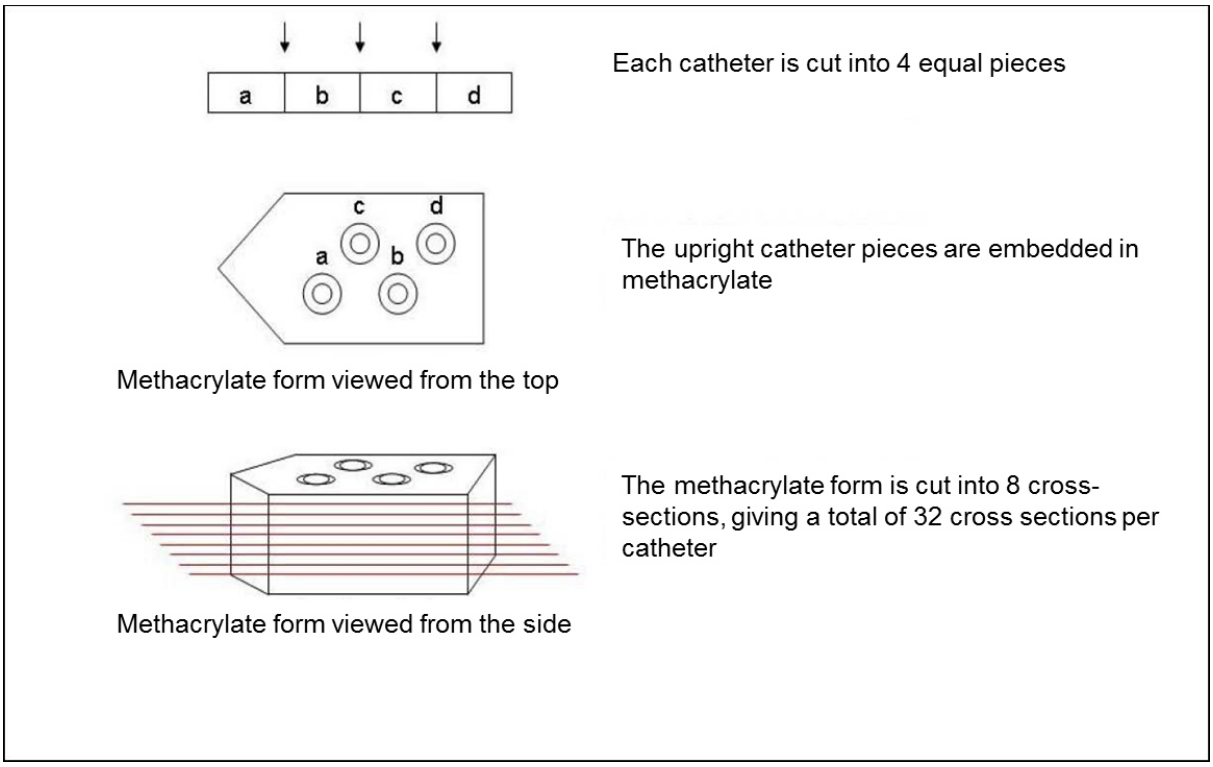
Supplement 2



S 2 Fig. Illustration showing the processing of the catheter samples in methacrylate. Each catheter is cut into four equal sections. These are upright embedded into methacrylate resin. The methacrylate block is sectioned into a total of 32 cross sections per catheter.

https://doi.org/10.1371/journal.pone.0221786.s002

12. Curriculum Vitae

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

13. List of Publications

Journal Article

Sutrave, S., Kikhney, J., Schmidt, J., Petrich, A., Wiessner, A., Kursawe, L., Gebhardt, M., Kertzscher, U., Gabel, G., Goubergrits, L., Affeld, K., Moter, A., 2019. Effect of daptomycin and vancomycin on *Staphylococcus epidermidis* biofilms: An *in vitro* assessment using fluorescence *in situ* hybridization. PLOS ONE 14, e0221786. https://doi.org/10.1371/journal.pone.0221786

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Poster Presentations

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Großhauser, J., Sutrave, S., Affeld, K., Moter, A., Reiter, K., Grosse-Siestrup, C., Kertzscher, U. Development of a novel catheter with infection-resistant exit-site. 23rd European Congress of Clinical Microbiology and Infectious Diseases (ECCMID). Berlin, Germany, 27 – 30 April 2013.

Sutrave, S., Schmiedel, J., Schulze, J., Petrich, A., Wiessner, A., Gebhardt, M., Göbel, U.B., Kertzscher, U., Gabel, G., Affeld, K., Moter, A. Fluorescence in situ hybridization (FISH) analysis of *Staphylococcus epidermidis* activity in an *in vitro* catheter biofilm model. EUROBIOFILMS 2011. Copenhagen, Denmark, 6 - 8 July 2011.

Sutrave, S., Blake, N., Giroux, M.J., Lanning, S., Martin, J.M., Talbert, L.E. Effect of rate and duration of starch biosynthesis on grain yield in wheat. Plant & Animal Genome Conference (PAG). San Diego, USA, 2005.

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