Diploma Thesis

Biofilm formation in central venous catheters - a prospective study performed with catheters from organ donors.

Submitted by

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Graz, 26.08.2016

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Graz, 26.08.2016 *Andreas Hoefel eh*

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Zusammenfassung

Einleitung.

In den letzten Jahren erlangte der zentralvenöse Zugang immer größere Bedeutung in der Medizin, insbesondere auf Intensivstationen. In Bezug auf die Verabreichung von vasoaktiven/aggressiven Medikamenten und großen Mengen Flüssigkeit sind zentralvenöse Katheter (ZVK) den peripheren klar überlegen. Allerdings können Infektionen jener zu schwerwiegenden Folgen mit hoher Mortalität führen. Ein allgegenwärtiges Problem heutzutage ist die Biofilm-Besiedelung von zentralvenösen Kathetern. Biofilm ist eine soziale Verhaltensform von Bakterien, die sie gegenüber Antibiotika äußerst resistent macht. Ziel zahlreicher Studien ist es gegenwärtig, neue Ansätze zur Prävention und Therapie von Biofilm und dessen Komplikationen zu finden.

Methoden.

Bei drei PatientInnen mit abgeschlossener Hirntoddiagnostik wurden im Zuge der Organentnahme liegende ZVKs entfernt und im Labor aufbereitet und in Segmente unterteilt. Die einzelnen Abschnitte der ZVKs wurden für die Isolation der gesamten bakteriellen DNA sowie für die Bestimmung der Koloniedichte mittels quantitativer Polymerase-Kettenreaktion (PCR), des Keimspektrums mittels Gen-Amplifizierung und Sequenzierung und des räumlichen Aufbaus der Biofilme mittels Fluoreszenz-in-situ-Hybridisierung (FISH) und Konfokalmikroskopie verwendet.

Ergebnisse.

Es konnten Bakterien auf den ZVKs nachgewiesen werden, die Zahlen variierten stark in Abhängigkeit der Katheteroberfläche. Es konnten keine Rückschlüsse auf die Zusammensetzung oder den räumlichen Aufbau der Biofilme gewonnen werden.

Schlussfolgerung.

Aufgrund mangelnder Ergebnisse unseres Experiments müssen in Zukunft weitere Versuche zur Gewinnung neuer Erkenntnisse unternommen werden. Prävention bleibt die Hauptsäule der Bekämpfung von Biofilmen.

Abstract

Introduction.

Over the last years, central venous access gained more importance in medicine, especially in intensive care units. Central venous catheters (CVC) are superior to their peripheral counterparts in terms of administration of vasoactive/aggressive substances and large fluid amounts. On the downside, infections of these devices can lead to serious complications with high mortality. Today, an omnipresent problem for physicians is biofilm formation in CVCs. Biofilm is a community behavior by bacteria that makes them much more resilient against antibiotics. It is the aim of many studies to find new approaches in order to prevent and treat biofilm occurrence and its complications.

Methods.

CVCs were extracted from three confirmed brain-dead patients during organ explantation and sent to a laboratory for further manipulation and division in segments. The segments were used for isolation of total bacterial DNA as well as for determination of colony density by quantitative PCR, determination of biofilm composition by gene amplification and sequencing and visualization of the biofilms' 3D structure by FISH and confocal laser microscopy.

Results.

Bacterial growth on the CVCs could be detected. However, the numbers depended heavily on the surface area. No information about biofilm composition and structure could be gained.

Conclusion.

Further experiments will have to be performed in future. Prevention remains the mainstay of biofilm management.

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List of abbreviations

AIP	auto-inducing peptide
CLM	confocal laser microscopy
cm	Centimeter
CRBSI	catheter related bloodstream infection
CRP	C-reactive protein
CVC	central venous catheter
E.coli	Escherichia coli
CVP	central venous pressure
EPS	extracellular polymeric substances
FISH	fluorescent in situ hybridization
IACP	implantable artificial cardiac pacemaker
ICU	intensive care unit
IMD	implantable medical device
IV	intravenous
P.aeruginosa	Pseudomonas aeruginosa
PCT	procalcitonin
PICC	peripherally inserted central catheter
qPCR	quantitative polymerase chain reaction
QS	quorum sensing
rRNA	ribosomal ribonucleic acid
S.aureus	Staphylococcus aureus
S.epidermidis	Staphylococcus epidermidis
TIPS	totally implantable port system
USA	United States of America

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1 Introduction

1.1 Implantable medical devices (IMD)

Modern medicine provides a vast number of different implantable medical devices (IMD) of great therapeutic value. They can increase the patients' quality of life, make the IV administration of drugs and fluid easier and help improving the outcome of certain medical conditions. In the USA alone, over five million catheters are inserted annually.(1)(2)

Even though the benefits of IMDs can be useful or even live saving there are some drawbacks that, amongst others, include the risk of contamination, biofilm forming and possibly infection. Per year, the five million catheters installed in the USA lead to 250.000 to 400.000 catheter-related blood stream infections (CRBSI) caused by bacteria or fungi/yeasts.(1,3)

A general overview of some of the many IMDs that are known to be potentially prone to these risks is made below.(1,4)

1.1.1 Central Venous Catheters (CVC)

Central venous catheters (CVC), also called central lines, are common devices in today's health care. They are of particular importance in intensive care units (ICU) where the administrations of large amounts of fluid in short time and numerous highly concentrated drugs are necessary. For that reason most modern CVCs come with multiple lumina. Central lines are also suitable for chemotherapy, hemodialysis, parenteral nutrition and the measurement of the central venous pressure (CVP).(1,5–7)

In general, a CVC is placed in a large vein with its tip usually resting in the superior vena cava. In order to minimize the risk of contamination and infection the whole procedure takes place under sterile circumstances. The most common sites for insertion are the internal jugular vein, the external jugular vein and the subclavian vein; preferably the right one of each of the mentioned. Another option for access is a peripheral vein like the basilic vein or the cephalic vein – the procedure is then called peripherally inserted central catheter (PICC). Locating the correct puncture site can be achieved through anatomical landmarks or ultrasonography guidance. After the spot for puncture is chosen and

sterilized, the Seldinger technique is utilized: skin and vein are penetrated with a trocar, through which a guidewire is carefully inserted into the lumen of the blood vessel. The trocar is withdrawn and a sheath is inserted over the guidewire. If needed, the puncture site can be extended by a small incision. The guidewire is removed with caution and the actual catheter can be installed into the vein through the sheath. Finally, the sheath is withdrawn as well and the CVC is fixed in place with a suture. The catheter tip should be placed in the superior vena cava about 2cm away from the right atrium of the heart. When using access through the right internal jugular vein for example, the advancing distance of the catheter is about 16-17cm in adults until the desired position is reached. An accurate positioning of the tip can be attained with an intracardiac electrocardiogram. Alternatively, the skin's puncture site can be separated from the spot the blood vessel is penetrated by a subcutaneous tunnel. Central lines installed this way are called 'tunneled'. For either method a chest x-ray should be performed later on to verify whether the catheter is still in place as well as to look out for possible complications like intravasal curling of the catheter, hematoma, pneumothorax or hematothorax. (1,2,6,8,5,7,9–12)

1.1.2 Totally Implantable Port System (TIPS)

In 1982, the newly developed totally implantable port system (TIPS), or just 'port', for central venous access was successfully tested in 30 cancer patients.(13,14) The port is a small reservoir chamber with a septum and an attached CVC that is surgically placed in the subcutaneous layer of the chest or the upper arm. The catheter usually rests inside the internal jugular vein, the subclavian vein or the cephalic vein and connects the chamber to the blood stream. Thus, the port is a completely implanted device with no part passing through the skin. Only when central venous access is needed, the port's septum is punctured through the skin with a specific needle.(1,13,15)

The TIPS is suited very well for IV application of chemotherapy since it can dwell for a long time and has a low rate of extravasation. However, the application of large amounts of fluid over short time can be problematic because of the small diameter of the port needle.(1,15)

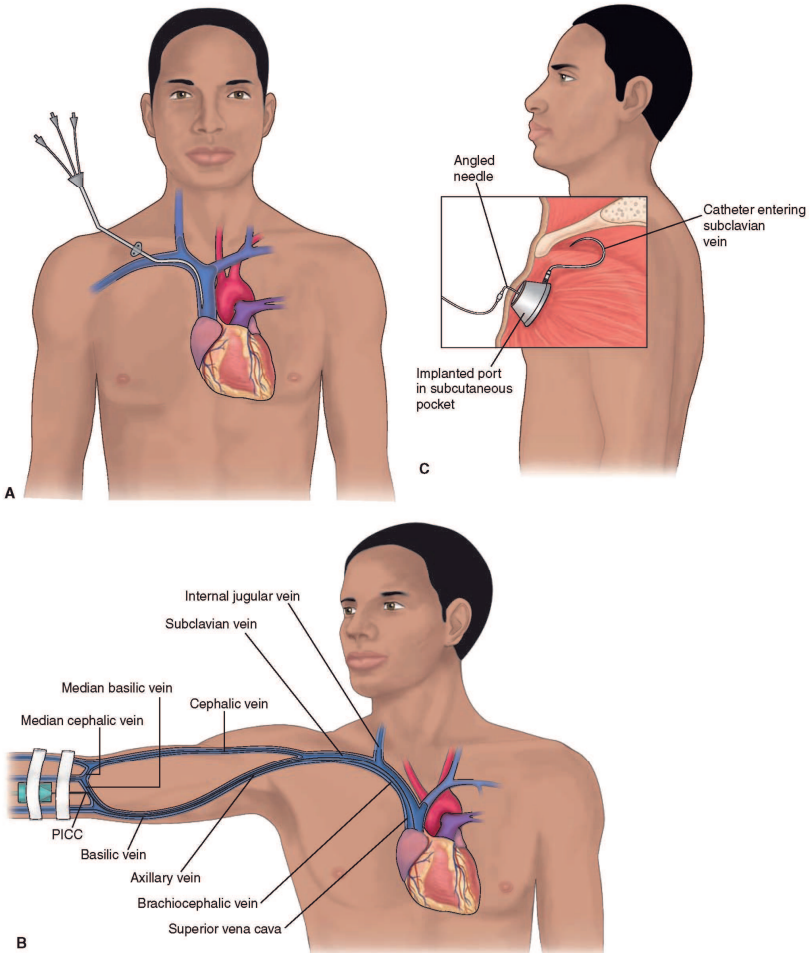


Figure 1 Different methods of central venous access (A: CVC, B: PICC, C: TIPS) (83)

1.1.3 Indications

Central venous access is indicated when peripheral venous access is not possible (like in hypovolemic shock), for infusion of large amounts of fluid, hypertonic solutions, vasoactive substances such as catecholamines and parenteral alimentation as well as for acute hemodialysis, the application of incompatible drugs via multiple lumina and the monitoring of CVP.(6,16)

1.1.4 Complications

The procedure of central venous catheterization involves a couple of serious complications, especially when the operator is less experienced. Thus, appropriate training and supervision by an experienced physician is crucial.(16)

Known complications are dysfunction, accidental arterial puncture, hematoma, pneumothorax, hematothorax, occlusion of the vessel, venous air embolism, puncture of nerves or, if cannulated on the left side, the thoracic duct, intraluminal dissection of the vessel, venous thrombosis and CRBSI.(10,12,16,17) Figure 2 shows a table taken from the 2015 paper 'Intravascular complications of central venous catheterization by insertion site' by Parienti, Mongardon, Mégarbane et al. that gives an overview on the frequency of complications. (18)

Table 3. Intention-to-Treat Pairwise Comparisons for the Trial Outcomes.

Outcome	Femoral versus Subclavian				Jugular versus Subclavian				Femoral versus Jugular			
	Femoral	Subclavian	Hazard Ratio (95% CI)*	P Value	Jugular	Subclavian	Hazard Ratio (95% CI)*	P Value	Femoral	Jugular	Hazard Ratio (95% CI)*	P Value
	number				*number*				*number*			
Catheters	875	878	—	—	984	981	—	—	1140	1145	—	—
Catheter-days	5198	5739	—	—	6573	6651	—	—	6658	7427	—	—
Primary composite outcome†	25	8	3.5 (1.5–7.8)	0.003	23	11	2.1 (1.0–4.3)	0.04	33	30	1.3 (0.8–2.1)	0.30
Bloodstream infection	11	4	3.4 (1.0–11.1)	0.048	13	6	2.3 (0.8–6.2)	0.11	15	21	0.9 (0.5–1.8)	0.81
Symptomatic deep-vein thrombosis	15	5	3.4 (1.2–9.3)	0.02	10	6	1.8 (0.6–4.9)	0.29	20	9	2.4 (1.1–5.4)	0.04
Secondary outcome												
Catheter-tip colonization	107	39	3.4 (2.4–5.0)	<0.001	104	42	2.5 (1.7–3.5)	<0.001	145	121	1.6 (1.2–2.0)	0.003
Deep-vein thrombosis‡	46	19	3.0 (1.7–5.3)	<0.001	69	20	3.1 (1.9–5.0)	<0.001	62	78	0.9 (0.7–1.3)	0.68
Major mechanical complications	6	18	0.3 (0.1–0.8)§	0.03	12	22	0.5 (0.3–1.1)§	0.09	7	13	0.5 (0.2–1.4)§	0.19
Arterial injury	4	1	—	—	2	2	—	—	4	2	—	—
Hematoma	0	1	—	—	4	1	—	—	0	4	—	—
Pneumothorax	NA	13	—	—	4	14	—	—	NA	4	—	—
Miscellaneous¶	2	3	—	—	2	5	—	—	3	3	—	—

* Values in this column are hazard ratios unless otherwise indicated. All hazard ratios were adjusted for stratification variables (intensive care unit and antibiotic use) and design variables (three-choice vs. two-choice scheme). The confidence intervals for hazard ratios are robust confidence intervals taking into account multiple catheters per patient. NA denotes not applicable.
† Some catheters had two events.
‡ The numbers of ultrasonographic evaluations in the three pairwise comparisons were 744 (398 in the femoral group and 346 in the subclavian group), 786 (399 in the jugular group and 387 in the subclavian group), and 955 (497 in the femoral group and 458 in the jugular group).
§ The value for major mechanical complications is an odds ratio computed by random-intercept logistic regression, rather than a hazard ratio.
¶ The specific miscellaneous mechanical complications for each pairwise comparison are listed in Table S8 in the Supplementary Appendix.

Figure 2 Comparison of complications due to CVC insertion by site.

Reference:(18)

1.2 Bacteria

Bacteria are one of the three domains that every cellular life form can be assigned to, the other two domains being eukaryotes and archaea.(19)

Bacteria are prokaryotes, which means that they do not store their genetic information (deoxyribonucleic acid, DNA) inside a nucleus but directly in the cytoplasm in form of one large circular chromosome.(19) They are unicellular organisms that reproduce by cell division and come in different shapes as figure 3 shows:

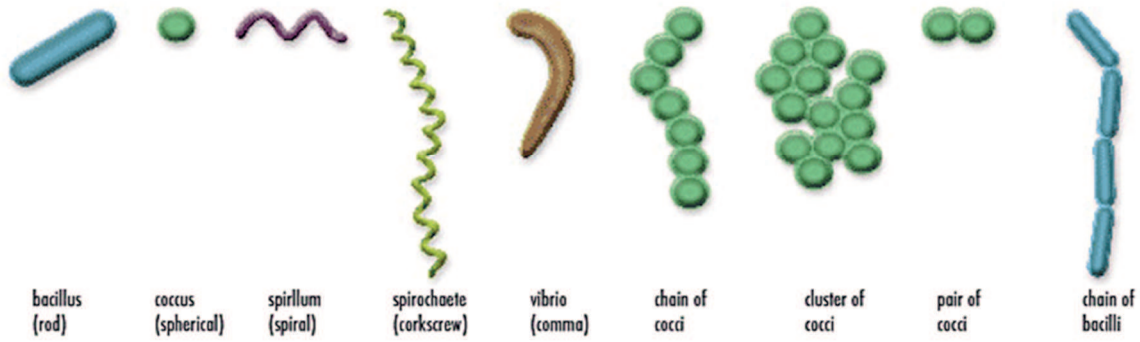


Figure 3 Bacterial shapes. Reference: (84)

Ways of classifying bacteria other than by morphology include their metabolic dependency on oxygen (aerobic, anaerobic, facultative anaerobic) and the structure of their outer layers, which influences their ability to be dyed utilizing the Gram's method (gram-positive, gram-negative).(19)

Although fulfilling numerous important functions on our planet, their relevance in medicine mostly comes from being a well-known cause for infection and disease. A prominent example is the plague, also called 'Black Death', an infectious disease which killed a third of Europe's total population (approximately 25 million deaths) within 5 years in the 14th century. The plague is caused by the bacteria Yersinia pestis, a gram-negative, facultative anaerobic bacillus.(19–22)

Table 1 shows several (pathogenic) bacteria and their respective assignment by the abovementioned classifications.

Bacteria	Form	Oxygen usage	Gram-	Notable diseases
Staphylococci	Coccus	Facultative anaerobic	Positive	Wound infection, endocarditis, sepsis
Streptococci	Coccus	Fac. anaerobic	Positive	Erysipelas, scarlet fever, endocarditis
Escherichia coli	Rods	Fac. anaerobic	Negative	Colitis
Neisseria	Coccus	Variable	Negative	Meningitis, gonorrhea
Corynebacterium	Rods	Fac. anaerobic	Positive	Diphtheria
Clostridium	Rods	Anaerobic	Positive	Gas gangrene, tetanus
Yersinia	Rods	Fac. anaerobic	Negative	Plague
Pseudomonas	Rods	Aerobic	Negative	Pneumonia
Vibrio (cholerae)	Vibrio	Variable	Negative	Cholera
Treponema	Spiral	?	Negative	Syphilis
Mycobacterium	Rods	Aerobic	Positive	Tuberculosis, leprosy

Table 1 Bacterial classification. Reference: (85–87)

1.3 Biofilm

When a medical device is implanted, a bridging of the natural physical barrier against the harmful environment takes place. Even though the effort by modern hygiene standards in health care institutions in the developed world to prevent contamination is big, a chance of spreading non-physiological substances or microorganisms into the body always persists. Essentially, biofilm is a result of community-based behavior by bacteria or fungi(23). They adhere to surfaces as well as to each other and produce extracellular polymeric substances (EPS) that form into a protecting matrix they live inside of. EPS account for 75-90% of a biofilm's mass and mostly consist of polysaccharides, proteins, phospholipids and extracellular DNA (eDNA), but also other substances.(1) This slimy matrix is accountable for the biofilms persistence towards antibiotic treatment and the host's immune system.(24) However, rate of growth, gene expression as well as other physiological

qualities are altered compared to planktonic microbes on a scale that makes biofilm partly non understood until today. Since biofilm can theoretically form on almost any surface, it is estimated that 60 to 80% of microbial infections in the developed world are linked to biofilm.(1,24–26)

1.3.1 Formation

Biofilm formation takes place in different stages: it starts when microbes adhere reversibly to a surface, known as stage of initial attachment. As Yousif, Jamal and Raad explain in their 2015 review(1), this initial adherence is influenced by a number of factors like temperature, pH and surface texture and chemical polarity. Also, sufficient nutrient availability in the environment is needed. (1,27–30) Additionally, metallic cations like magnesium, potassium, calcium, and iron play important roles in biofilm formation by P.aeruginosa, E.coli, S.aureus and S.epidermidis.(1,31–33)

Also, genetic factors influence adherence. S.aureus and other stems of staphylococci for example were shown to be able to bind to platelets, thrombin, fibrinogen, fibrin, collagen and other components that are widely present in a human host. The abovementioned components are very likely to sooner or later accumulate in an indwelling catheter, which leads to its colonization. (1,34–36)

Following this abovementioned stage of reversible attachment, irreversible attachment follows. Microbes will now reinforce their adherence to the surface, which is, for example, promoted by type I pili in Escherichia Coli and type IV pili in Pseudomonas aeruginosa.(1,37–39) Eventually, the bacteria community will proliferate and usually grow into pillar or mushroom formations. At this point, the production of EPS will initiate in order to form a protecting matrix coat for the microbes. Inside these so-called microcolonies, nutrient transport takes place through complex fluid-filled channels. The abovementioned can be summarized as the stage of maturation. In the final stage called dispersion, microbes detach from the microcolony to reproduce in another place. The cycle then restarts. The detachment may be promoted by environmental signals and could explain why contaminated IMDs only sometimes lead to bacteremia and sepsis rather than every time.(40,41)

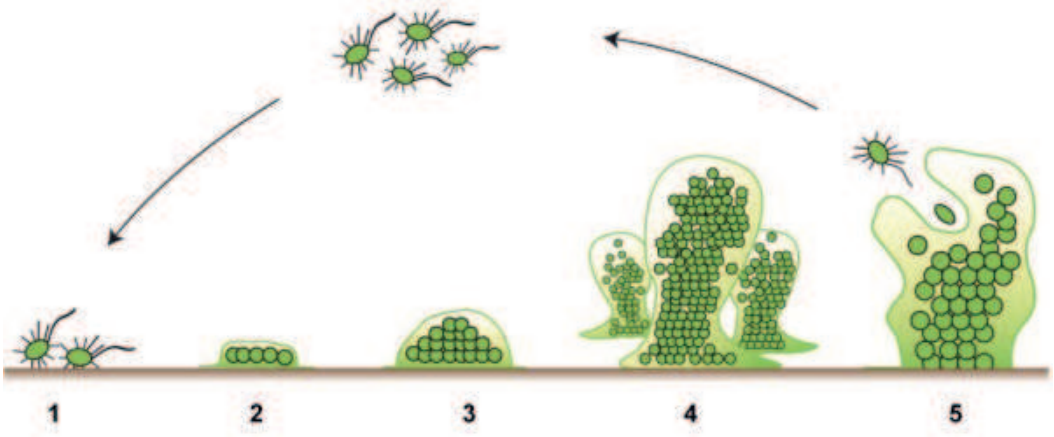


Figure 4 Biofilm formation and dispersion. Reference: (88)

1.3.2 Quorum Sensing (QS)

An excellent example for the complexity of biofilm is quorum sensing (QS). Quorum sensing is a microbial form of social behavior within a biofilm where bacteria sense their colony density and, in dependence of this density, produce and receive molecular signals in order to regulate the expression of certain genes. Thus, the gene expression in biofilm-bacteria is altered in comparison to planktonic bacteria. The aim is to ensure a sufficient cell-density before certain beneficial traits like resistance to the host's immune system as well as to antibiotics is developed. Thus, it has been concluded that a higher amount of cells makes a biofilm more virulent. (1,24,25,42–45) Interestingly, cells that detach from a biofilm and re-enter planktonic state also resume their original sensitivity to antibiotic agents.(46)

All bacteria that utilize QS produce the abovementioned signal molecules. With a growing cell-density the accumulation rate of the signal molecules increases. When a certain concentration is reached, these molecules react with compatible bacteria and induce genetic alteration, for example in favor of establishment of resistances or production of virulence factors. Also, the production of even more signal molecules is triggered this way, which is called positive feedback. While gram-negative bacteria use acyl homoserine lactones for QS, gram-positive bacteria utilize different peptides.(46,47)

S.aureus for example utilizes the Agr system (figure 5). In simplified terms, the membrane protein AgrB exports an altered version of the signal peptide AgrD, also called an auto-inducing peptide (AIP). When a certain threshold of this AIP is reached, it binds to AgrC, which is also found in the membrane of S.aureus. This triggers the phosphorylation of

AgrC and, in further consequence, of the regulator AgrA. This activated form of AgrA then reacts with RNA, which leads to alteration in the expression of different genes. Other factors like the amount of extracellular glucose can also influence the activity of the Agr QS system. High acitivity of the Agr system promotes increased virulence - this is the case particularly in acute infections. When a CVC is colonized with biofilm, the activity of Agr was found to be less. One important virulence factor in S.aureus driven by Agr QS is alpha-toxin, which, in high concentrations, can lead to severe tissue damage. (47,48)

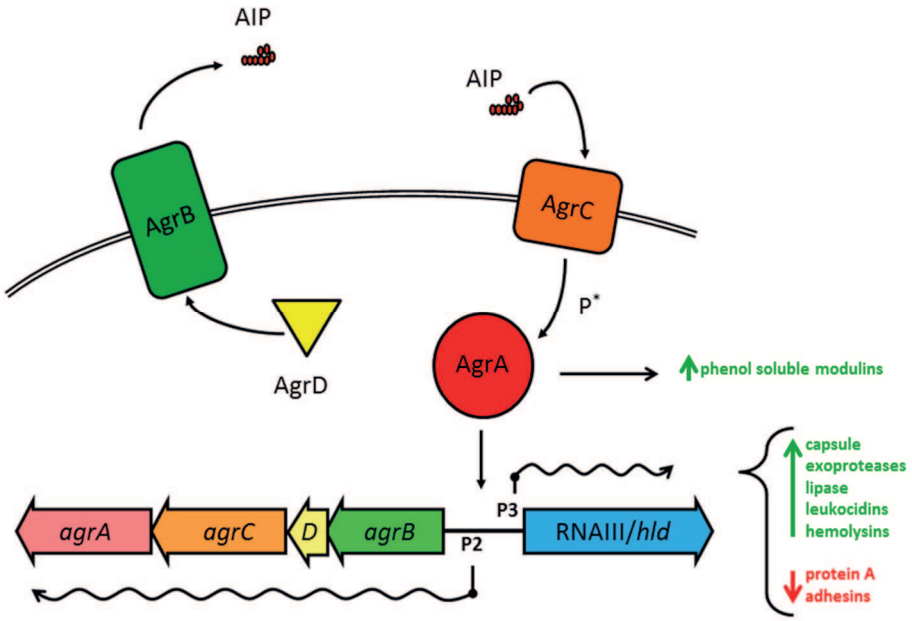


Figure 5 Quorum sensing (Agr) in Staphylococcus aureus

Reference/source: (89)

Not only the high density of inhabitants characterizes biofilms but also the diversity of those. A gram of biofilm in dental plaques for instants contains approximately 10^{11} cells including more than 700 species. With this amount of different bacteria, communication and regulation mechanisms in order to conserve interspecies balance seem necessary. The production of peptides called bacteriocins may play a role since those produced by one species were found to be cytotoxic to other species – even amongst species that are all

assumed to be main colonizers in certain settings. However, even between different species the interactions can be of constructive nature as well. (24,49–52)

1.3.3 Pathogen spectrum

A great number of bacterial species is able to form biofilms. Also, generally speaking, a biofilm can theoretically develop in many tissues. A very pathogenic, thus relevant representative is Staphylococcus aureus. It is known to actually form biofilms on many different surfaces or tissues, potentially causing osteomyelitis, septic arthritis, pneumonia, endocarditis, infections of skin and soft tissue and others. Most of these infections can lead to blood stream infection and sepsis. The infestation of a CVC, which is frequently caused by S.aureus, can lead to CRBSI.(24,48,53,54) Pseudomonas aeruginosa plays a substantial role in cystic fibrosis and is also known to cause chronic biofilm related infections of the prostate, the middle ear, open wounds and, again, through venous catheters.(54) Even usually harmless bacteria as S.epidermidis is known to cause infections of joint implants and CRBSI due to its ability to form biofilm on these surfaces.(55) Table 2 shows more relevant bacteria.

Other biofilm-forming bacteria
Streptococcus pneumonia, pyogenes, vididans
Coagulase-negative Staphylococci
Enterococcus faecalis, faecium
Enterobacter
Escherichia coli
Klebsiella
Reference: (1,23,56,57)

Table 2 Other biofilm forming bacteria

1.4 Catheter related bloodstream infections (CRBSI)

1.4.1 Biofilm dispersal

Once a biofilm has developed on a catheter surface, there is an elevated risk of CRBSI. Whether or not a CRBSI occurs is determined by many factors. An important step towards a blood stream infection and sepsis is biofilm dispersal. Biofilm breakdown is regulated by QS. The mechanisms found in S.aureus will serve as examples. These mechanisms are mediated by proteases as well as nucleases, but also by environmental factors like nutrient availability.(46)

S.aureus was found to produce 10 different proteases, which, especially at high activity, were found to disintegrate the biofilm by degrading EPS. When Agr is activated, the making rate of these proteases is enhanced. Furthermore, toxin production and therefore virulence is enhanced whereas the production of surface factors is reduced. This leads to the conclusion that proteases promote biofilm dispersal.(46,58–61)

S.aureus also produces two extracellular nucleases. One was shown to play important roles in the bacteria's survival against neutrophil extracellular traps, DNA produced and released by neutrophils to capture pathogenic cells. The nuclease activity reaches low levels especially during biofilm development.(46,62–64)

When nutrient availability is low, the so called stringent response can be triggered. It is a complex system that decreases the metabolic rate of microbes to make them less vulnerable to starvation.(65)

Other triggers for biofilm dispersal might be dispersin B. Dispersin B, which was first found in an Actinobacillus species, was observed to be capable of activating dispersal in certain S.epidermidis and S.aureus biofilms, even though no (version of) dispersin B was found to be produced by themselves.(66,67)

1.4.2 Complications and diagnosis

CRBSI is a serious complication that is associated with a significant risk of death(68) since it may lead to sepsis. Sepsis is a condition defined by the presence of infection with systemic manifestation of the same infection.(69) The systemic symptoms may present themselves in many ways as shown in table 3.

General	Temperature < 36°C (central) or > 38.3°C Tachycardia Tachypnea Altered mental state Edema or positive fluid balance Hyperglycemia without previously known diabetes mellitus
Inflammatory	Leukocytosis/leukopenia Left shift in white blood count Elevated CRP or PCT in plasma
Hemodynamic	Arterial hypotension
Organ dysfunction	Decreased oxygenation index Oliguria Elevated serum creatinine levels Prolonged blood coagulation time Ileus Thrombocytopenia Hyperbilirubinemia
Tissue perfusion	Hyperlactatemia Prolonged recapillarisation time Reference: (69)

Table 3 Signs of sepsis.

1.5 Biofilm management

Biofilms gain enhanced resistance to many antibiotic agents and to the host's immune system. Because of this, an important part of biofilm management is to prevent its formation in the first place. The following chapter will give an overview of current strategies for prevention and therapy of biofilm as well as future approaches.

1.5.1 Hygiene aspects

In their 2011 guidelines for the prevention of intravascular catheter-related infections(70), O'Grady, Alexander, Dellinger et al. emphasize the importance of hygienic precautions in preventing CRBSI.

Strong recommendations include, among others, the following (table 4).

Action	Grade of recommendation/evidence
Proper training and education of healthcare staff members that work with CVCs	IA
Frequent reevaluation of the staff's education	IA
Risk assessment for installation of a CVC	IA
Avoidance of femoral vein access for a CVC (in adults)	IA
Usage of a CVC with the lowest number of lumens possible	IB
Sterile circumstances and antiseptic skin preparation during CVC installation	IB/IA
Usage of sterile and transparent dressing for the CVC	IA
Replacement of dressing after 7 days or if looking dislocated/soiled	IB
Usage of antiseptic agents for skin before change of dressing	IA
Removal of CVC as soon as it's not mandatorily needed anymore	IA
Removal of CVC that was not definitely installed under sterile conditions	IB Reference:(70)

Table 4 Hygiene measures for CVC management and their strength of recommendation (IA: strong recommendation, good evidence; IB: strong recommendation, some evidence; Reference: (70)

1.5.2 Catheter-lock-technique

The idea behind the catheter lock technique is based on the observation that in long-term catheters biofilm tends to form inside its lumen. Antimicrobial substances like ethanol or various antibiotics (either specifically against one sensitive species/stem or in terms of a broadband cover) are included into the catheter-lumen for certain periods of time (the exact spans vary through literature). Occasionally, an anticoagulant substance like heparin is also contained. Catheter lock technique was shown to be a useful addition to systemic antibiotic therapy. (70–73)

1.5.3 Antimicrobial agents

Antibiotics are the first line therapy in acute bacterial infections. There are various different mechanisms of action. Bactericidal agents like beta-lactam-antibiotics directly attack bacterial cells by interfering with cell wall synthesis with the goal of killing them. Bacteriostatic agents like tetracyclines on the other hand inhibit the bacteria's protein biosynthesis and thus their proliferation, allowing the host's immune system to kill them on its own.(74,75)

Bacteria inside a biofilm develop advanced resistance to many of these substances. It was observed, that decreased antibiotic agent sensitivity due to biofilm is only temporary. Bacteria who detach from a biofilm and re-enter planktonic state also lose their resistance. However, this must be differentiated from heritable resistance like the production of beta-lactamase, which is not bound to biofilm.(76)

Thus, antibiotics used against biofilm require proper tissue-penetrating characteristics and should be rather acid-stable, since the pH in infected tissue can drop because of the increased oxygen-consumption. For example, Rifamycin SV was shown to meet these requirements.(71,77) The importance of considering the pH-dependent efficiency of different agents was also demonstrated by Lebeaux et al. in 2014: gentamicin, which is known to be more potent in an alkaline environment, was shown to be more effective in killing S.aureus, P.aeruginosa and E.coli in vivo when the basic amino acid L-arginine was added.(72) Also, combinations of multiple substances were observed to be more effective than monotherapy. Local antibiotics, like in catheter-lock-technique, can be added to the combination.(23,71,73)

Regarding venous catheter-related biofilm formation and CRBSI, a prophylactic systemic administration of antibiotic drugs is not recommended.(23)

1.5.4 Catheter removal

When a catheter of any kind is suspected to be origin to an infection, removal of the catheter seems like an almost intuitive reaction. However, a liberal practice of this 'play it safe'-approach requires resources and could lead to a quick increase in costs. Another argument to not remove a suspicious CVC immediately is the vascular accessibility. When access to a large, central vein is needed for further treatment, the installation of another CVC will be hardly evitable. However, the associated risks for mechanical complications

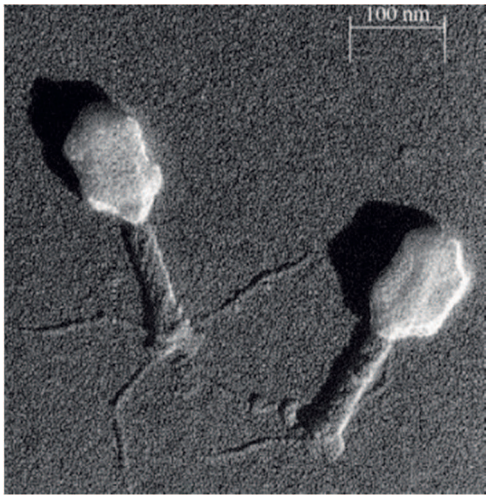
could, especially in patients where venous access is complicated, outweigh the anyhow controversial benefit of (early) removal. Therefore, individual clinical judgment considering as many factors as possible is suggested rather than following strict algorithms for leaving/removing a catheter. (56,70,78) Exceptions include S.aureus and Candida biofilms, where the pathogens' high virulence makes the removal of the CVC the superior option.(23)

1.5.5 Future approaches

With current treatment options, IMD-associated biofilm formation poses a problem for doctors, patients and economics. Due to the enhanced resistance mechanisms of biofilm bacteria in comparison to planktonic bacteria, numerous antibiotic substances, which would be the first line therapy in acute bacterial infections, are not effective enough for sufficient treatment. CRBSI originating from biofilm can lead to sepsis and increased mortality. Early removal and replacement of a suspicious CVC however increases economic costs and carries the risks of mechanical complications due to the re-installation, especially in patients where vascular accessibility is poor.(1,7)

One interesting approach currently in development is phage therapy. As Motlagh, Bhattacharjee and Goel explain in their 2016 paper 'Biofilm control with natural and genetically-modified phages'(79), this kind therapy utilizes bacteriophages, ubiquitously occurring viruses that target prokaryotes for their own reproduction. (Figure 6 & 7) They are known for integrating their own genome into certain bacteria (the selectivity varies through different sorts of bacteriophages) and exploiting their host's replication. Bacteriophages will go through two states in their cycle: virulent and temperate state. Virulent phages will lyse the host from the inside to set free the newly produced viruses. Temperate phages will allow the host bacteria to proliferate by cell division, reproducing with them. Both mechanisms could be utilized for treatment: while virulent phages are effective in killing bacteria, phages in temperate state could be used to modify the bacteria's genome to make them more prone to antibiotics and the human immune system. Also, bacteriophages in general were observed to produce substances that are able to break down a biofilm's EPS matrix itself.(79)

Another part of research aims towards production of new, potentially more efficient antibiotic substances. For example, FYL-67, a newly developed oxazolidinone antibiotic, was found to be effective in inhibition of biofilm formation.(80)



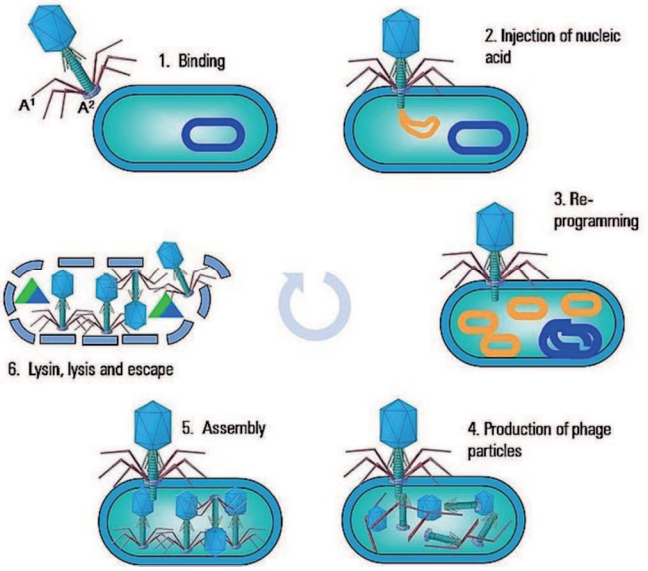


Figure 6 (left) microscopic image of two bacteriophages. Reference: (90)

Figure 7 (right) cycle of phage reproduction exploiting a prokaryotic host. Reference: (91)

1.6 Management of catheter related bloodstream infections

CRBSIs are potentially life-threatening complications of CVC and port installation. They can pass into sepsis, which is associated with high mortality.

Shah, Bosch, Thompson et al.(81) suggest that for successful management of CRBSI a certain diagnosis is required. Signs of catheter related bloodstream infection include all the abovementioned signs of sepsis as shown in table 3. Visually screening the vascular access sites for obvious infection should be performed. If signs of systemic inflammation persist and no evidence of non-catheter related infection can be gained, catheter removal is to be taken into consideration at least.(81) However, if CRBSI of a long-term CVC, which is associated with endocarditis, septic thrombosis et cetera, occurs, immediate removal of the CVC is recommended. Microbiological analysis of the tip of a removed catheter should be performed. Whether or not the suspected CVC is removed, blood cultures (perhaps taken from the potentially colonized catheter as well as from a peripheral venous access) should be performed in patients with suspected infection.(81)

Empiric administration of vancomycin/daptomycin as well as agents effective against P.aeruginosa, gram-negative rods and candida is recommended until further results from microbiological analysis are available. With the results available, treatment adjustment

should be performed accordingly. Antibiotic treatment duration can range from one to two weeks, depending on the pathogen.(81)

2 Materials and methods

Our study's goal was to investigate the patterns of microbial colonization on an explanted CVC by sequencing of the 16S rRNA gene. For this example, the CVC was separated into three different segments: extracorporeal-extravascular, intracorporeal-extravascular & intracorporeal-intravascular) (Figure 8). Another goal was to confirm possibly existing biofilms with fluorescent in situ hybridization (FISH) and confocal laser microscopy (CLM).

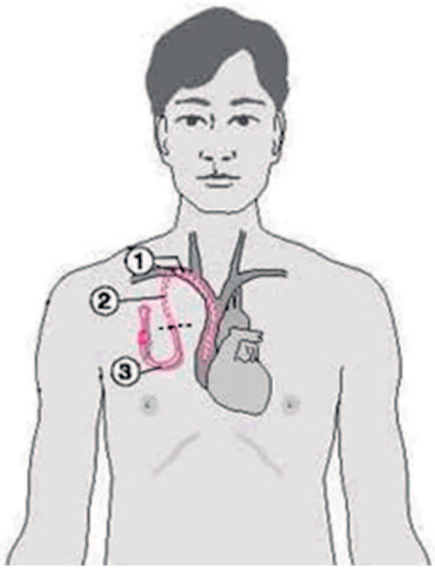


Figure 8 segments of a CVC:
1) intracorporeal-intravascular; 2) intracorporeal-extravascular; 3) extracorporeal-extravascular
Reference: (92)

2.1 Patients

Table 5 shows the inclusion criteria for our study:

Confirmed Cerebral death *(with completed and positive brain death diagnosis)*
Context of organ explantation
One or more indwelling CVCs
Male or female gender
Age between 18 and 99 years

Table 5 inclusion criteria for the study

As seen above, our study included confirmed brain-dead patients with indwelling CVCs that were suited for organ donation. Also, for comparison purposes, one aerobic and one anaerobic blood culture of 16ml each was drawn during organ explantation from the CVC as well as from a peripheral venous access site.

The acquisition of the CVC was performed immediately after organ explantation by surgical extraction of the catheter and its surrounding tissue. Neither any harm for the organ recipient nor any disfigurement of the brain-dead patient was to be expected and neither of them occurred.

3 (three) patients met the criteria and were included to the study.

2.2 Materials

Our study utilized blood cultures (aerobic & anaerobic, central & peripheral) of 16ml each and CVCs withdrawn from confirmed brain-dead organ donors.

In the first step, the extracorporeal part of the catheter was cut off by a transplantation surgeon approximately 1cm from the skin entry and stored in a sterile plastic bag without any additives at 4°C. Then the remaining, indwelling CVC including the surrounding tissue was extracted carefully and then stored in a sterile additive-less plastic bag at 4°C as well. Within 4 (four) hours after extraction, the samples were transferred to the laboratory, where the removal of the human tissue from the intracorporeal part of the CVC was performed cautiously without damaging the catheter's structures. All catheters were

separated into three parts: extracorporeal-extravascular (from now on abbreviated with D for distal), intracorporeal-extravascular (M for medial) and intracorporeal-intravascular (P for proximal). All segments had an approximate length of 5cm each. All CVCs, except CVC1, were then stored for 10 days at -20°C. Storing conditions of CVC1 are not known. Table 6 and figures 9 and 10 show the CVCs' segments and relevant data.

Catheter name	Patient (insertion site)	Storing conditions	Figure
CVC1P	Patient A (unknown)	Unknown	9
CVC2D	Patient B (jugular)	-20°C for 10 days	10A
CVC2M	Patient B (jugular)	-20°C for 10 days	10B
CVC2P	Patient B (jugular)	-20°C for 10 days	10C
CVC2aD	Patient B (femoral)	-20°C for 10 days	10D
CVC2aM	Patient B (femoral)	-20°C for 10 days	10E
CVC2aP	Patient B (femoral)	-20°C for 10 days	10F
CVC3D	Patient C (unknown)	-20°C for 10 days	10G
CVC3M	Patient C (unknown)	-20°C for 10 days	10H
CVC3P	Patient C (unknown)	-20°C for 10 days	10I

Table 6 CVC data

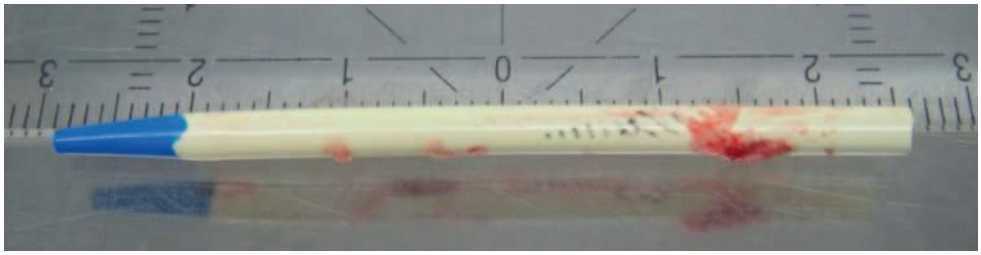


Figure 9 CVC1P

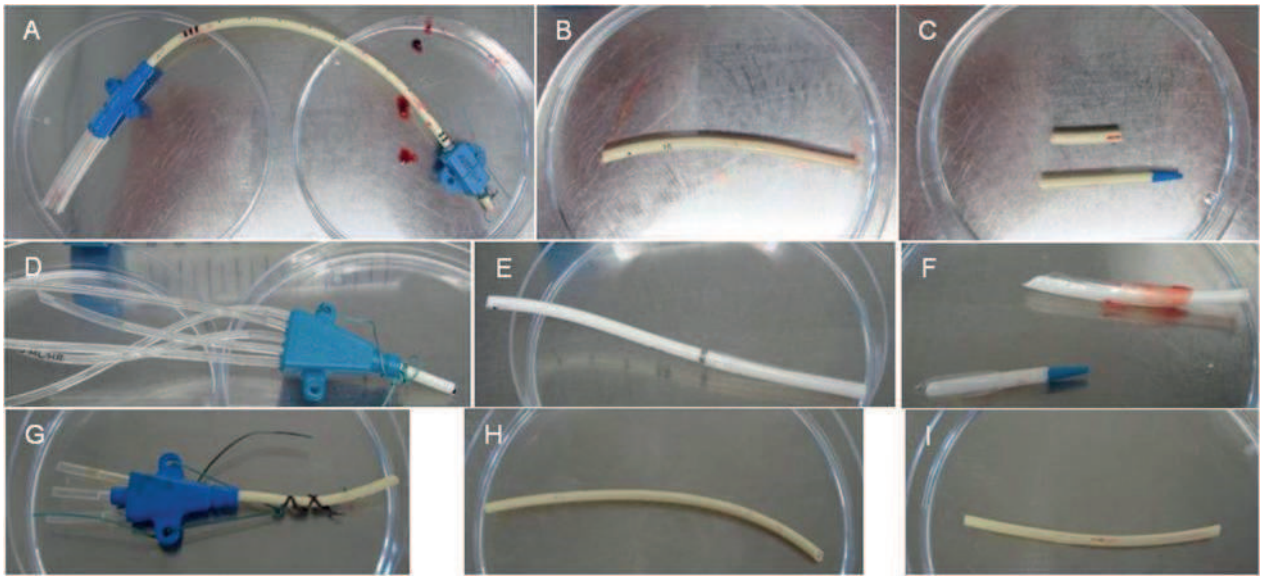


Figure 10 Segments of CVC2, CVC2a and CVC3

2.3 Methods

In the first step, approximately 1cm of every CVC segment was cut off and chopped into smaller pieces with a sterile scalpel. This material was used for isolation of the total bacterial DNA. The CVC pieces were directly transferred into the lysing-tube of the DNA isolation kit. MOBIO PowerBiofilm DNA Isolation Kit was utilized since it was specifically designed for DNA isolation from biofilm bacteria.

2.3.1 Determination of colony density

In order to determine the colony density on the CVC segments, a quantitative PCR (qPCR) was performed to identify the amount of copies of the 16S rRNA gene per cm catheter. This allows making assumptions about the number of bacteria on the CVC segments. The primers used for this experiment were the forward-primer Unibac II-515f and the reverse-primer Unibac II-927r. Three independent runs were performed. Each sample was measured twice. The data was analyzed with the statistics program SPSS.

2.3.2 Determination of biofilm composition

To determine the bacterial composition of the biofilms, the 16S rRNA gene was amplified and sequenced. The forward-primer 27f and the reverse-primer 1492r were used. The advantage of this primer set is the fact that a fragment of 1465 base pairs can be reached, which is suitable to differentiate bacterial species from each other. The experiment was performed with several different annealing temperatures and primer/template concentrations. Also, a different primer set within the 16S rRNA gene was tested as well.

2.3.3 Determination of biofilm structure

In order to conceive an idea of the three-dimensional structure of the biofilm, sections of the CVC segments were hybridized with FISH-probes and analyzed with CLM afterwards. In the first step, small sections of the CVC segments were made with a scalpel and fixed overnight in a 4% paraformaldehyde solution to prevent detachment of the biofilm during FISH treatment. For the hybridization, a mixture of EUB I, EUB II and EUB III was utilized. Also, FISH probes specifically for S.aureus and S.epidermidis were used as well. In the last step, CLM was used to detect bacteria and possibly biofilms.

3 Results

3.1 Colony density

The qPCR showed that the number of copies of the 16S rRNA gene per cm catheter was strongly depending on the diameter of the catheter segment and therefore on the surface area. As table 7 shows, the number of gene copies varied between catheters and catheter segments.

Catheter name	Catheter segment, gene copies per cm catheter		
	Distal	Medial	Proximal
CVC1	-	-	$1.03x10^7$ ($4.15x10^5$)
CVC2	$4.43x10^4$ ($9.71x10^3$)	$3.87x10^6$ ($1.95x10^4$)	$3.28x10^7$ ($5.58x10^6$)
CVC2a	$1.06x10^5$ ($2.08x10^4$)	$7.94x10^6$ ($5.29x10^4$)	$2.02x10^6$ ($1.53x10^5$)
CVC3	$5.75x10^7$ ($6.29x10^6$)	$1.87x10^6$ ($1.92x10^5$)	$6.00x10^6$ ($9.45x10^5$)

Table 7 16S rRNA gene copies per cm catheter as expression of microbial abundance on the catheter segments, as detected with qPCR (standard deviations in brackets)

3.2 Biofilm composition

The determination of bacterial composition of biofilms on our catheters was performed by amplification and sequencing of the 16S rRNA gene for which the total DNA isolated from a catheter piece of 1cm in length was utilized. The primers 27F (forward) and 1492R (reverse) were used to extend the length of the acquired fragment of the gene to a maximum.

Even though annealing temperature and template/primer concentration as well as primer sets were modified, no suitable material for sequencing could be obtained with PCR.

3.3 Biofilm structure

No data about the three-dimensional structure of biofilms on our catheters could be obtained, since neither biofilms nor single bacteria were found with CLM.

4 Discussion

Since central venous access becomes more and more important for various forms of diagnostics and treatment even outside of ICUs, the prevalence of biofilm formation inside the IMDs used for this kind of vascular access increased significantly. Biofilm however can lead to systemic infection (CRBSI), sepsis and eventually death.
250,000 - 400,000 cases of CRBSI were reported in the USA in 2002, consuming $296 million to $2,3 billion. An estimated 18,000 – 23,000 cases of CRBSI occurred in intensive care settings across the USA in 2014. (1,2,70,82) This demonstrates the necessity of further research for early detection and treatment.

Our study addressed the finding of a way for detection and understanding of biofilm. We were able to show that quantitative PCR can be used for detection of bacterial cells in CVCs, although it is accompanied by a big effort. Also a qPCR cannot differentiate between living and dead bacterial cells, which makes it less suitable for clinical use since living cells only are relevant for patients.
We were not able to find an explanation for the failure of the PCR-experiment. It is possible, that the DNA concentration of the DNA-isolate was too low because of a lack of a sufficient number of bacteria. This could be caused by a too short indwelling time of the catheters. Also, a previous systemic antimicrobial therapy could have affected the amount of bacteria on the CVCs' surfaces. The usage of an unsuitable DNA isolation kit could be another possible explanation for the results. The different results between the qPCR and PCR experiment could be explained by the enhanced sensitivity of the qPCR.
The shorts catheterization time was possibly the cause for the lack of biofilm formation, which then led to a failure of visualization. It was shown previously, that CLM is generally suitable for visualizing the 3D structure of biofilms on catheter surfaces. Like the qPCR, it is very time consuming and expensive and therefore not suited for routine clinical testing.

Aside from detection methods, further strategies against biofilm and CRBSI will have to be developed because of the high risk for the patients as well as the high costs for the health care systems. Currently, there are three options to proceed against biofilm: prevention, treatment and removal of the populated CVC.

Prevention of biofilm formation can be achieved by obeying high hygiene standards while implantation of a new catheter as well as the handling of an indwelling one. The idea behind prevention is not to let a catheter be populated by pathogens in the first place. Critical measures include sufficient instruction of staff in contact with CVCs as well as reevaluation of those skills in appropriate intervals, careful consideration of patients and installation sites that are suited for a CVC implantation, usage of catheters with as much lumens as necessary but as less as possible, frequent replacement of dressing and usage of antiseptic agents when replacing as well as early removal of CVCs that are no longer needed for treatment or were not implanted under sterile circumstances. Implementation of many of these measures will require initial financial effort and time.
Another form of prevention is the catheter lock technique, where antimicrobial agents dwell inside a catheter to interfere with bacterial attachment and biofilm formation.

Treatment of biofilm is currently problematic and thus a large research field. Biofilm has mechanisms to gain resistance to a number of treatment options like, for example, many antibiotics. However, some antibiotics offer promising results in in vitro experiments. Rifamycin SV and Gentamicin were shown to be effective against biofilm in some settings.

When prevention and treatment fail, catheter removal is the last option.

5 Conclusion

Our study presented mixed results. While qPCR can be used to detect bacterial cells on a CVC, this method is unable to differentiate between living and dead cells, which makes it less valuable for clinical use. We were not able to gain further information about composition and structure of biofilms, possibly because the indwelling time of the CVCs was too short for sufficient biofilm formation. Further experiments similar to our one but with adapted study design could be performed in future for more promising results. However, methods used over the course of our study (qPCR, PCR, FISH, CLM) are all accompanied by large effort and/or costs, making them rather unsuitable for ordinary clinical use.

Overall, prevention appears to be the most sufficient way to handle biofilm. While new potent antibiotic drugs could be developed in the future to decrease patient mortality due to biofilm and its consequences, prevention might be the only method that lowers the costs for the health care system at the same time. Catheter removal is neither cost efficient nor save for the patient, since the possibly necessary re-implantation of a new CVC carries the risk of complications during installation. Also, as long as preventing mechanisms are not implemented ideally, reformation of biofilm is likely to occur.

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