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Abstract

Biofilm formation plays a key role in development of catheter-associated bacteremia especially during the long-term catheterization. Despite the high mortality rate from these infections, origin and structure of catheter-associated biofilms remains poorly described. Most of the previous study addressed the issue of biofilm formation and detection using conventional cultivation-dependent methods. In this study, a multiphasic approach was applied for characterizing biofilms in central venous catheters (CVCs) that were removed from the patient's body. The 16S rRNA gene copies cm^{-1} CVC on different parts of explanted catheters were analyzed using a quantitative polymerase chain reaction (qPCR) approach. The biofilms at the intravasal CVC sites were visualized using fluorescence *in situ* hybridization (FISH) and confocal laser scanning microscopy (CLSM). The microbial diversity was resolved using 16S rRNA gene clone libraries. qPCR approach revealed that the gene copies cm^{-1} CVC were depended on the catheter part and on the type of catheter. Diverse but highly individual compositions of the biofilm community were found in the catheters, consisting of hospital/human-related strains (*Staphylococcus aureus* or *Staphylococcus epidermidis*) and environmental-associated strains (*Brucella* sp., *Herbaspirillum seropaedicae*). In addition several clinically relevant strains of *Staphylococcus aureus, Stenotrophomonas maltophilia,* and *Pseudomonas aeruginosa* were tested for i) biofilm formation on CVC sections and in a specially developed *in vitro* plant and ii) viability by treating them with *in-house* developed antimicrobial substances. Better understanding of colonization structure and diversity within the biofilms on the CVC surfaces should facilitate prevention and adequate treatment of the CVC-associated bacteremia.

Kurzfassung:

Die Biofilmbildung spielt hauptsächlich bei Langzeit-Katheterisierung eine entscheidende Rolle im Auftreten von Katheter-assoziierten Infektionen. Trotz der hohen Sterblichkeit bleiben der Ursprung und der Aufbau dieser Biofilme unbekannt. Viele Studien beschäftigen sich mit der Bildung und der Detektion von Biofilmen, durch Anwendung von kultivierungsabhänigen Methoden. In dieser Studie wurden durch den

Einsatz von multiphasischen Methoden Biofilme auf Zentralvenenkathetern (ZVKs), die von Patienten/Innen entfernt wurden untersucht. Die Anzahl an 16S rRNA Gen Kopien cm^{-1} ZVK wurde auf unterschiedlichen Teilen explantierter Katheter mit Hilfe der quantitativer Polymeranse Ketten Reaktion (qPCR) untersucht. Die Biofilme auf der intravasalen Seite der ZVKs wurden mittels Fluoreszenz *in situ* Hybridisierung (FISH) und Konfokal Mikroskopie (CLSM) untersucht. Die mikrobielle Diversität wurde mit Hilfe von 16S rRNA Gen Klonbibliotheken aufgeklärt. Die qPCR Analyse zeigte, dass die Anzahl an 16S rRNA Gen Kopien cm^{-1} ZVK abhängig vom Katheter-Abschnitt und Kathetertyp ist. Eine diverse aber individuelle Zusammensetzung wurde entdeckt, die menschliche/krankenhaus-verwandte Stämme (*Staphylococcus aureus*, *Staphylococcus epidermidis*) als auch umwelt-assoziierte Stämme (*Brucella* sp., *Herbaspirillum seropaedicae*) enthielt. Zusätzlich wurden einige klinisch relevante Stämme von *S. aureus*, *Stenotrophomonas maltophilia* und *Pseudomonas aeruginosa* auf i) Biofilmbildung auf ZVK-Schnitten sowie in einem speziell entwickeltem Biofilm-Bildungs-Reaktor getestet, ii) und die Lebensfähigkeit gegenüber einer speziellen im Haus entwickelter Substanz getestet. Ein besseres Verständnis über die Bildung von Biofilmen auf ZVKs soll Vorbeugung und entsprechende Behandlung ermöglichen.

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Introduction

Definition of biofilms, their ecological role and occurrence.

Biofilms can be defined as mixture of different bacteria which are irreversibly connected to the surface they stick on and embedded in a matrix that is in many cases polysaccharide (Donlan 2002). Biofilms represent the oldest strategy of a symbiotic community on earth. The discovered fossilized biofilms were older than 3.5 billion years (De Carvalho 2007). An outstanding amount of bacteria (up to 99%) live in a community organized as biofilm. Biofilms can occur in different ecosystems and fulfill specific ecosystem functions. Basically, they are very important in degrading organic matter and pollutants, and cycling of sulfur, nitrogen and many metals (Davey & O´Tool 2000). Biofilm formation is an efficient way of life and, therefore, they can be found on both environmental and human-created surfaces e.g. regions of high radiation - nuclear power plants (De Carvalho 2007). Microorganisms living in the biofilm are protected by the produced matrix and adapt to harsh environmental conditions. Within this matrix the cells are protected from environmental stresses, have the ability to colonize various niches and can stay in nutrient poor areas (Jefferson 2004). The environmental stress protection plays a key role in bacterial resistance to antibiotics and cell destructive agents e.g. H_2O_2 (Costerton *et al.* 1999). This protective function makes the removal of biofilms from the human created surface such as medical indwelling devices a challenge.

Biological and biochemical aspects of biofilm formation.

Microorganisms that are involved in the biofilm formation.

Many human infections are associated with biofilm-forming bacteria. The variation of bacteria depends on the infection. For instance in dental caries the common bacteria are acidogenic Gram-positive cocci (Costerton *et al.* 1999). Especially, catheters used on different parts of the body can show completely different taxonomic composition and structural patterns (Donlan 2001).

The most important representatives in the central venous catheters (CVCs) are the coagulase-negative staphylococci, *Staphylococcus aureus*, *Enterococcus faecalis*, *Klebsiella pneumonia* and *Candida albicans* (Donlan 2001). *S. epidermidis* belongs to coagulase negative *Staphyloccus*. Despite the fact *S. epidermidis* belongs to the natural microbiota of the human skin, it can cause pyogenic infections, especially in patients with indwelling devices (Helewa & Embil 2007). *S. aureus*, a coagulase positive *Staphylococcus* species, has nearly the same environment as *S. epidermidis*: nasal passage or axillae (Foster 1996). *Enterococcus faecalis* is a Gram-positive bacterium that inhabits human intestine tract and may colonize other vertebrates and non-vertebrates, but also different unspoiled food. As a facultative or opportunistic pathogen it causes endogenous inflammations, especially in immune suppressed persons (http://www.bvl.bund.de/SharedDocs/Downloads/06_Gentechnik/ZKBS/01_Allgemeine_Stellungnahmen_deutsch/02_Bakterien/Enterococcus_faecalis.pdf?__blob=publicationFile). *Klebsiella pneumonia* is a Gram-negative bacterium which can cause different infections: blood stream infections, pneumonia, meningitis or wound infections. *K. pneumonia* can be detected in the human intestine or in the human stool. Infections risk by *K. pneumonia* is higher for patients undergoing medical procedures (catheter, breathing machines) than for healthy people (http://www.cdc.gov/HAI/organisms/klebsiella/klebsiella.html). *C. albicans* belongs to the taxonomic group of yeasts. *C. albicans* lives on the skin or in the mucous membrane. *C. albicans* is one member of 20 *Candida* species which can cause infections known as candidiasis (http://www.cdc.gov/fungal/diseases/candidiasis/).

Characteristics of ground surface materials relevant for biofilm formation.

The effectiveness of microbial attachment to a specific surface depends on different properties of the surface and microbes. Typical bacterial adaptation is formation of fimbriae. They have a strong effect on hydrophobicity by having a high amount of hydrophobic amino acids. Therefore, they play a key role by overcoming the initial repulsive electrostatic forces (Donlan 2002).

The ground surface characteristics are important for the attachment of microorganisms and, therefore for the biofilm formation. Porosity, hydrophobicity, charge, roughness and surface functional groups effect bacterial attachment to the surface (Treter & Macedo 2011). With an increased roughness the shear forces are reduced and with a larger surface the attachment of bacteria is easier (Donlan 2002). MacKintosh *et al.* (2005) has shown that the hydrophobicity play a significant role in attachment. Especially, *S. epidermidis* prefers to attach to hydrophobic surfaces. A general problem of interaction between bacterial cell and surface is lack of standardized methods for analysis (Donlan 2002).

Chemical composition of biofilms.

The polysaccharide matrix consists of different types of macromolecules primarily produced by microorganisms. The protective layer also known as matrix or extracellular polymeric substance has many advantages for the cells living in this environment. The matrix consists up to 97% of water. The main macromolecules in the biofilm are different types of polysaccharides. The majority of the polysaccharides are polyanionic because of the uronic acids (D-glucuronic acid, D-galacturonic acid, D-mannuronic acid) or ketal-linked pyruvate (Sutherland 2001). Some exopolysaccharide substances may interact with metal ions, divalent cathions, proteins, DNA lipids and humic substances (Donlan 2002). Beside the polysaccharides, other molecules are incorporated in the biofilm depending on the habitat (Donlan 2002). In medical indwelling devices, fibrin, red blood cells and platelets are often incorporated in the biofilm (Donlan 2001).

The architecture of a biofilm depends on environmental conditions (Stooley *et al.* 2002). In cultivation studies, the biofilm formed mushroom-like or undefined structures in an area with low laminar flow whereas in regions with higher laminar flow the biofilm formed filamentous streamers (Stooley 2002).

Stages of biofilm formation and cell communication within the biofilm.

The formation of biofilm is divided in five stages (Fig. 1). In the initial step, when bacteria attach to the surface, they are capable of releasing from the surface again and become planktonic cells. At this stage, the bacteria are able to move on the surface and it is not predetermined whether the cells start to form a biofilm. In the next step, the bacteria produce exopolysaccharides and attach irreversibly to the ground material. In the third step, they form a typical biofilm with colony/water channel structure. In the last steps, the biofilm may release its parts or planctonic cells (Stooley 2002).

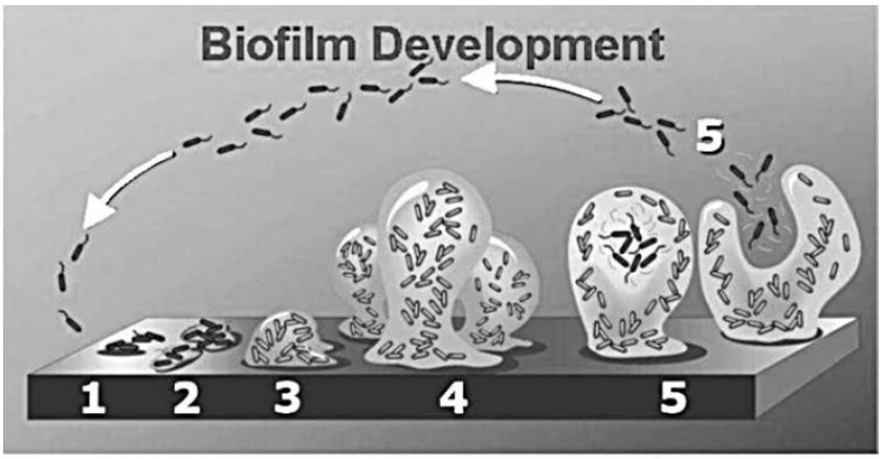

Figure 1 Stages of biofilm formation (Stooley 2002). 1) Initial attachment, 2) Polysaccharide production, 3-4) Biofilm formation, 5) Mature biofilm – release of planktonic cells or biofilm parts.

Communication between microorganisms during biofilm formation plays a key role for this process (Li & Tian 2012). In study of *Pseudomonas aeruginosa,* a knocked *lasI* genes led to only 20% biofilm thickness compared to the wild type strain (Stooley 2002). Gram-positive and Gram-negative bacteria have different communication systems. Gram-negative bacteria use the *lux* communication system. The gene *luxI* encodes for an autoinducer synthase, which produces acyl-homoserine lactone (AHL). By binding on the receptor in the cytoplasm and reaching a critical threshold, the operon is activated for luciferase (Waters & Bassler 2005). Gram-positive cells communicate with each other using modified oligopeptides and a “two-component” type membrane-bound histidine kinase (Waters & Bassler 2005). The signaling is achieved by phosphorylation cascade. This cascade influences the activity of DNA-binding transcriptional regulatory protein (Waters 2005). The communication *C. albicans* in biofilm of is similar to bacteria.

They also use different molecules released in the surrounding. Farnesol and tyrosol are the key molecules that are involved in *C. albicans* communication (Mallick & Bennett 2013).

Central venous catheters (CVCs) and their colonization by microorganisms.

History of CVC invention and application.

The invention of catheters was one of the most important findings in medical history. The development of CVCs for modern healthcare applications would not be possible without invention of intravenous catheters. The function of the first intravenous catheters was for the blood transfer. Experimentation by transferring blood from one person to another was done for the first time in 1492 (Rivera *et al.* 2005).

The rise of intravenous transfusions began in 1658, when Christopher Wen invented the first infusion device. The new infusion device opened the prospective for further animals-animals and animal-humans blood transfusion studies. In 1795 Philip Syng Physick came up with the idea to perform a human-to-human transfusion. But it was not reported whether he tested his idea on humans. Dr. James Blundell used this idea and performed several successful blood transfusion experiments (Rivera *et al.* 2005).

In the 19th century important devices, especially hollow-needles and syringes were improved. The First World War boosted again the improvement of these devices and new devices were developed (Rivera *et al.* 2005). Parallel to the development of new materials and the improvement peripheral intravenous catheters, Forssman described in 1929 an experiment that he did on his own body. He punctured his arm vein and put a plastic tube to his heart. In 1950, Aubaniac reported about the puncturing of the subcalvian vein for the first time (B. Braun Melsungen AG 2002).

Catheter types and their characteristics.

The types of CVCs, which are used in daily clinical routine, depend on the purpose. The advantage of a CVC compared to a peripheral intravenous catheter is that these types of

catheters can be used for long-term treatment of patients. The long-term treatment is often associated with chemotherapy or dialysis. Other applications are the quick administration of drugs or the administration of irritating or toxic drugs. Among the advantages of CVC application are the possibility to give high volumes of different substances, to prevent a collapse of the peripheral vessels and application of solutions with high osmolality (B. Braun Melsungen AG 2002).

Nowadays different types of CVCs are available. The three main types of catheters are: i) tunneled catheters, ii) non-tunneled catheters and iii) peripherally inserted central catheters (Figure 2). Tunneled catheters, which were named by implantation method, are used for long term catheterization (http://www.plymouthhospitals.nhs.uk/ourservices/clinicaldepartments/Documents/PHNT%20CVAD%20Guidelines%20%20November_%202011.pdf). The tunneled catheters are placed in the subclavian vein. The middle part of the catheter is in the tissue. In the past few years the rise of implantable port catheters improved the patient´s living by being more discrete and easier for maintenance (http://www.plymouthhospitals.nhs.uk/ourservices/clinicaldepartments/Documents/PHNT%20CVAD%20Guidelines%20%20November_%202011.pdf). This type of catheters consists of chamber sealed with self-sealing silicone membrane, a catheter connector, which brings the catheter and the port together, and the catheter itself. The implantable port can be placed under the skin of the chest or arm (Smith-medical 2009).

In contrast, when a quick access to large blood vessel is needed, normally a non-tunneled catheter is a catheter of choice (Mickley 2002). The peripherally inserted central venous catheter is placed in a bigger peripheral vena. The cechalic or the basilica veins are typical insertion sites. After insertion, the catheter is further introduced in the distal superior vena cava or the cavoatrial junction, or the superior vena cava (www.jointcommission.org/assets/1/6/CLABSI_Toolkit_Tool_1-3_Pictures_of_Central_Venous_Catheters_F.pdf).

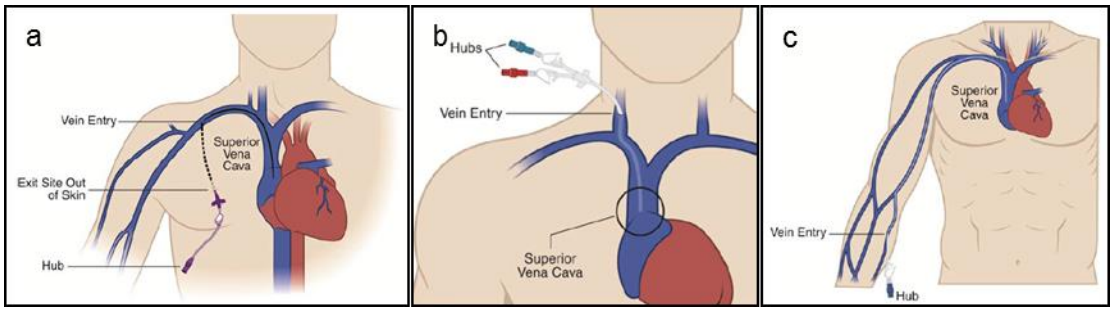

Figure 2 Different implantation methods used for catheter insertion (www.jointcommission.org/assets/1/6/CLABSI_Toolkit_Tool_1-3_Pictures_of_Central_Venous_Catheters_F.pdf). a) Tunneled catheter; b) non-tunneled catheter; c) peripherally inserted central venous catheter.

The catheter's material is a very important issue because it defines the fastness of bacterial attachment to the catheter. Standard materials for the production of CVCs are: polyethylene, polyvinylchloride, polyurethane and silicone. These materials are used for the production of haemodialysis CVCs. Among all materials, silicone is the best one due to its low thrombogenic characteristics (Mickley 2002). Bacteria have different preferences in colonizing material surfaces: *S. epidermidis* attaches to teflon very effectively, whereas *S. aureus*, *E. coli* and *P. aeruginosa* succeed in attaching to polyurethane (Treter 2011). To make the catheter materials more resistant to bacterial colonization and biofilm formation, manufacturing companies coat the catheters with different antimicrobials. These antimicrobial substances are shown in Table 1. The effectiveness of the different coatings is controversial. A meta-study of 56 publications of 16512 catheters with 11 different coatings showed that coated catheters reduce the absolute risk of getting an infection by 2% (Lei *et al.* 2013). In contrast, the other meta-study of silver-impregnated catheters revealed that there is no reduction of blood stream infections and catheter colonization (Chen *et al.* 2014).

Table 1 Antimicrobial coatings used by different manufacturers (Elliott 2005).

Antimicrobial	Surface coated	Manufacturer or distributor
Minocycline and rifampicin	External and internal	Bio-guard Spectrum™ Cook Spectrum, Cook Critical Care, Bloomington, Ind., U.S.A.
Chlorhexidine and silver sulphadiazine	External	Arrowguard Blue™, Arrow International, Reading, Pa., U.S.A.
Silver and platinum particles in a carbon-based polyurethane*	External and internal	Vygon (UK) Ltd, Gloucester, U.K.
Benzalkonium chloride	External and internal	Becton Dickinson (UK) Ltd, Swindon, U.K.
BZC-heparin bonded	External and internal	AMC Thromboshield™ Baxter, Irvine, Calif., U.S.A.

*Only available as a PICC (peripherally inserted central catheter).

Implantation of CVCs in human body – implantation sites and methods

Insertion of CVC depends on many factors. Different skin alterations (scars, burns or infections) or extraordinary anatomy may influence the decision of CVC insertion site. In clinical routine, there are seven different insertion sites: internal jugular vein, subclavian vein, external jugular vein, basilar vein, common femoral vein and axillary vein cephalic vein (Beutlhauser 2012). The internal jugular vein (70%) and the subclavian vein (19%) are the mainly used insertion sites in German hospitals (Beulhauser 2012). In Germany, the internal jugular vein is mainly used because it is i) easy to find; ii) fixed by a big muscle behind; and iii) always filled with blood in lying position. An alternative insertion site is the subclavian vein. The advantages of this vein are the quickly insertion and the longer area between the skin barrier. Moreover, usage of this vein reduces the infection risk (Beutlhauser 2012).

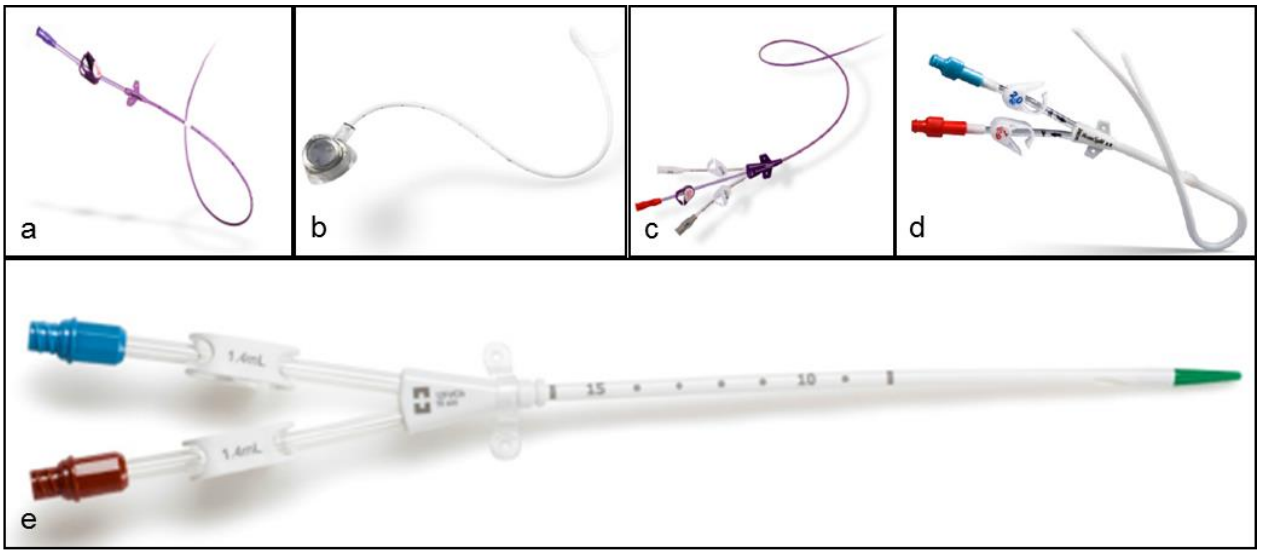

Figure 3 Types of CVCs for different applications (www.jointcommission.org/assets/1/6/CLABSI_Toolkit_Tool_1-3_Pictures_of_Central_Venous_Catheters_F .pdf). a) Power Hickman ® Tunneled Central Venous Catheter, b) PowerPort ® Vuw Implantable Port, c) PowerPICC ® Catheter, d) HemoSplit® Long-Term Dialysis Catheter, e) MAHURKAR™ Elite Dialysis Catheter.

The external jugular vein and the common femoral vein are very rare implantation sites in 5% and 2% of all cases, respectively (Beutlhauser 2012). The advantage of using the external jugular vein is the implantation of the CVC without ultrasonic guidance (Mickley 2002). Implantation of the CVC in the common femoral vein reduces the risk of pneumothorax, haemothorax, cardiac arrhythmia and pericardial tamponate but this site has a higher chance of a catheter-associated bacterial colonization (Mickley 2002). Based on the high infection risk, the common femoral should be the second choice (Consensus Statement, OIG 2011).

Different implantation methods have been developed in the last decades: a) surgical venous incision; b) catheter-through-neddle technique; c) catheter over needle-technique; d) catheter-through canulla technique; and e) guidewire or seldinger technique (B. Braun Melsungen AG 2002).

Surgical venous incision should only be applied by specialists when the long-term catheterization (Silastik-Catheter, Broviac-Catheter) is needed. To place the catheter by using this method, the vein should be opened via small section, then the catheter is placed, and finally the vein and the skin are closed via suture (B. Braun Melsungen AG 2002, Beutlhauser 2002).

For the catheter-through-needle technique, the catheter is placed in the correct position in the vein by puncturing the vena cava with a steel needle. Disadvantage of this method is formation of hematomas because of the loose connection between the puncture hole in the vein and the catheter. Another disadvantage is the possibility that parts of a broken catheter tubes can lead to a catheter embolism (B. Braun Melsungen AG 2002).

For the catheter-over-needle technique, the catheter is placed around a steel needle. With the steel needle the vein gets punctured and then the needle has to be removed. The advantage of this method is that there is a reduced hematoma formation. The disadvantage of this method is large diameter of the needle that makes the implantation of the catheter more difficult and without a guidewire it is very hard to place the catheter in a curved vein (B. Braun Melsungen AG 2002).

The catheter-through-cannula (B. Braun Melsungen AG 2002) technique or Braunülentechnik (in German) was developed in 1960s (Beutlhauser 2009). When this method is applied, the blood vessel gets pre-punctured using an intravenous catheter which consists of a plastic cannula and a steel needle (in German: Braunüle). After punctuation, the needle is removed and the plastic cannula stays in the vein. In the next step, the catheter is air-tightly connected to the cannula. After placing the catheter in the correct position, the cannula gets removed. The cannula prevents the off-shearing of catheter parts. This method is the standard method used in clinical procedures or in emergency cases (B. Braun Melsungen AG 2002).

The guidewire technique or Seldinger technique, which was developed in 1953, is similar to the catheter-through-cannula technique (B. Braun Melsungen AG 2002). For this method, the vein gets punctured using a steel needle of a small diameter. The next step is inserting a flexible wire through the needle in the vein. Afterwards, the needle has to be removed and the catheter is put over the guidewire in the vein. The guidewire stabilizes the catheter and helps to bring the catheter in the correct position. The advantage of this method is that there is almost no hematoma formation. This method is one of the best methods in hospitals for all applications, despite the difficult implantation and contamination risk (B. Braun Melsungen AG 2002).

CVC related blood stream infection (CRBSI).

The CVC related blood stream infections (CRBSIs) are a serious problem when catheters are implanted in a patient. The CRBSI is a bacteremia caused by bacterial colonization of an intra-venous catheter (Fletcher 2005). Statistically, 60% of all nosocomial infections are based on catheter related infections (Consensus Statement, OIG 2011). The estimated mortality rate is between 3% and 25%. In the USA, nearly 100.000 deaths are reported because of CRBSI. The CRBSIs are also related to an economic problem. The treatment costs increase by 34.500 to 65.000 US$. In Austria, approximately 6500 CRBSI are reported annually (Consensus Statement, OIG 2011).

Different methods are established in clinical routine to remove biofilms from the catheter. A bacteria-free catheter is essential to avoid a constant source of infection. Therefore, treatment of catheter-biofilms using different types of antibiotics are applied for successful bacteria reduction. A combination of different antibiotics is often used for treatment. Rifampin and fluoroqinolone are antibiotics that are often used as single substance or in combination for treatment of CRBSI because of their capability to penetrate the polysaccharide matrix (Donlan 2011). A common method is the antimicrobial lock technique (ALT). This method is very effective in destroying bacterial and fungal cells in the catheter (Donlan 2011). A combination of vancomycin and heparin were often used as ALT solution but failed in biofilm destruction. In contrast, minocycline and EDTA removed bacterial and fungal biofilms in an animal model (Sousa *et al.* 2011). A disadvantage of this method is high concentration of antibiotics (5-6 mg/ml) that can have a toxic effect to the patient when this solution diffuses into the blood system (Donlan 2011).

Modern methods of biofilm treatment in the CVCs try to avoid antimicrobial chemicals. Ethanol, chelating agents and taurolidine-citrate are validated in human studies, whereas biofilm dispersants, bacteriophages, nitric oxide and enzyme inhibitors and quorum-sensing inhibitors are not validated in human studies (Donlan 2011).

Prevention approach is suggested to be the best way to avoid biofilm formation (Donlan 2011). Several criteria are essential to avoid colonization of the catheter and biofilm formation: aseptic insertion of the catheter, its regular cleaning and usage of

disinfectants (Consensus Statement, OIG 2011). These criteria are underlined by a study of Longmate *et al.* (2011). This surveyed emerging of CRBSI when different actions were introduced in hospital routine. These actions included aseptic techniques, a mixture of 2% chlorhexidine and 70% ethanol as a skin disinfectant, the avoidance of the femoral vein as catheter insertion site, CVC insertion checklists and other actions. Combining all these actions resulted in a decrease of 3.38 CRBSI per 1000 CVC days to 0.46 between year 1 and 3. Between year 1 and 4 the incidence fell from 3.38 to 0 CRBI per 1000 CVC days.

Detection of CVC-associated bacteria in biofilms.

Cultivation-based methods.

Cultivation-based methods are standard methods that are used for detecting microorganisms on catheter surfaces. An important criterion is whether the catheter remains in the patient or has to be removed. An *in-situ* catheter only allows investigation of the catheter lumen by using endoluminal brush or different blood culturing methods. One of the blood culturing methods is the differential-time-to-positivity-test (DTP) (Consensus Statement, OIG). For the endoluminal brush methods, a small nylon bristle (8mm long) is introduced in the catheter and put to the front end of the catheter. The bristle is placed in a tube with phosphate buffered saline (PBS) and by ultra-sonication (44 KHz, 1 minute) and vortex (15 seconds) steps the bacteria are released (Kite *et al.* 1997). Bacterial suspensions are spread on 5% on blood agar plates and are incubated overnight at 37°C. The catheters are considered as significantly colonized when colony counts exceed 100 colony forming units per milliliter (CFU ml^{-1}). The drawback of this method is that only bacteria of the inner side of the catheter can be detected (Kite 1997).

The at-least-invasive-test is a differential-time-to-positivity-test (DTP). It is an advancement of traditional quantitative blood culture. This method is based on the blood taking from the CVC and from the periphery vein. Blot (1998) has shown that the amount of bacteria in a culture is dependent on the time to positivity and the length of the lag-phase. A sensitivity of 100% was reached at a cut-off time of 120 minutes. Furthermore, the same bacterium species has to be detected in the peripheral and central blood

culture (Consensus statement, OIG 2011). The workflow of this method is relatively simple. Before starting with blood taking, skin and the catheter hub are disinfected in a relatively short period (Raad *et al.* 2004, Bouza *et al.* 2007). To avoid contamination of antimicrobial drugs, the first aliquot of taken blood (10 ml) might be discarded from the catheter (Raad *et al.* 2004). The blood taking from the catheter should be done simultaneously. New methods allow an automated sample management by using machine-controlled culture detectors. These machines record the positivity of the blood culture in regular periods by measuring the consumed oxygen and the produced carbon dioxide by measuring fluorescence. The fluorescence increases proportional to the increasing carbon dioxide and the decreasing oxide in a vial (https://www.bd.com/ds/technicalCenter/clsi/clsi-9000bc2.pdf). According to Sabatier *et al.* (2013), many studies had problems in taking blood cultures from the peripheral vein. The handling with blood cultures is often related to frequent contamination. Furthermore, this method is tedious for the laboratory personnel and is costly (Bouza *et al.* 2007). Another technical issue is coupled with storage conditions of the blood samples. Schwetz *et al.* (2013) were able to show that storage of blood samples for 24 hours at room temperature led to false negative DTP results. Schwetz *et al.* (2013) recommended sample storage at 4°C.

A removal of the catheter extends the possibilities of investigation by different semi- and quantitative methods. One semi-quantitative test is the Maki test (Maki *et al.*1977).The principle of this method is cultivation of bacteria on the outer side of the catheter by rolling them over a 5% sheep-blood agar plate. The incubation is carried out at 37°C. Although this method has up to 90% sensitivity (Consensus Statement, OIG 2011), it has many drawbacks. The specificity of this method is only 55% (Consensus Statement, OIG 2011). The disadvantages of the Maki method are the cross contamination of exit site infections during catheter removal and the low detection cut-off of 15 CFU per 6 cm of rolled catheter tip (Kite 1997). According to Kite (1997), Maki method is less sensitive (82%) and less specific (66%) compared to the endoluminal brush method (Sensitivity: 95%; Specificity: 84%). The Brun-Buisson method is also frequently applied when the catheter is removed. Bacteria are removed from the catheter by centrifugation using the catheter tip and 1 ml of water. In the next step, 0.1 ml of this cell suspension are applied

on a 5% horse-blood agar plate. Agar plates are incubated at 37°C (Brun-Buisson *et al.* 1987). Sensitivity of this method is 92% whereas specificity is 98% (Consensus Statement, OIG 2011).

In general, cultivation-dependent methods have different disadvantages when compared to cultivation-independent methods. The application of cultivation-dependent methods is time consuming for lab personnel. One important issue is dormancy of microbial cells. These cells are alive but do not grow under *in-vitro* conditions. Especially, microbial cells in biofilms are often considered being dormant (Sousa 2007).

Cultivation-independent methods.

Development of cultivation-independent methods improved detection and identification of different biofilm-associated infections (Hall-Stooley 2002). Amplification of the microbial marker genes using polymerase chain reaction (PCR) is one of the most applied methods to investigate infections without cultivation (Hall-Stooley 2012). This type of testing dramatically increased in the past decades compared to cultivation-based approaches (Hall-Stooley 2012). An advance of the PCR method is the multiplex PCR. With this type of PCR it is possible to detect different bacteria in one sample simultaneously. Multiplex PCR provides a reduction in sample material and higher throughput by reduction of PCR rounds. The design of highly specific primers for multiplex PCR is crucial to avoid cross reactions and primer dimers (Sager 2015). The quantitative amplification of the marker genes (qPCR) helps to detect very low amounts of the microbial DNA. The qPCR system gives the possibility to monitor the amplification cycle over timer whereas in a conventional PCR only the end-point can be monitored (Almeida *et al.* 2007). For the qPCR, the detection limit lies beyond the diagnostic relevance which could be a disadvantage of this system. A technical problem of the qPCR is the PCR inhibiting substances in complex samples (Hall-Stooley 2012). One drawback of qPCR is the impossible differentiation between living cells and dead cells which may be relevant in diagnostic (Hall-Stooley 2012). All PCR methods rely on the detection of marker genes which are specific to certain taxa. For *S. aureus* it is the cryptic sequence *pSA422* or the *mecA* gene. These two gene loci can be used for

detection of common *S. aureus* and for methicillin-resistant *S. aureus* (MRSA). For detecting MRSA alone, the molecular *mecA* gene in combination with *nucA* target gene is applied (Reischl & Rabenau 2015).

Total DNA isolation followed by either clone libraries or deep sequencing methods gives a deeper insight in the community structure (Larsen *et al.* 2008, Zhang *et al.* 2013). Sequencing approaches in combination with different other cultivation-independent methods have the ability to reveal possible unknown relationships to pathogens or harmless colonizers (Larsen *et al.* 2008).

Fluorescence *in situ* hybridization (FISH) allows the visualization of bacteria in their natural environment. This method enables a visual discrimination between prokaryotic and eukaryotic organisms depending on their taxonomy. Prior to application of FISH probes, the specificity, the sensitivity and the tissue penetration should be considered. The FISH probes are DNA fragments of 15-30 bases that are labeled with a fluorescent dye. The FISH probes target microbial 16S rRNA microbial sequences (Moter & Göbel 2000). Different fluorescent dyes allow simulatenous application of different probes and, thereby, detection of different taxa of microorganisms. The disadvantage of FISH for biofilms study is the detection limit that is described by Larsen *et al.* 2008. Due to the low abundance of bacteria in the biofilm sample the group was only able to detect bacteria in one out of 18 catheter samples. A faster method is the peptide nucleic acid fluorescence *in situ* hybridization (PNA FISH). The PNA FISH probes consist of achiral, uncharged polyamide backbone of repetitive units of N-(2-aminoethyl). The length of the probes is approximately 15 bases. Due to their length, the probes easier penetrate through the cell wall (Almeida *et al.* 2007). Regarding biofilms, PNA FISH probes are more efficient for detecting bacteria because of better matrix penetration (Almeida *et al.* 2007). In contrast to conventional FISH, the advantage of PNA FISH is the application of unfixed samples. The handicap of PNA FISH is a limited number of specific probes which reduce the application possibilities (Hall-Stooley *et al.* 2012). PNA FISH is often applied with Gram-staining and acridine orange leucocyte cyotpin (Gram-AOLC) test. The Gram-AOCL test is based on cell wall penetration of different dyes which enable to differentiate cells in blood samples. Gram-AOCL has a straightforward workflow. After sample-preprocessing, a monolayer of microorganisms and leucocytes is heat fixed on a

microscope slide. Afterwards, the cells are stained using acridine organe or Gram stain solution and visualized via ultraviolet or light microscopy (Kite *et al.* 1999). GRAM-AOCL is an inexpensive and quick method and need very low amounts of blood obtained from the catheter (Kite *et al.* 1999). The disadvantage of this system is the sample taking due to possible blocking of the catheters samples (Kite *et al.* 1999). Sensitivity and specificity of GRAM-AOCL are 99% and 96%, respectively (Kite *et al.* 1999). Gram-AOLC test combined with PNA FISH is successfully used for detecting of CRBSI. Wagner *et al.* (2013) showed that the combination of these two methods reduces the detection of a CRBSI by 2-8 days.

Multi-locus PCR and electrospray ionization mass spectrometry can also be used for investigation of bacterial composition in a biofilm. The workflow starts with a PCR using different primers targeting wide range specific regions of a genus. Typical target regions are i) 16S rRNA gene; ii) species-specific; iii) house-keeping genes; iv) antibiotic resistance genes. The amplified DNA fragments are separated using electrospray ionization mass spectrometer and assigned to specific genera (Hall-Stooley *et al.* 2012).

In general, cultivation-independent methods have many advantages compared to cultivation-independent methods with regard on limitations. Nowadays, the economical aspect of test application is of special interest. Using PCR-based approaches of MRSA screening, a cost reduction can achieve of 35US$ per test (Zitterkopf 2008).

Both cultivation-dependent and cultivation-independent approaches have their advantages and limitations. With the combination of these approaches an effect of the single method limitation can be successfully reduced. Keeping all the benefits and drawbacks of all methods in mind, analyzes of biofilm related catheters should be done using both cultivation-dependent and cultivation-independent approaches.

Material and Methods

Samples description

The first catheter set for trial experiments consisted of five different central venous catheters (CVC). The CVCs were obtained from the Medical University of Graz (Graz, Austria) in April 2014 and were labeled according to patient specific information. Patient specific information was assigned to internal SampleID (Table 2). Detailed information about health status of the patients and reason for catheterization were not available. All intracorporal intravenous parts of the catheters were removed under sterile conditions by pulling the catheter out of the patient's body and were either placed in nutrient medium or put in single plastic boxes and stored for 2 month at 4°C.

Table 2: Samples internal labelling and anonymized patient information.

Internal sample ID	Patient	Figure
UBT:T1D	Patient A	Figure 4A
UBT:T2D	Patient B	Figure 4B
UBT:T3D	Patient C	Figure 4C
UBT:T4D	Patient C	Figure 4D
UBT:T5D	Patient D	Figure 4E

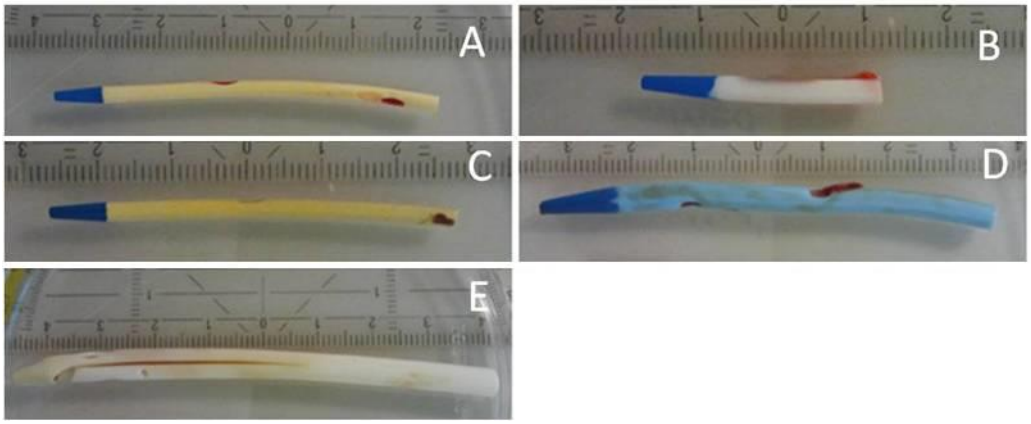

Figure 4: Tips of the catheters used in the trial experiments. Photographs show intravascular intravenous catheter fragments sampled.

The second set of catheters was obtained from the Medical University of Graz (Graz, Austria) in December 2014. In contrast to the catheters shown in Figure 4A-E these

samples were removed from the patients by explantation to avoid biofilm destruction of the outer biolfilm layer. The catheters shown in Table 3 were sectioned in three main parts: i) hub; ii) middle part; and iii) tip. The hub is an extracorporal extravasal part of the catheter, which in these experiments termed as distal (D). The middle or intracorporal extravasal part, is assigned to medial (M). The third part - the tip or intracorporal intravasal part- is termed as proximal (P) (Table 3). Except for CVC1, these catheters were stored at -20°C for 10 days prior to further processing.

Table 3 Internal samples labelling and storage conditions

Internal sample ID	Original labelling	Storage conditions	Figure
CVC1D	154437	Not known	Figure 5
CVC2D	56 Jug Hub	-20°C, 10 days	Figure 6 A
CVC2M	56 Jug Med	-20°C, 10 days	Figure 6B
CVC2P	56 Jug Spitze	-20°C, 10 days	Figure 6 C
CVC2aD	56 Hub Leiste	-20°C, 10 days	Figure 6 D
CVC2aM	56 Med Leiste	-20°C, 10 days	Figure 6 E
CVC2aP	56 Spitze Leiste	-20°C, 10 days	Figure 6 F
CVC3D	57 Hub	-20°C, 10 days	Figure 6 G
CVC3M	57 Med	-20°C, 10 days	Figure 6 H
CVC3P	57 Spitze	-20°C, 10 days	Figure 6 I

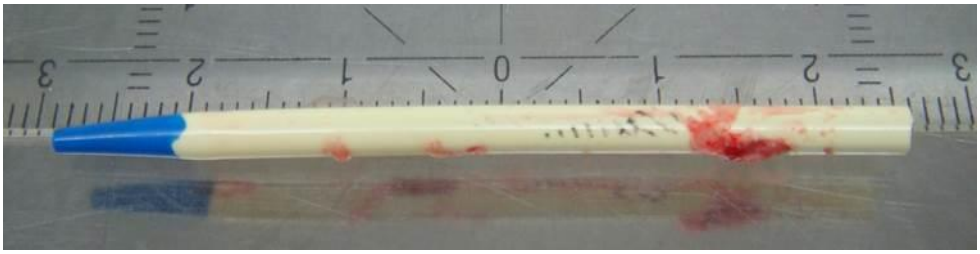

Figure 5: Tip of CVC1. Photographic image of CVC1.

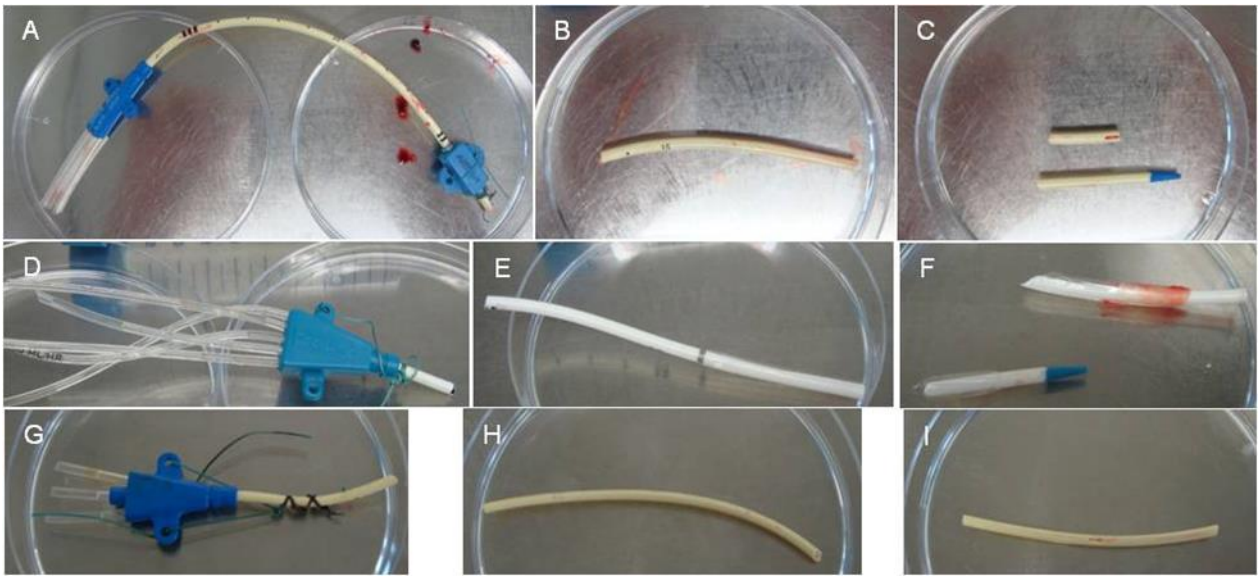

Figure 6: Sections of the catheters obtained from Medical University of Graz. Photographs show intravascular intravenous catheter fragments sampled. Sample IDs are listed in Table 2.

Method for investigation of the community structure

Total community DNA isolation

Fast DNA Spin Kit for Soil

DNA isolation for samples UBT:T1D-UBT:T5D was done using MP FastDNA MP FastDNA SPIN Kit for Soil (MP Biomedicals, Solon, OH, USA). 1 cm of each CVC was sectioned and was cut into 1 mm pieces, and placed directly in the Lysing Matrix E tube. The further steps were conducted according to the provided protocol. The negative control (UBT: TNKD) was prepared by washing the scissors, which were used for CVCs sectioning with 978 µl of PBS. The PBS aliquot was directly collected in the Lysing Matrix E tube.

PowerBiofilm DNA isolation Kit

The PowerBiofilm DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA) was used for samples UBT:T1D-UBT:T4D and CVC1-CVC3. 1 cm of CVC was cut in small pieces and was placed directly in PowerBiofilm Bead Tube. The DNA isolation was done according to the standard protocol with few variations. Namely, 350 µl of BF1 was

added. Cell disruption was achieved by using the MP FastPrep-24 (MP Biomedicals, Solon, OH, USA). For MP FastPrep-24 following settings were used: grinding at 4.0 – 5.0 m x sec^{-1} for 30 seconds. 100 µl of BF3 were used. DNA elution from the column was done using either 100 µl or 50 µl of provided elution buffer.

DNA quantification

The isolated total-community DNA and PCR products were quantified using NanoDrop 2000c Spectrometer (Thermo Scientific, Walthem, MA, USA). The elution buffer of MP FastDNA SPIN Kit for Soil (MP Biomedicals, Solon, OH, USA), PowerBiofilm DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA) or the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) were used as a blank, respectively.

Quantitative PCR (qPCR)

Primers and target genes

To determine the amount of bacteria in the CVC fragments, a quantitative PCR was conducted using the isolated total community DNA. The forward primer Unibac II-515f (Sequence 5´-3´: GTGCCAGCAGCCGC) and the reverse primer Unibac II-927r (Sequence 5´-3´: CCGTCAATTYMTTTGAGT) (Lieber *et al.* 2003) were applied for amplification of a 412 basepair fragment in the16S rRNA gene.

Polymerase and amplification conditions

The microbial 16S rRNA gene fragment was amplified using 2x Kapa Sybr Fast qPCR Mastermix Universal (VWR, Radnor, PA, USA) in 10µl reaction volume. Detailed sample mixture and qPCR program are shown in Table 4 and Table 5. qPCR was performed on the Rotor-Gene 6000 (Corbett Research, Mortlake, Australia).

Table 4 Reaction mixture used for qPCR.

Component	Volume [µl]/sample
2x Sybr Green Mastermix	5.0
Unibac II-515f, 10 pmol/µl	0.5
Unibac II-927r, 10 pmol/µl	0.5
H_2O	3.0
DNA-Template	1.0

Table 5 qPCR program used for amplification.

Step	Time, sec	Temperature,°C
Initial denaturation	300	95
Amplification (40 cycles)	20	95
	15	54
	30	72

Measurements and replicates

Quantification of the microbial 16S rDNA was done for samples UBT:T1D, UBT:T2D, UBT:T3D and UBT:T4D in two independent amplification runs, in which each DNA aliquot of was run in triplicate. For CVC1-CVC3, three independent runs were conducted, where each sample was measured in duplicate per run). As non-template control, DNA/RNA-free water (Roth, Karlsruhe, Germany) was amplified in triplicate or duplicate in each run, respectively.

Amplification value and reaction efficiency were calculated using following equations:

$$10^{\left(-\frac{1}{M-value}\right)} = Amplification\ value$$

$$\left[10^{\left(-\frac{1}{M-value}\right)}\right] - 1 = Reaction\ efficiency$$

The amplification value 2 and the M-value -3.322 indicate the most efficient amplification. Reaction efficiency in percent was calculated by multiplying the reaction

efficiency by 100. All PCR products were analyzed using gel-electrophoresis with 0.8% agarose gel.

Statistical analysis of gene copies/cm CVC and CFU/ml

Computer-assisted analysis was done to compare the performance of different DNA isolation kits, the abundance of microbial genes in different CVCs as well as to determine the re-isolation efficiency of the modified vortexing method and the ultrasonication method. The data were analyzed using PASW Statistics 18 version 18.0.0. (Polar Engineering and Consulting, www.winrap.com). The gene copy numbers, which were measured by qPCR, were extrapolated to the final amount of gene copy numbers per CVC centimeter and imported into the PASW Statistics program. Data were tested for normal distribution using Shapiro-Wilk test. The significance of the difference between the MO BIO PowerBiofilm DNA isolation Kit and the MP FastDNA spin Kit for soil were analyzed using the Student T-test (for normally distributed data) or Mann Whitney test (for not-normally distributed data). The difference between the catheters CVC1-CVC3 was analyzed using Mann-Whitney-U test. The differences between the different sections of one catheter and different catheters of one section were analyzed using One-way-ANOVA and Krustal-Wallis test. Data were considered statistically significantly different when P-value < 0.05.

The mean values of CFU ml^{-1} and standard deviations were calculated using Microsoft Excel 2010 (Microsoft Cooperation 2010). The CFU ml^{-1} was calculated using the Formula 2. Standard deviation was determined using the function in Excel Software.

16S rRNA gene amplification and clone libraries

Microbial 16S rRNA gene clone libraries were constructed for analyzing the community pattern on different catheter sections. The clone libraries were constructed for samples UBT:T1D-UBT:T5D and the negative control (UBT:TNKD).

16S rRNA gene amplification

The 16S rRNA gene was amplified using the forward primer 27f (Sequence 5´-3´: AGAGTTTGATCMTGGTCAG) and the reverse primer 1492r (Sequence 5´-3´: TACGGYTACCTTGTTACGGACTT) according to Jiang *et al*. (2006). PCR reaction was set up in a total volume of 50µl with a primer concentration of 10 pmol/µl each and

ready-to-use 5x Taq-&Go™ Ready-to-use PCR Mix (MP Biomedicals, Solon, OH, USA). As template, 1µl of total community DNA was used. Two negative controls were conducted using DNAse-free water (Roth, Karlsruhe, Germany) as a template. The applied PCR program is shown in Table 6.

Table 6: PCR program 27/1492

Steps	Temperature, °C	Time, sec
Initial denaturation	95	300
30 cycles	95	30
	50	30
	72	90
Final extension	72	600
Cooling	15	∞

Gel-electrophoresis of PCR products and/or total DNA

5 µl of PCR product or total community DNA was mixed with 6x Loading Dye. PCR products were tested on 0.8% agarose gel. The power supply was operated at 100 volts for 60 to 90 minutes depending on the gel size. Gels were stained in a water-ethidiumbromid solution (0.0001%) for 30 minutes. GeneRuler 1kb DNA (Waltham, MA, USA) ladder mix was used as a standard for the electrophoresis. Gel images were acquired by a Gel Doc 2000 (Bio-Rad, Hercules, CA, USA).

PCR products amplification purification

Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) was used for purification of PCR products. All steps were conducted according to the manufactures protocol. DNA was eluted with 50 µl of DNAse-free water.

Cloning

pGEM-T Easy Vector Systems (Promega, Madison, WI, USA) was used for ligation of the amplified 16S rRNA gene fragment into the cloning vector.

Ligation

The amount of insert was calculated according to the equation provided in the standard protocol (Formula 1).

$$\frac{Amount\ of\ vector\ [ng] \times Insert\ lenght\ [kb]}{Vector\ length\ [kb]} \times \frac{Insert}{Vector}\ molar\ ratio = Amount\ of\ insert\ [ng]$$

Formula 1: Calculation of insert in nanogramm used per ligation.

50 ng of vector was applied for ligation. The insert length for this ligation was 1.4kb. The vector length was 3kb. The insert to vector ratio was 3:1. According to this calculation, 70 ng of insert should be applied in the ligation mixture. Three samples had to be diluted to reach the appropriate concentration. The amount and concentration of insert are shown in Table 7.

Table 7: Plasmid dilutions for ligation.

Sample ID	DNA concentration [ng/µl]	Dilution	Amount of insert used for ligation [µl]	Final insert concentration used for ligation [ng]
UBT: T1D	72.2	-	1.0	72.2
UBT: T2D	73.8	-	1.0	72.2
UBT: T3D	121.2	1:2	1.2	72.6
UBT: T4D	125.2	1:2	1.2	75.0
UBT: T5D	179.5	1:4	1.6	71.8
UBT: TNKD	67.3	-	1.1	74.0

Ligation mixtures for standard reaction, positive control and background control was set according to manufacturer's protocol (Table 8).

Table 8: Ligation mixture

Reagent	Standard Reaction [µl]	Positive Control [µl]	Background Control [µl]
2x Rapid Ligation Buffer	5	5	5
pGEM-T easy vector (50ng)	1	1	1
PCR Product	a	-	-
Control insert DNA	-	2	-
T4 DNA Ligase	1	1	1
DNA/RNA free dH_2O to a final volume of	10	10	10

Transformation of ligated DNA fragment into competent cells

Chemically competent cells of *Escherichia coli* NEB 5-alpha (New England Biolabs, Ipswich, MA, USA) were thawed on ice as specified in the standard protocol. 25 µl of thawed cells were used for each transformation. To reach the appropriate concentration (1 pg – 100 ng), ligated DNA was 1:2 diluted using RNA-free H_2O. 0.5 µl of diluted ligation-mixture was applied. The transformation and regeneration was done according to the standard protocol. Regeneration was done for 1 hour at 37°C in 950 µl LB medium. After regeneration, samples were diluted with SOC-medium in ratio 1:10 and 1:100 according to the manufacturers protocol. 100 µl of diluted cell suspension was plated on LB agar medium containing ampicillin (Roth, Karlsruhe, Germany) (final concentration, 100 µg ml^{-1}), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (VWR Inernational Ltd., Lutterworth, Leics, UK) (final concentration, 80 µg ml^{-1}), and isopropyl β-D-1-thiogalactopyranoside (Roth, Karlsruhe, Germany) (final concentration 0.3 mM). Plates were incubated over night at 37°C.

Screening for positive transformants

Positive transformants (white colonies) were picked using sterile toothpicks and streaked out on LB agar medium containing ampicillin (100 µg ml^{-1}). For each CVC sample, 20 positive transformants were selected. Plates were incubated over night at 37°C. For colony PCR, cell material was resuspended in 10 µl RNA/DNA free dH_2O (Roth, Karlsruhe, Germany). Cell suspension was heated for 5 minutes at 95°C. Afterwards, cell suspension was centrifuged for 1 minute at 12000 x g. 1 µl of supernatant was used as a template for PCR. The PCR was conducted using forward primer USP (Sequence 5´-3´: GTAAAACGACGCCCAGT) and reverse primer RSP (Sequence 5´-3´: CAGGAAACAGCTATGACC). PCR reaction was set up in a total volume of 50 µl with a primer concentration of 10 pmol $µl^{-1}$ each, ready-to-use Taq-&Go™ Ready-to-use PCR Mix (MP Biomedicals, Solon, OH, USA). Negative control was conducted with DNAse-free water (Roth, Karlsruhe, Germany). PCR program for colony PCR with USP/RSP primer set in shown in Table 9.

Table 9: PCR program USP/RSP

Step	Temperature,°C	Time, seconds
Initial Denaturation	95°C	300
30 cycles	95°C	50
	50°C	50
	72°C	90
Final extension	72°C	600
Hold	15°C	∞

The amplified products were examined using gel-electrophoresis as described above.

Sanger sequencing and analysis

PCR products preparation for sequencing

In total, 120 clones of CVC UBT:T1D-UBT:T5D and UBT:TNKD (20 clones per CVC) were subjected to sequencing. PCR products were prepared for sequencing by purification using Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) and diluting the purified PCR samples to a concentration of 40 ng μl^{-1}. Forward primer (USP) was diluted to a concentration of 5 ng μl^{-1}. 10 µl of template-DNA were mixed with 4 µl of diluted primer. Sequencing was conducted by LGC Genomics (Berlin, Germany).

Computational analysis of Sanger sequencing data

DNA sequencing data were manually truncated using SeqScanner version 2 (Applied Biosystems. 2012). BLASTn search was done towards high similar sequences (megablast). The reference RNA sequences database (refseq_rna) and the nucleotide-collection database (nr/nt) of the NCBI server were used as reference databases. The search-output with the highest maximal score and the highest similarity was considered. Taxonomic information at the species level was considered when similarity was ≥ 97%.

Methods for investigation of the colonization pattern

Biofilm fixation and storage

Sample preparation, fixation and storage in phosphate buffered saline (PBS)/ethanol solution

The paraformaldehyde fixation was done according to Grube *et al*. (2009). For biofilm fixation, 1cm of each CVC was cut using scissors (Aesculap BC 110R Iris Fadenschere), placed into a 1.5 ml Eppendorf tube and washed with phosphate buffered saline (PBS). PBS was removed, a mixture of 1 vol. PBS : 3 vol. 4% paraformaldehyde (Roth, Karlsruhe, Germany) (PFA) was added and the sample was incubated for 4 hours at 4°C. After fixation, PFA was removed and CVC-sample was washed 3 times using PBS (1^{st}: rinse, 2^{nd}: 5 minutes, 3^{rd}: 10 minutes). PBS was removed and 1 vol. PBS : 1 vol. DNA-standard grade ethanol (Merck, Germany) was added. Samples were stored at -20°C. For confocal laser scanning microscopy (CLSM) cross-sectional segments and longitudinal CVC-sections were obtained using a razor blade or scissors.

Sample preparation, fixation and storage in PBS/glycerol solution

This fixation was done according to Brileya *et al.* (2014) without using ethanol as cryo-preservative. The preparation and fixation were carried out same as mentioned in the previous section. Instead of PBS/ethanol mixture, a 1 vol. PBS : 1 vol. glycerol (Roth, Karlsruhe, Germany) mixture was added. Samples were stored at -20°C. Sample preparation for CLSM was done as mentioned in the previous section.

Modified paraformaldehyde fixation method for CVC-sections

Original CVC-samples were sectioned using a disposable scalpel (Swann-Morton, Sheffield, England) and directly placed in 4% paraformaldehyde (PFA) (Roth, Karlsruhe Germany) for approximately 12 hours at 4°C. After fixation, PFA was removed and the sample was washed 3 times with phosphate buffered saline (PBS) (1^{st}: wash, 2^{nd}: 3 minutes, 3^{rd}: 7 minutes). Instead of PBS/ethanol mixture, a 1vol. PBS: 1 vol. glycerol (Roth, Karlsruhe, Germany) mixture was added. Fixed CVC-sections were stored at -20°C.

Sample preparation and fixation of CVC sections in acrylamide

Testing the polyacrylamide behavior to the three dehydration steps and the embedding handling a biofilm-free CVC sectioned sample was embedded in Rotiphorese gel 30 (Roth, Karlsruhe, Germany). To prepare the acrylamide mixture, 1 ml of Rotiphorese Gel 30 was diluted to a concentration of 20% and was mixed with 5 µl of ammonium persulafate (APS) 10% and 1 µl of tetramethylethylenediamine (TEMED). 100 -150 µl of the mixture was put on a CVC section placed in the microscope slide with cavity (depth of cavity, 1.5 mm) (Thermo Fisher Scientific, Waltham, MA, USA). The polyacrylamide was capped with a cover glass treated with a gel repellant (Acryl-Glide Glass Plate coating, Amresco, USA). The treated cover glass was removed after maximal 10 minutes. Robustness of polyacrylamide was tested via dehydration steps of 50%, 80% and 96% of ethanol according to Grube *et al.* (2009).

The experiment was repeated with sections of UBT:T3D. The dehydration steps were not conducted. Instead the polyacrylamide gel was covered with SlowFade with DAPI (Thermo Scientific, Waltham, Mam USA) for 30 minutes. A cover glass was placed on the gel and sealed with nail polish.

Fluorescent *in situ* hybridization (FISH)

CVC-sections were hybridized with rRNA-targeting FISH probes (Genexpress, Wr. Neudorf, Austria) specific to different classes according to the protocol of Grube *et al.* (2009). Additionally a universal set of bacterial probes was used. Hybridization temperature was 41°C. Hybridization was done for 180 minutes. All FISH probes and stringency conditions are listed in Table 10.

Table 10: FISH probes and stringency conditions

FISH-Probe	Sequence (5´-3´)	Specificity	Reference	Formamide concentration, %[a]	Fluorescent dye
ALF968	GGTAAGGTTCTGCGCGTT	*Alpha proteobakterien*	Neef (1997)	45	Cy5
Gam42a	GCCTTCCCACATCGTTT	*Gamma proteobacterium*	Manz *et al.* (1992)	45	Cy5
Gam42a competitor	GCCTTCCCACTTCGTTT	*Beta-proteobacterium*	Manz *et al.* (1992)	45	-
Bet42a	GCCTTCCCACTTCGTTT	*Beta-proteobacterium*	Manz *et al.* (1992)	45	ATTO488
NONEUB-Cy5	ACTCCTACGGGAGGCAGC	-	Wallner *et al.* (1999)	45	Cy5
NONEUB-ATTO488	ACTCCTACGGGAGGCAGC	-	Wallner *et al.* (1993)	45	ATTO488
LGC354A	TGGAAGATTCCCTACTGC	*Firmicutes*	Meier *et al.* (1999)	45	FITC
LGC354B	CGGAAGATTCCCTACTGC	*Firmicutes*	Meier *et al.* (1999)	45	FITC
LGC354C	CCGAAGATTCCCTACTGC	*Firmicutes*	Meier *et al.* (1999)	45	FITC
EUB338	GCTGCCTCCCGTAGGA	Most bacteria	Amman *et al.* (1990)	15	Cy3
EUB338II	GCAGCCACCCGTAGGTG	*Planctomycetales*	Daims *et al.* (1999)	15	Cy3
EUB338III	GCTGCCACCCGTAGGTG	*Verrucomicrobiales*	Daims *et al.* (1999)	15	Cy3
NONEUB338	CTCCTACGGGAGGCAG	-	Amman *et al.* (1990)	15	Cy3

[a]The FISH probes, which should be applied at the same stringency conditions, were mixed in equimolar ratios.

After hybridization, CVC-sections were placed onto microscope slide with cavity (depth of cavity, 1.2 mm) (Menzel-Gläser, Thermo Fisher Scientific, Waltham, MA, USA) and covered using SlowFade Gold Antifade Mountant (Thermo Scientific, Waltham, MA USA).

Confocal laser scanning microscopy (CLSM)

Confocal laser scanning microscopy was done using Leica TCS SPE/DM5500Q confocal laser microscope (Leica Microsystems, Mannheim, Germany). The microscopy was operated using three different lasers of 488nm, 532 nm, 635 nm for FITC/ATTO488-, Cy5- and Cy3-labelled FISH probes, respectively. The lasers of 405nm, 488nmand 532nm were operated at an intensity of 40%. The laser of 635nm was operated at an intensity of 60%. The acquisition wavelength for FITC/ATTO488-labelled probes was around 525 nm, Cy-3 at 568 nm and Cy-5 with 666 nm. Samples, which were treated using Slow Fade with DAPI, were at 405 nm. The images were acquired with a z-stack size of 0.8 nm and a frame average of 3. For higher resolution the z-stack size was reduced to 0.5 nm and a frame average of 4. Images were processed using the maximum projection tool of the Leica software.

Biofilm formation on CVCs

A set of experiments were conducted to address the issue of biofilm formation in/on CVC by single and multiple clinical strains and its possible treatment. These experiments included colonization of sectioned tubes and intact CVCs using the biofilm formation plant.

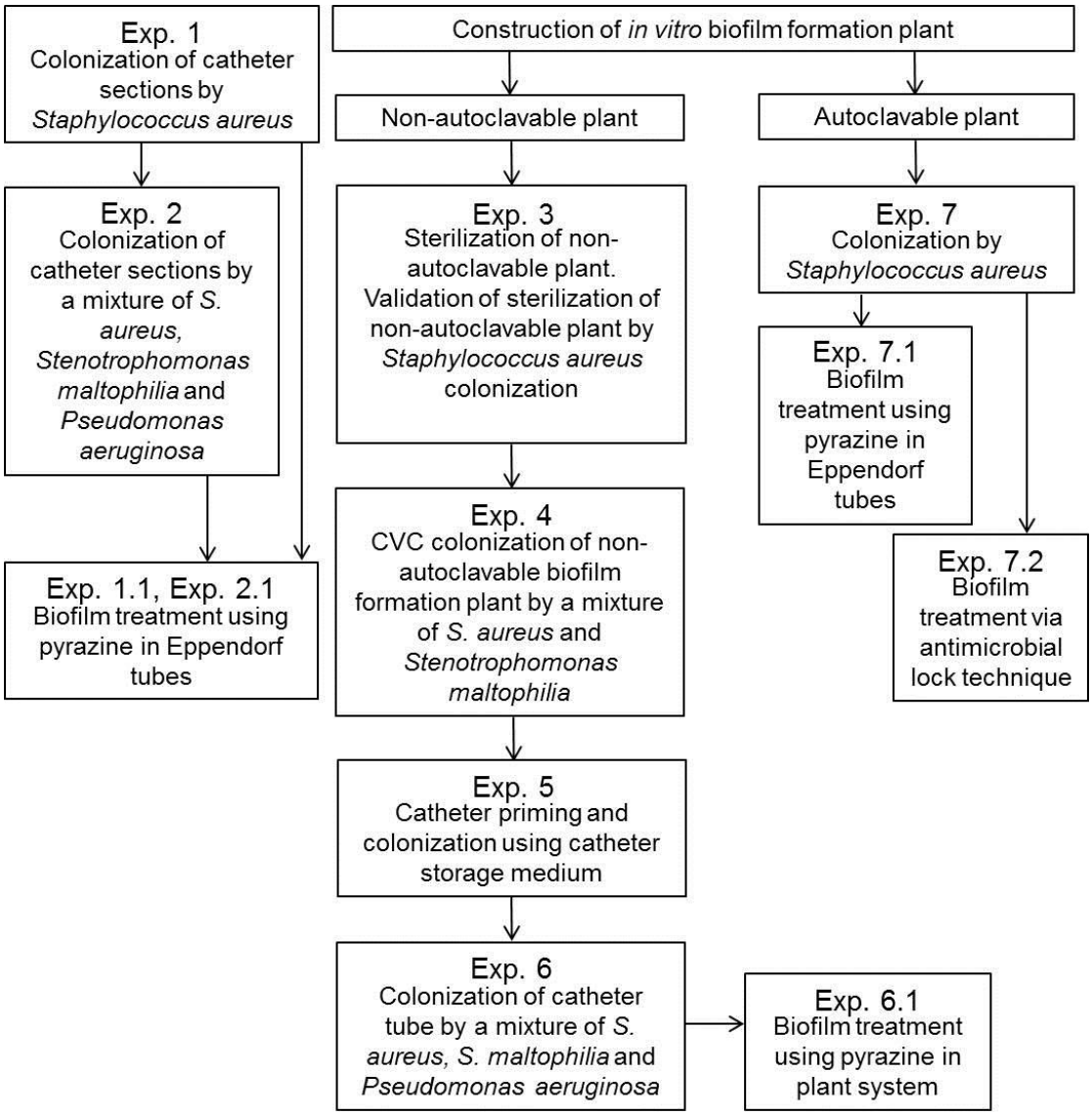

Figure 7: Workflow showing different experiments (Exp.) including catheter sections and biofilm formation plant.

Catheters used for colonization experiments

SplitCath III Catheter (Medcomp, Harleysville, PA, USA) and Softline (Medcomp, Harleysville, PA, USA) catheter types were used to study *in vitro* biofilm formation. Furthermore, colonization experiments were conducted using catheter sections of a Large-Bore Multi-Lumen Central Venous Catheterization Set (Arrow International Inc., Reading, PA, USA). This catheter has a chlorhexidine and sulfadiazine coating to prevent biofilm-formation.

Colonization experiments using different catheter sections

Experiment 1: Colonization of catheter sections by *S. aureus*

1-2 mm sections of the intracorporal and extracorporal extravasal part of a SplithCathIII and a SoftLine, respectively, were incubated using a *S. aureus* ATCC 25923 overnight culture (ONC) (8.24×10^7 CFU ml^{-1}). The number of living cells in the ONC were determined by plating the ONC using PBS in a dilution series (10^{-1}-10^{-7}). 50µl of diluted bacterial culture were spread out on LB agar plates and incubated at 30°C overnight. Colonies were counted manually using a colony counter (schuett count, schuett-biotec GmbH, Göttingen, Germany). CFU ml^{-1} was calculated using Formula 2.

Formula 2 Calculation of colony forming units per milliliter

$$\frac{cfu}{ml} = \frac{number\ of\ colonies \times dilution\ factor}{Plated\ volume\ [ml]}$$

Catheter sections were placed in a 12-well plate (greiner bio-one, Kremsmünster, Austria) and covered with 1 ml ONC. The plate was incubated for 24 hours at 37°C. CVC sections were directly subjected to pyrazine treatment.

In the repeated experiment 1-2 mm sections of the intracorporal part of SplithCathIII were placed in a 48-well plate (one section per well) and were covered with a mixture of *S. aureus* ATCC 25923 of an OD_{600} of 1 ($3.20 \times 10^8 \pm 3.27 \times 10^7$ CFU ml^{-1}). The plate was placed at 37°C for 24 hours under static conditions. As before, the CVC sections were directly subjected to pyrazine treatment.

Experiment 1.1: Pyrazine treatment of catheter sections colonized by *S. aureus*

Samples were transferred to a new 12-well plate containing 1ml PBS and 2, 4 and 10 µl of pyrazine (Sigma-Aldrich, St. Louis, MO, USA). 12-well plates were closed with the provided lid and sealed using parafilm. Negative control was conducted without pyrazine. CVC sections were incubated for 24 hours at 37°C. In the modified protocol, 12-well plates were exchanged to Eppendorf tubes. The results of the pyrazine treatment were evaluated using a viability test described below.

In the repeated experiment a pyrazine concentration of 0.2%, 0.4%, 0.6% and 0.8% were used for treatment of colonized CVC sections. The treatment solution was

prepared in a volume of 1ml using sterile PBS. The catheter sections were directly placed in the Eppendorf tube. CVC sections were treated for 24 hours for 30°C at 120 rpm.

Experiment 2: Colonization of catheter sections by a mixture of *S. aureus*, *Stenotrophomonas maltophilia* and *Pseudomonas aeruginosa*

The intracorporal tubes of a SplithCathIII and a Large-Bore Multi-Lumen Central Venous Catheterization Set were sectioned using a scalpel in 0.5 to 1 mm sections. Sections were transferred into a 12-well plate and covered with 2 ml of a bacterial cell suspension. The cell suspension was prepared by inoculating 25 ml of LB medium using *S. aureus* ATCC 25293 / *S. maltophilia* (internal strain ID: e-p3=R 3089) / *P. aeruginosa* (internal strain ID: QC 14-3-8) ONC (OD_{600} = 4.98) to reach the desired optical density of 0.002. The plate was kept under static conditions for 5 days at 37°C.

The experiment was repeated using a mixture of equal volumes of *S. aureus*, *S. malophilia* and *P. aeruginosa* with an OD_{600} of 0.002 each. The CFU ml^{-1} of each strain at an OD_{600} of 0.002 was: *S. aureus* $6.10x10^5 \pm 4.97x10^4$, *P. aeruginosa* $1.67x10^6 \pm 2.49x10^5$ and *S. maltophilia* $3.60x10^6 \pm 8.16x10^4$. The CVC sections were placed in a 48-well plate (one section per well) and were covered using 1 ml of the mixture. The plate was incubated at 37°C for 5 days under static conditions.

Experiment 2.1: Pyrazine treatment of catheter sections colonized by a mixture of *S. aureus, S. maltophilia* and *P. aeruginosa*

The sections were placed in a sterile 1.5 ml Eppendorf tube containing 1 ml PBS and 4 µl pyrazine and were shaken at 120 (rotations per minute, rpm) at 30°C for 24 h. The negative control was conducted by placing CVC sections in PBS without pyrazine. The results of the pyrazine treatment were evaluated using a viability test described below.

In the repetition of the experiment a pyrazine concentration of 0.2%, 0.4%, 0.6% and 0.8% were applied on the colonized CVC sections. The treatment solution was prepared in sterile PBS in a volume of 1ml. The CVC sections were transferred in the treatment solution and were treated at 120 rpm and 30°C for 24h.

Viability test of bacteria on CVC sections after pyrazine treatment

Pyrazine treatment efficiency was tested by placing the treated CVC sections in 20ml TSB-medium overnight at 30°C on a shaking plate (120 rpm). Afterwards, optical density measurement was done using the Eppendorf Bio Photometer (Eppendorf, Hamburg, Germany). If bacterial growth was visually detectable, the ONC was diluted in a ratio 1:2 or 1:10 using PBS prior to the measurement. Negative control and treated samples were diluted in the range of 10^{-1} to 10^{-7} with PBS, if bacterial growth was detected. 50µl of these dilutions were applied on tryptic soy broth (TSB) (Roth, Karlsruhe, Germany) or LB plates. In the treated samples with no detectable growth, 50µl of sample was applied on TSB-plates. Plate incubation was done at 30°C overnight. CFUs were counted manually using colony counter (schuett count schuett-biotec GmbH, Göttingen, Germany). In the first experiment a statistical analysis was not done. The data were compared whether the pyrazine treatment removed all bacteria from the sample or not.

In the repeated experiments CFU ml^{-1} obtained from different samples were analyzed statistically.

Colonization experiments using *in-vitro* biofilm formation plant

Construction of *in-vitro* biofilm formation plant

An experimental plant was constructed to study the biofilm formation *in vitro* and to simulate biofilm formation by single (or mixed) bacterial strains on different catheters (Figure 8). The plant consisted of the following parts: medium-flask (F), silicone tubes (diameter, 4mm) (black lines), a peristaltic pump (PP) (Masterflex L/S Economy Drive, Cole-Parmer, Waltham, MA, USA), a three-way-valve (BD Connecta Luer-Lok, BD Connecta, Becton, Dickinson and Company, Franklin Lakes, NJ, USA), a cap with a self-sealing membrane (M). The open tube (OT) (broken line) plugged different parts of a CVC together. The circular flow (C) allowed applying a specific amount of pyrazine, which was pumped into the “small” circular flow. The screw connection (SC) linked the catheter hub to the system. Two modifications of the plant were used for the *in-vitro* experiments – a non-autoclavable and an autoclavable modification.

Non-autoclavable plant

The non-autoclavable plant (Figure 8) consisted of different non-autoclavable parts, which are used in routine medicine (BD Connecta Luer-Lock, cap with self-sealing membrane). The connections between the fittings and the cap of the medium flask were sealed using technical glue (UHU Max repair, Bühl, Germany).

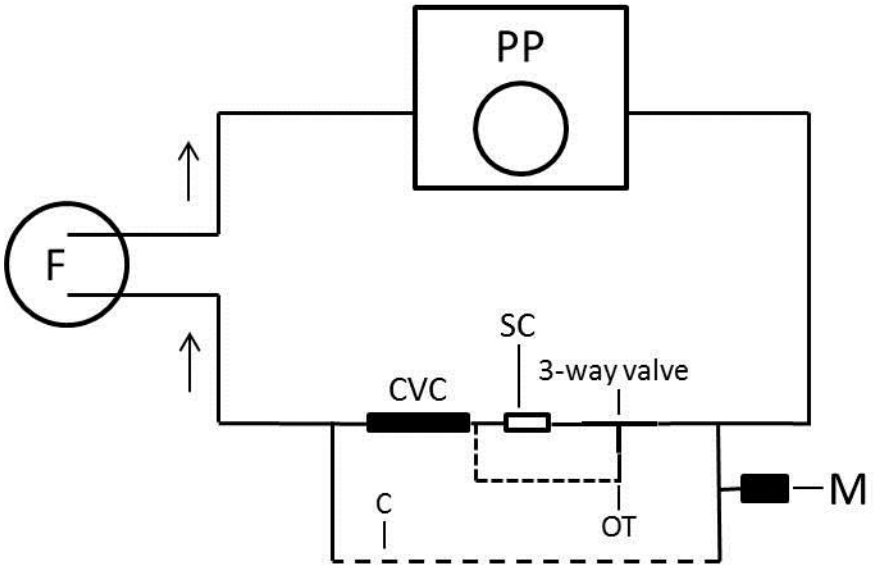

Figure 8: Scheme of an artificial non-autoclavable catheter colonization plant. The plant consisted of medium-flask (F), silicone tubes (black lines), a peristaltic pump (PP), a three-way-valve and a cap with a silicone membrane (M).

Experiment 3: Sterilization of non-autoclavable *in-vitro* biofilm formation plant and validation of sterilization method

To avoid microbial contamination, the plant was sterilized using sodium hypochlorite. Water in the medium flask was discarded and the residual water in the tube system was collected. The dilution series (10^{-1}-10^{-7}) were done using PBS to count the number of viable bacteria by plating on LB agar plates. The pre-sterilization control was done using 200 ml sterile water. The water was circulated for 40 minutes in the tube system. The peristaltic pump (Masterflex L/S Economy Drive, Cole-Parmer, Vernon Hills, Illinois, USA) was operated at setting 3. Afterwards a sample was taken from the pre-sterilization control. 50 µl of a dilution series (10^{-1}-10^{-7}) were plated out on LB agar plates. Sterilization was conducted by applying 200 ml of a 6% sodium hypochlorite (Roth, Karlsruhe, Germany) in the tube system for 40 minutes. The sodium hypochlorite

was removed and the tube system was washed three times, 5 minutes each, using 400 ml sterile water. After each washing step, 50 µl of the washing water was streaked out on LB agar plates. All plates were incubated at 30°C for two days.

To analyze sterilization efficiency, the plant tube system was colonized with *S. aureus*. A 500 ml flask (Duran, DURAN Group GmbH, Wertheim/Main, Germany) was filled with 399 ml TSB medium and was inoculated with 1 ml of ONC of *S. aureus* ATCC 29253. The OD value was not determined in this experiment. The flask was placed on a magnet stirrer with heating module (IKA RCT basic safety control, IKA®-Werke GmbH & Co. KG, Staufen, Germany). The magnet stirrer was operated at 37°C at 90 rpm. Peristaltic pump was operated at the setting 1.5 for 5 hours. The first sample was taken 15 minutes after inoculation. Five additional samples were taken every hour. Peristaltic pump was switched off after the last sampling. Samples were diluted with PBS in a dilution series (10^{-1} - 10^{-6}). 50 µl of different dilutions were plated out on LB agar plates. The medium was removed from the tube and the system was rinsed with sterile water followed by sterilization using 6% sodium hypochlorite for 40 minutes. Sterilization solution was removed from the system and residual disinfectant was removed by rinsing the system using sterile water. A sample was taken out of the water and 50 µl was plated out on LB agar plate. The agar plates were incubated at 30°C overnight.

Experiment 4: CVC colonization of non-autoclavable biofilm formation plant by a mixture of *S. aureus* and *Stenotrophomonas maltophilia*

To analyse the colonization pattern of two-species biofilm, a 500 ml flask (Duran, DURAN Group GmbH, Wertheim/Main, Germany) containing 398 ml LB-Medium (Luria-Miller) (Roth, Germany) was inoculated with both ONC of *S. aureus* and *S. maltophilia*, 1 ml each. The CVC colonization was done twice. At the first colonization survey, the optical density of *S. aureus* and *S. maltophilia* was 6.75 and 3.76, respectively. The biofilm formation plant was operated at 37°C for 24 hours using the peristaltic pump setting of 1.5. The experiment was repeated with *S. aureus* (OD_{600}: 1.83) and *Stenotrophomonas maltophilia* (OD_{600}: 3.76). The biofilm formation plant was placed in a 37°C incubator and run for 72 hours using the peristaltic pump setting of 1.5. For easier handling of the CVC, the CVC-carrier was partly reconstructed. The reconstructed handle reduced contamination due to easier disassembling (Figure 9).

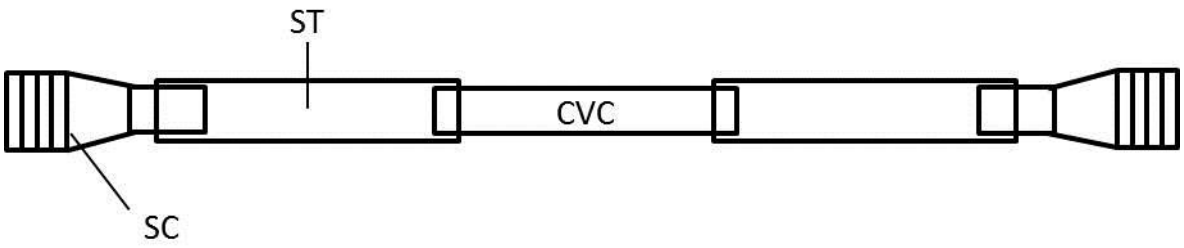

Figure 9 Modified CVC carrier. The modified holder consisted of two screw connections (SC) on left and the right side of the holder, the silicone tube (ST) and the CVC.

CVC removal and sample processing

The CVC and the conjunctions between the tube and the CVC were swabbed using 6% sodium hypochlorite. Finally, the CVC was removed from the plant. In the first experiment, the remaining medium in the CVC was removed and the CVC were directly used for re-isolation of bacteria. In the repeated experiment, non-attached cells in the CVC were removed by rinsing the lumen using PBS. At one side of the CVC carrier, a 5 ml syringe was placed containing 2 ml of PBS. Planctonic cells were removed by slowly pressing the sterile PBS through the CVC. To avoid cross contamination, the outer surface of the CVC was swapped with 99% ethanol.

Re-isolation of biofilm bacteria from the CVC by a modified vortexing method

For re-isolation of biofilm bacteria that colonized CVC, 1 cm of CVC was cut off and cut longitudinal in half. The CVC was placed into Eppendorf tube and 1 ml PBS was added. Cells were detached by intensive shaking using a FastPREP-24 (MP Biomedicals, Santa Ana, CA, USA). The device was set to speed 5.0 m s^{-1} for 20 seconds. 50 µl were applied on LB agar plates. Plates were incubated at 37°C overnight.

Re-isolation of biofilm bacteria from the CVC by ultrasonication method

The settings for the ultra-sonication were set according to Donlan *et al.* (2001). In contrast, the ultrasonicator was operated at 35 kHz (Sonorex super RK 102H, Bandelin electronic GmBH & Co. KG, Berlin, Germany). The samples were repeatedly treated three times for 30 seconds of ultrasonication and 30 seconds of vortexing.

Viability test of biofilm bacteria re-isolated from the CVC sections

To test the viability of the re-isolated bacteria from the CVC sections different dilutions (10^{-1}-10^{-7}) were made using sterile PBS. 50 µl of cell suspension were spread on LB agar plates using a drigalsiky spatula. The plates were incubated at 37°C overnight.

The second plating technique, which was used for the viability test, was based on trickling of bacteria dilution on an agar plate. 10 µl of bacterial suspensions of different dilutions were pipetted on an agar plate and put in a slope position. Plates were incubated overnight at 37°C. CFUs were counted manually using a colony counter.

Experiment 5: Catheter priming and colonization using catheter storage medium

CVC priming

To analyse the biofilm formation by bacteria, which were isolated from a clinical catheter sample (UBT: T4D) a clean catheter was primed using the storage medium of a removed catheter. 4 cm of SplithCathIII was mounted in the modified CVC carrier. The connections between the CVC and the tube were sealed with parafilm. Priming solution was prepared by mixing sterile LB medium and catheter storage medium in equal volumes. 2 ml of priming mixture was transferred with a 5 ml syringe through the CVC in a second 5 ml syringe to reach a homogenous distribution of priming solution in the CVC (Figure 10). CVC was primed at 37°C for 21 hours.

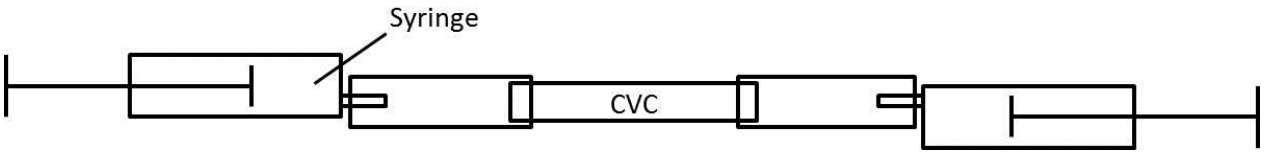

Figure 10 Setup for catheter priming. Syringes containing the priming solution were placed on both ends of the catheter.

CVC mounting and starting of biofilm formation plant

CVC priming solution was removed from the CVC. The CVC carrier was mounted in the biofilm formation plant. 200 µl of priming solution was transferred into the running plant. The plant was placed in a 37°C incubation room and operated for 7 days using the peristaltic pump setting of 1.5.

CVC removal and sample processing

CVC was gently rinsed with 2ml sterile PBS to remove planctonic cells. Parafilm was removed and outer surface of the CVC was swapped with 96% ethanol. CVC was sectioned with a scalpel for the FISH paraformaldehyde fixation. CFU ml^{-1} of medium was determined via plating of dilutions as described above.

Experiment 6: Colonization of catheter tube by a mixture of *S. aureus, S. maltophilia* and *Pseudomonas aeruginosa*

400 ml LB medium was inoculated with a mixture of *S. aureus* ATCC 29253 / *S. maltophilia* (internal strain description: e-p3=R 3089) / *P. aeruginosa* (internal strain description: QC 14-3-8) to an optical density of 0.002. The bacteria mixture was prepared by picking one colony of each strain and transferring it into LB medium. The mixture was incubated overnight at 30°C at 120 rpm. The calculated volume of ONC was directly transferred into the medium under sterile conditions. The pump setting was 1.5. The plant was kept at 37°C for 14 days.

The CVC carrier was adapted to the experiment. This modification allowed the easier removal of one catheter, whereas the other CVC could stay in the system and be used for treatment experiments (Figure 11).

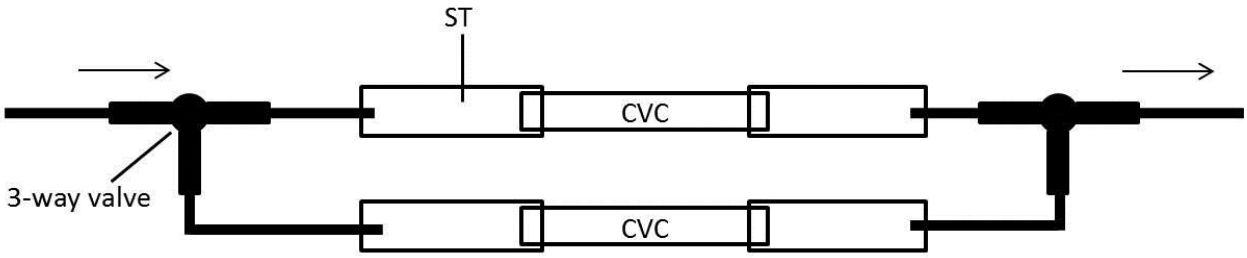

Figure 11 Modified CVC carrier with two parallel catheters. The modified CVC carrier setup allowed the removal of one catheter and the treatment of the other. Abbreviations: ST, silicone tube.

Prior to CVC removal, the silicone tubes and the CVC - surface were rinsed with 6% sodium hypochlorite. Residual disinfectant was washed away with sterile PBS buffer. Prior to demounting the silicone tubes from the CVC, planctonic cells were removed by gentle rinsing 2 ml PBS with a syringe through the catheter. 1 cm of catheter was cut using a sterile scalpel and bacteria were isolated by using shaking method as described

above. 50 µl of suspension, which was prepared by diluting cell suspension with PBS (10^{-1}-10^{-7}), were spread on LB agar plates.

Experiment 6.1: Pyrazine treatment of *S. aureus*-, *P. aeruginosa*- and *S. maltophilia*-colonized catheter tubes

Treatment was done by applying a solution of 6% pyrazine in the circular flow cycle (Figure 8). To assure that the desired concentration of pyrazine is reached in the tube system, the desired amount of pyrazine was adjusted to the volume of the circular flow. The inner volume of the system was calculated to a total volume of 9.5 ml. The calculated amount of pyrazine (57 µl) was mixed with PBS to a total volume of 0.5 ml. The solution was brought into the system through the self-sealing membrane. Pyrazine was applied in the pump system for 24 hours.

Experiment 7: Colonization of autoclavable plant by *S. aureus*

To reduce the risk of contamination, the non-autoclavable plant was partly re-constructed (Figure 12B). The re-constructed plant should reduce possible sources of contamination due simplified setup. All non-autoclavable parts were removed. The silicone tubes were connected to a metal multiple distributors for bottles. Two three-way plug connections were used to connect the circuit for pyrazin treatment (C) and the main tube system. The tubes on the multiple distributors and other connections were fixed with zip ties.

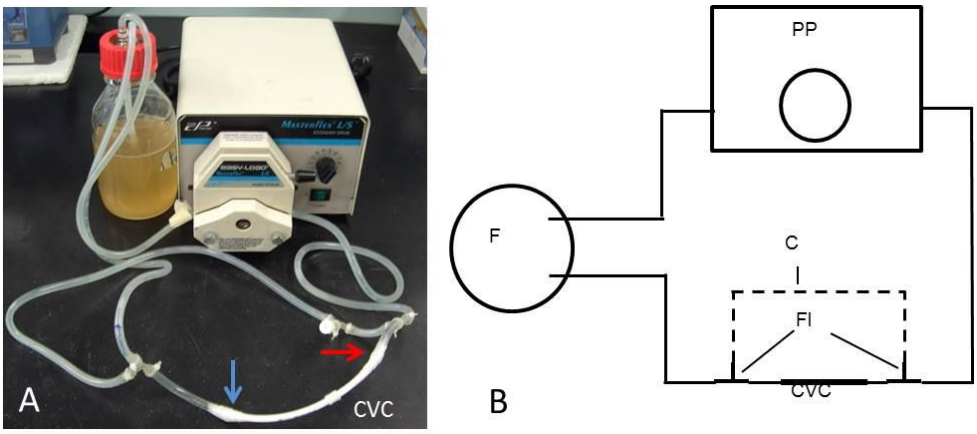

Figure 12 Image and scheme of autoclavable catheter colonization plant. A: Image of autoclavable plant. Red and blue arrows indicate constantly sealed and opening sites, respectively. B: The plant consisted of following parts: flask for medium (F), peristaltic pump (PP), three-way flange (Fl) and a circuit for pyrazine treatment (C).

The intracorporal part of SplithCathIII (approximately 16 cm long) was tightly mounted on one side of the tube system. The other side of the catheter was plugged to the silicone tube of the biofilm formation plant. To avoid leaking the conjunctions, of both ends were sealed with parafilm (Figure 12A).

The culture medium was prepared by inoculating 400 ml LB medium with a *S. aureus* ATCC 25923 ONC to an optical density of 0.002 (9.33±2.64*105 CFU ml^{-1}). The flask was placed on a magnetic stirrer. The setting of the peristaltic pump was 1.5. The system was placed in the 37°C incubation room for 5 days. The whole culture medium was exchanged on the fifth day after sampling procedure. To compare the growth conditions in the plant system and a stirring-only system, a growth control was conducted. The growth control, 400 ml LB medium was inoculated using the same ONC and optical density. The 500 ml flask was placed on a magnetic stirrer at 37°C.

CVC sampling from the autoclavable *in-vitro* biofilm formation plant

10 samples were taken from the catheter, the culture medium and the growth control each. The first sample was taken 2 minutes after inoculation (T_0). Further samples were taken every 24 hours. Sampling of the medium from the biofilm formation device and the growth control were done by taking 1 ml medium under sterile conditions. Before removing the parafilm, the surface was swabbed using 96% ethanol. Than the surface of the catheter was disinfected using 96% ethanol and 1 cm of the catheter was cut. For cutting, flamed scissors and forceps were used.

Re-isolation of biofilm bacteria from the catheter was done by using the shaking method as described above. CFU ml^{-1} was determined using the plating method and the trickling method as described above.

Experiments 7.1 and 7.2: Pyrazine treatment of *S. aureus*-colonized catheter tubes

Antimicrobial lock technique

To reach similar treatment-conditions as applied in medical routine different concentrations of pyrazine were locked within the CVC. The treatment solution contained 0.4%, 0.5% or 0.6% pyrazine in PBS. Sterile PBS was applied on the CVCs as negative control. Solutions were prepared in a total volume of 5 ml in 15 ml tubes

(Sarstedt, Nürnbrecht, Germany). To get an emulsion of pyrazine, the tubes were shook for 20 seconds at 3.0 m s^{-1} using a MP FastPrep with a CoolTeenPrep 6x 15 ml attachment. Negative control was treated same as the positive treatment. The emulsion was immediately taken up with a syringe and put in the tube system (Figure 13). The solution was pressed into the CVC and was locked with a clamp. The CVCs were incubated under static conditions at 37°C for 19 hours. Re-isolation of bacteria was done using the shaking method as describes above.

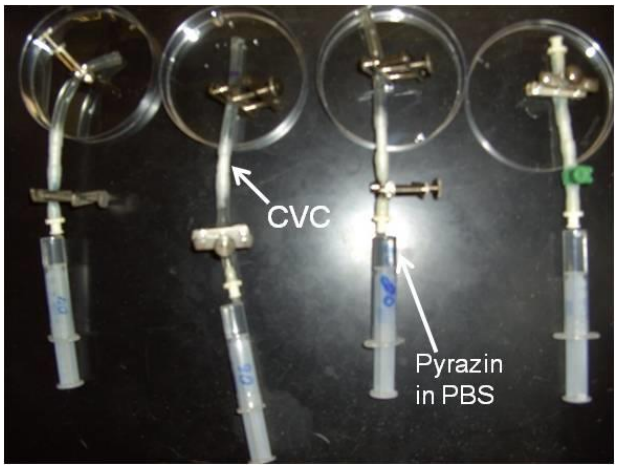

Figure 13: Catheter treatment with pyrazine/PBS solution applied as antimicrobial lock solution. Premixed pyrazine/PBS solution for biofilm treatment was applied on the catheter by injecting the solution into the tubes.

Biofilm treatment with pyrazine in Eppendorf tubes

To compare the treatment efficiency between the antimicrobial lock technique and the treatment in Eppendorf tubes, 0.5 cm of CVC were placed in 2 ml Eppendorf tubes containing 0.4%, 0.6% or 0.8% of pyrazine treatment solution. A negative control was conducted using PBS. Treatment solution was prepared as described above. Eppendorf tubes were placed in a shaker (120 rpm) at 37°C for 19 hours. Re-isolation of bacteria was done by shaking as describes above. The results were compared by number of cells grown on agar plates.

Results

All quantitative and qualitative methods, which we used in this study were adapted to CVC-associated biofilm samples and examined for their efficiency.

Quantification of bacteria in CVC-associated biofilms

Establishing the appropriate methods for CVC investigation using qPCR

DNA isolation using MP Fast DNA spin Kit for Soil showed higher DNA yield than isolation using the MoBio Power Biofilm DNA isolation Kit. Results are shown in Table 11.

Table 11: Total amount of DNA isolated using MP Fast DNA Spin Kit for Soil and Mo Bio Power DNA isolation Kit.

Sample ID	DNA amount [ng] isolated with MP Fast DNA spin Kit for Soil	DNA amount [ng] isolated with MO BIO PowerBiofilm DNA Isolation Kit
UBT: T1D	1470	740
UBT: T2D	1040	560
UBT: T3D	1170	940
UBT: T4D	1370	760
UBT: T5D	1480	-
UBT: TNKD	1120	-
CVC1	-	500
CVC2D	-	265
CVC2M	-	255
CVC2P	-	715
CVC2aD	-	325
CVC2aM	-	475
CVC2aP	-	360
CVC3D	-	290
CVC3M	-	405
CVC3P	-	395

Statistical analysis of the data showed that the of gene copy/cm CVC were statistically higher ($P<0.05$) when bacterial DNA was isolated using the PowerBiofilm DNA Isolation Kit (Figure 14). Based on this data, all following catheters were isolated using MoBio PowerBiofilm DNA Isolation Kit.

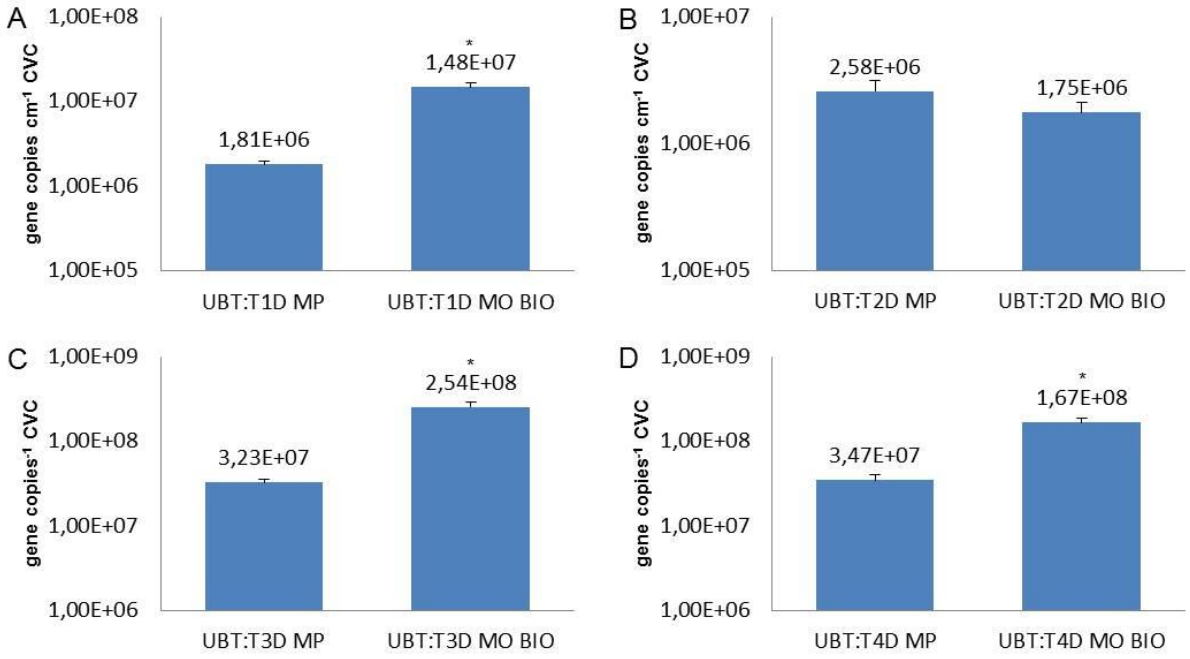

Figure 14: Statistical analysis of 16S rDNA gene copies per cm of CVC detected by qPCR. Sample UBT:T1D (A), UBT:T3D (C) and UBT:T4D (D) showed significantly higher numbers of gene copies per cm of CVC. Error bars indicate the standard deviation. Asterisks denote the significant differences (P < 0.05) between the two kits. Abbreviations: MP - PowerBiofilm DNA Isolation Kit, MO BIO - MoBio PowerBiofilm DNA Isolation Kit.

Amplification of 16SrRNA gene with Unibac II-515f and Unibac II-927r primers resulted in two products of different length (Figure 15-Figure 16): 600bp and 400bp, respectively. The negative control in the first run showed a false positive result whereas the negative control in the second run was negative (Figure 16).

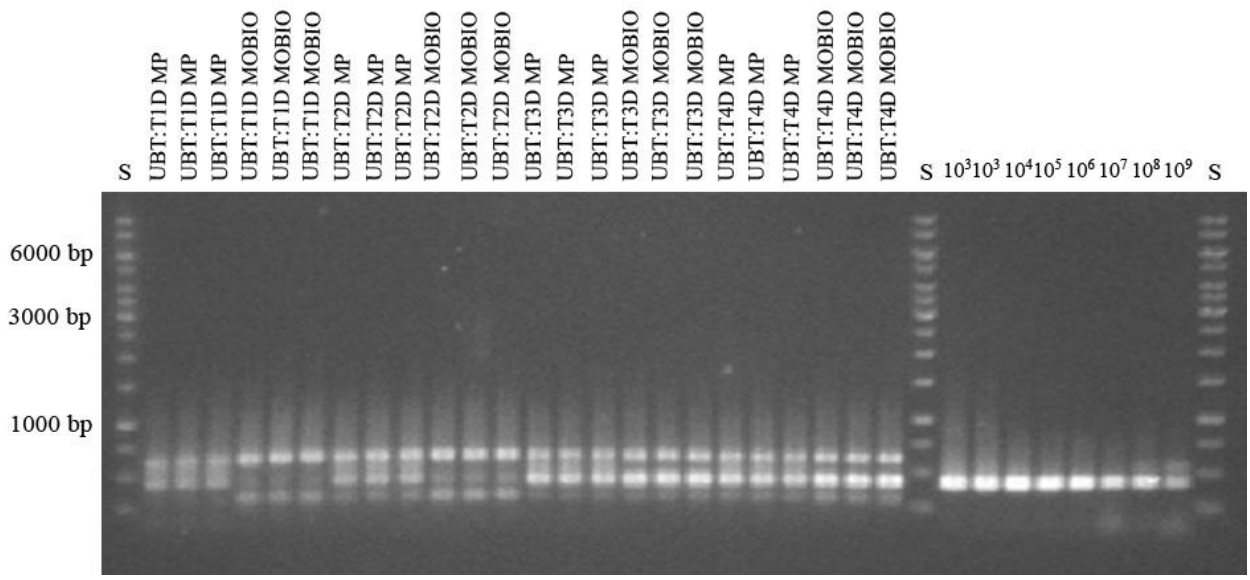

Figure 15: Gel electrophoresis image of qPCR products of the first run obtained from CVC total community DNA with Unibac II-515f/ Unibac II-927r primers. IDs are specified in the upper part of the image for each sample respectively. Abbreviations: S, GeneRuler 1kb DNA Ladder. 10^{-3}-10^{-9}, qPCR standard dilutions.

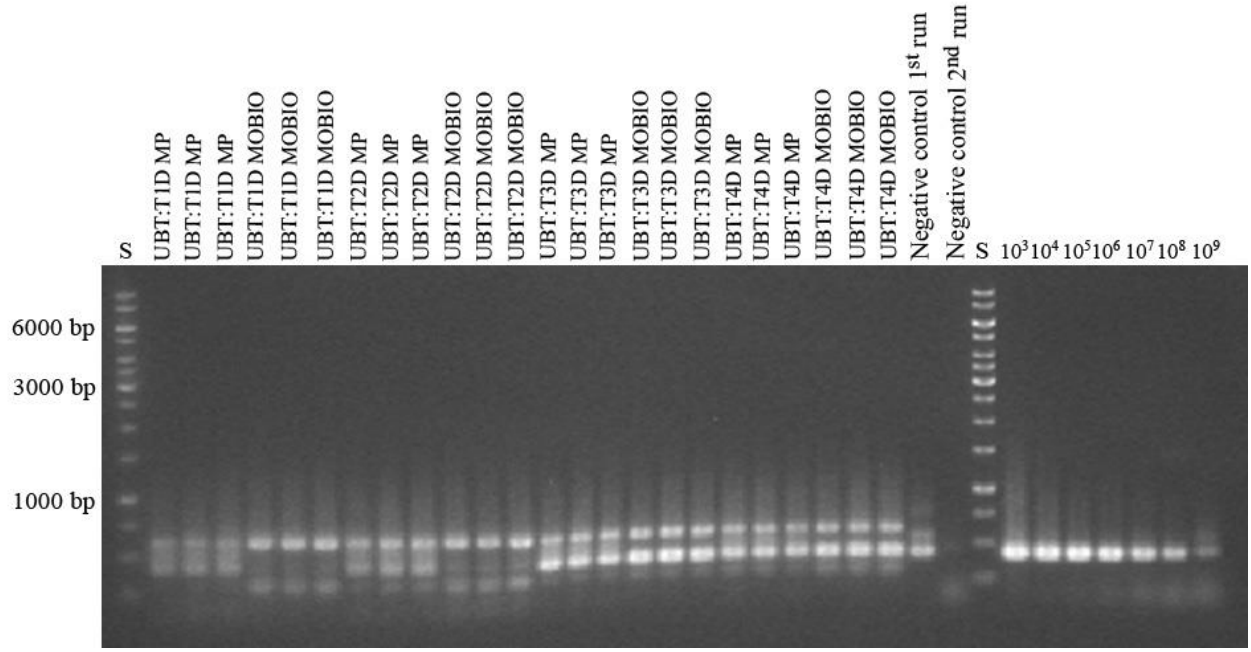

Figure 16: Gel electrophoresis image of qPCR products of the first run obtained from CVC total community DNA with Unibac II-515f/ Unibac II-927r primers. IDs are specified in the upper part of the image for each sample respectively. Abbreviations: S, GeneRuler 1kb DNA Ladder. 10^{-3}-10^{-9}, qPCR standard dilutions.

Quantification of bacteria in catheter sections

Statistical analysis of the different catheter sections showed that the number of gene copies/cm CVC strongly depends on catheter type and part within the catheter. The negative control showed low numbers of gene copies (run1: 148 gene copies μl^{-1}; run2:

213 gene copies μl^{-1}; run3: 355 gene copies μl^{-1}) and subtracted from the values of CVC samples. Prior to analysis of the catheters, all three runs were analyzed for normal distribution and presence of outliers. The statistical analysis showed 12 outliers, which were included to avoid loss of data (Figure 17B), which resulted in a higher standard deviation (Figure 17A). The standard deviation in CVC1 was lower when compared to the other three catheters. The highest bacterial density was observed for CVC3 (2.18 ± 0.6210^8 gene copies/cm CVC). CVC2 and CVC2a, which were explanted from one patient, showed different numbers of gene copies/cm CVC. All means and standard deviations are shown in Table 12.

For the analyzed CVCs, bacterial abundances were differently distributed between catheter parts. CVC2 showed the highest gene copies/cm CVC in the proximal end ($3.28\pm0.56x10^7$ gene copies/cm CVC), followed by the medial part ($3.87\pm0.02*10^6$ gene copies/cm CVC) and the distal part ($4.43\pm0.97*10^4$ gene copies/cm CVC) (Figure 18A). In CVC2a the highest number of gene copies/cm CVC was detected in the medial part ($7.94\pm0.05*10^6$ gene copies/cm CVC), followed by the proximal part ($2.02\pm0.15*10^6$ gene copies/cm CVC) and at least the distal part ($1.06\pm0.21*10^5$ gene copies/cm CVC) (Figure 18B). In CVC3 the highest number of gene copies/cm CVC were detected in the distal part ($5.75\pm0.63*10^7$) gene copies/cm CVC), followed by the proximal part ($6.00\pm0.95*10^6$) gene copies/cm CVC). The medial part had the lowest number of gene copies/cm CVC ($1.87\pm0.19*10^6$) gene copies/cm CVC) (Figure 18C).

Table 12: Microbial abundance in different CVC parts expressed in 16S rRNA gene copies/cm CVC as detected by qPCR. Standard deviations are shown in brackets.

Catheter ID	Catheter part, gene copies/cm CVC		
	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	$5.75x10^7$ ($6.29x10^6$)	$1.87x10^6$ ($1.92x10^5$)	$6.00x10^6$ ($9.45x10^5$)

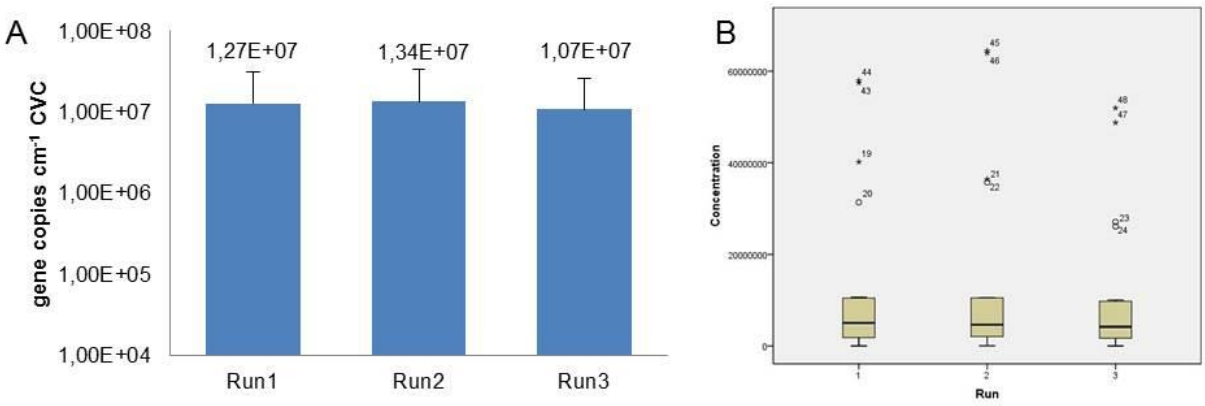

Figure 17: 16S rRNA gene copy numbers per PCR run. A: Graph showing the mean values and standard deviations of 16S rRNA gene copy numbers for each PCR run and. B: SPSS exported image showing the outliers (dots and asterisks).

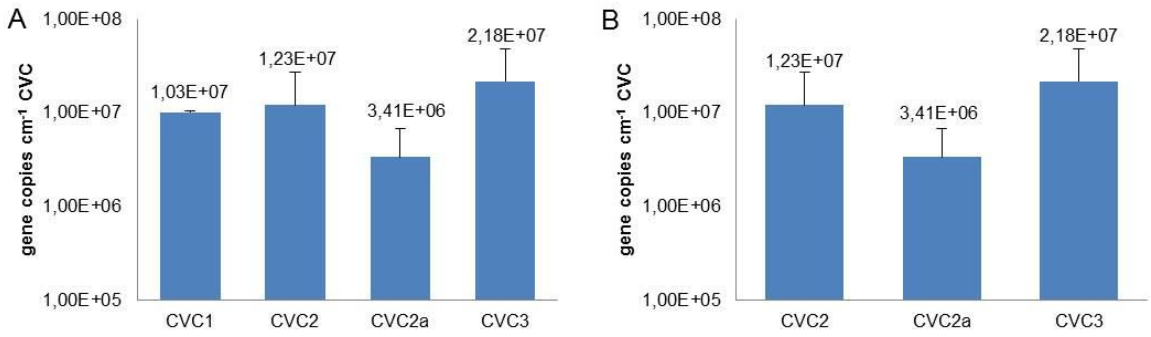

Figure 18: 16S rRNA gene copy numbers per PCR run. A: Graph showing the mean values and standard deviations of 16S rRNA gene copy numbers for each CVC including CVC1 and. B: without CVC1.

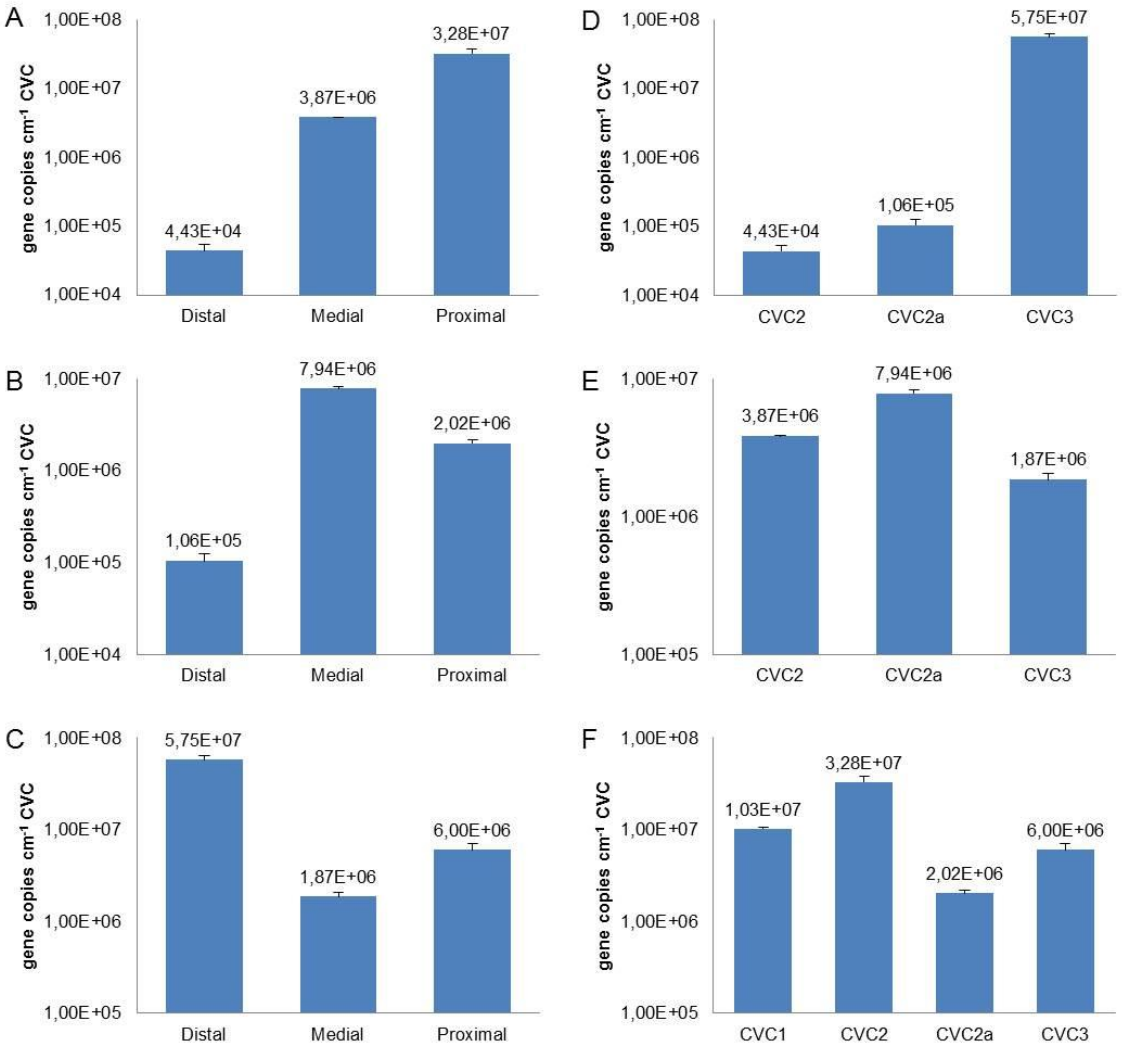

Figure 19: 16S rRNA gene copy numbers within each catheter and distal, medial and proximal part of each catheter. A-C: Graphs showing the gene copies cm^{-1} CVC mean values and standard deviations within the different catheter parts (A: CVC2, B: CVC2a and C: CVC3). D-F: Comparison of gene copies cm^{-1} CVC obtained for the distal, the medial and the proximal part of different catheters (D: distal part, E: medial part, F: proximal part).

In contrast to the previous qPCRs, the standards applied together with CVC1-CVC3 showed a smear and all negative controls showed a false positive result (Figure 20-Figure 22).

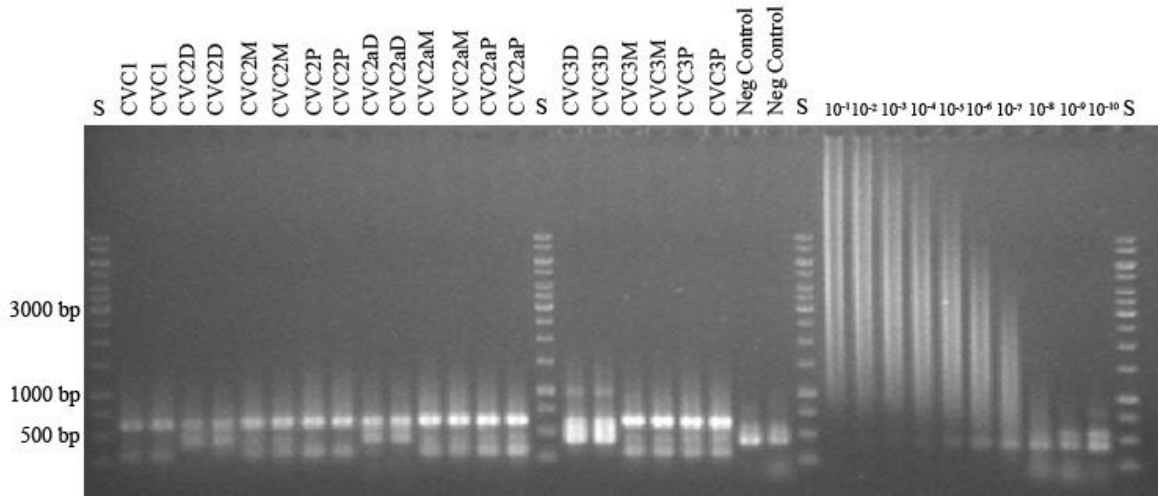

Figure 20: Gel electrophoresis image of qPCR products of the first run obtained from CVC total community DNA with Unibac II-515f/ Unibac II-927r primers. IDs are specified in the upper part of the image for each sample respectively. Abbreviations: S, GeneRuler 1kb DNA Ladder. 10^{-3}-10^{-9}, qPCR standard in different dilutions.

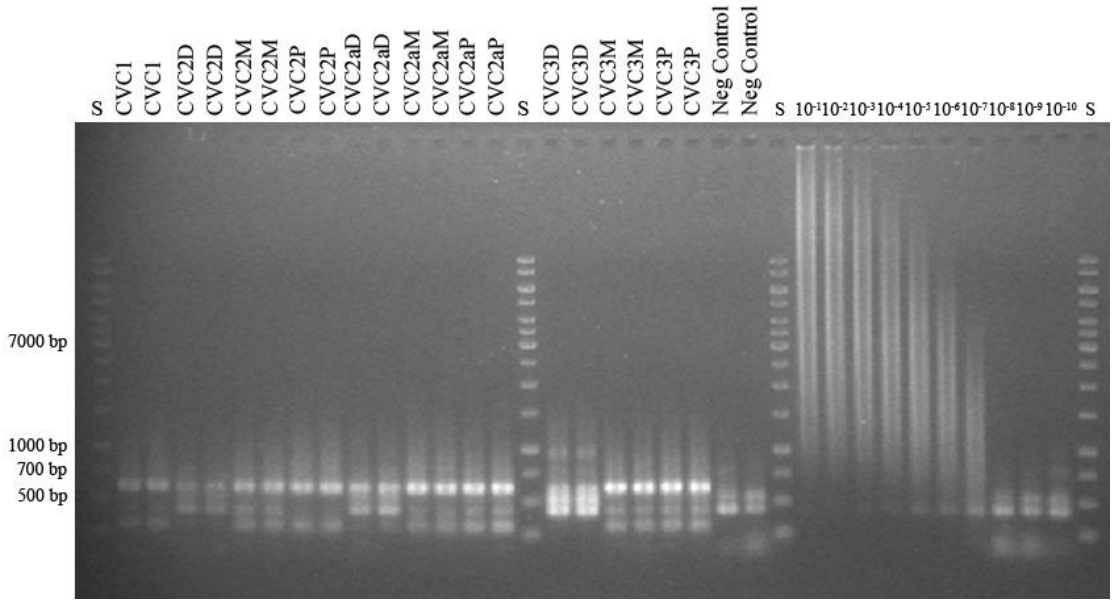

Figure 21: G Gel electrophoresis image of qPCR products of the second run obtained from CVC total community DNA with Unibac II-515f/ Unibac II-927r primers. IDs are specified in the upper part of the image for each sample respectively. Abbreviations: S, GeneRuler 1kb DNA Ladder. 10^{-3}-10^{-9}, qPCR standards in different dilutions.

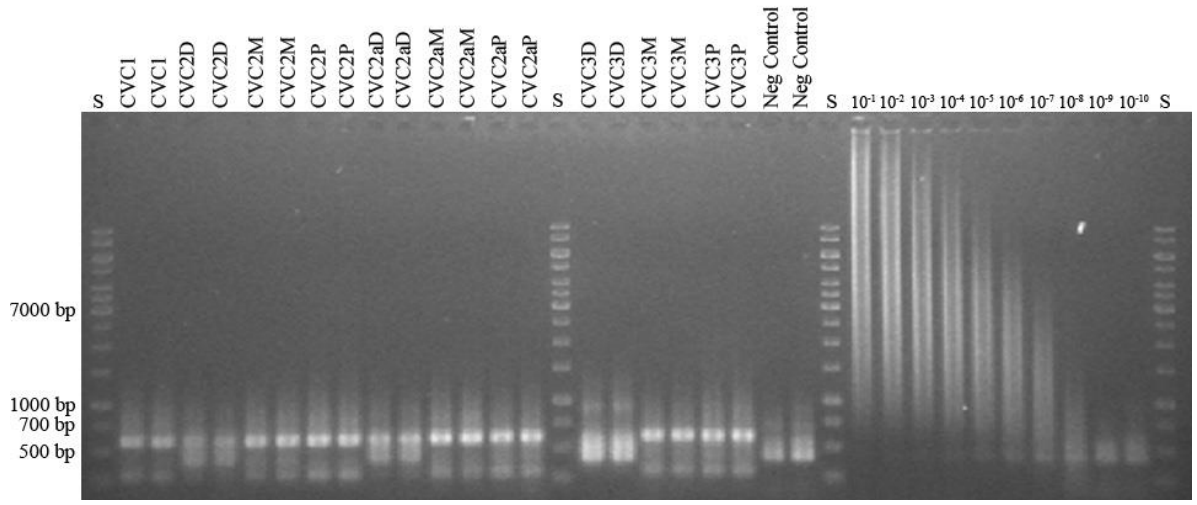

Figure 22: Gel electrophoresis image of qPCR products of the third run obtained from CVC total community DNA with Unibac II-515f/ Unibac II-927r primers. IDs are specified in the upper part of the image for each sample respectively. Abbreviations: S, GeneRuler 1kb DNA Ladder. 10^{-3}-10^{-9}, qPCR standard in different dilutions.

Comparison of the catheter removed from the patient´s body by non-invasive method (Set 1) and by explanation (Set 2) showed that the catheter removed by the non-invasive method had higher gene copies/cm CVC than the explanted CVC (Set 2) (Figure 23A). A comparison of all CVCs showed that the highest gene copies/cm CVC were on UBT:T3D (2.55±0.39*10^8 gene copies/cm CVC) and UBT:T4D (1.67±0.22*10^8) gene copies/cm CVC) (Figure 23B).

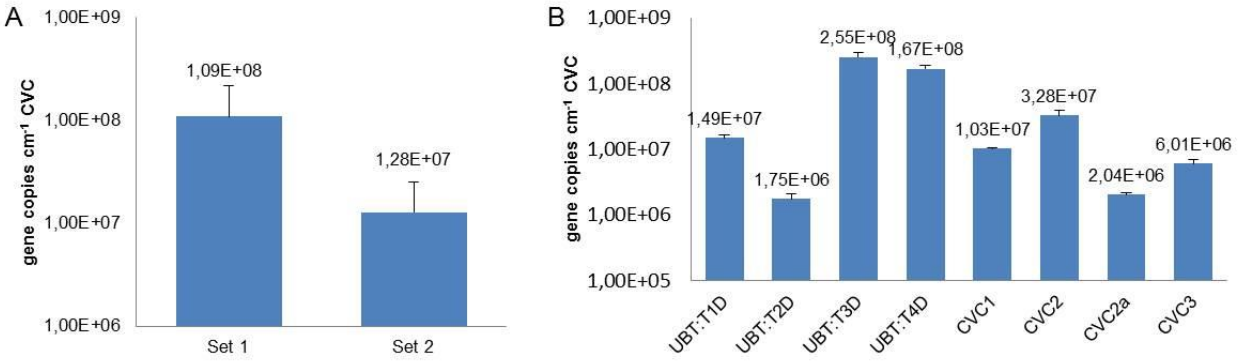

Figure 23: Abundance of microbial 16S rRNA gene copy numbers for two different catheter sets by qPCR. A: catheter from April 2014 (Set 1) and catheter from January 2015 removed by explanation. B: Graph showing the mean values and standard deviations of 16S rRNA gene copy numbers of catheters from Set 1 and Set 2.

In conclusion, based on these results isolation of total community DNA using the Mo Bio PowerBiofilm Kit outperformed the isolation using MP FastDNA Spin Kit for Soil. The

number of gene copies/cm CVC was strongly dependent on 1) the part and the type of the catheter; 2) the retention time, in which the catheter remained in the donor.

Analysis of community composition of CVC-associated biofilms

Establishing the appropriate methods for CVC investigation using 16S rRNA gene amplification and clone libraries

Total-community DNA, which was isolated using the MP Fast DNA Spin Kit for Soil, showed no product in each eluate on agarose gel (Figure not shown). However, the total community DNA was used as a template for the PCR using the 27f/1492r primer set. A product size *in silico* was 1465 bp. The PCR product of approximate size of 1500 bp was obtained for all five CVC samples and the negative control. The negative control showed a slight band revealing a false positive result (Figure 24).

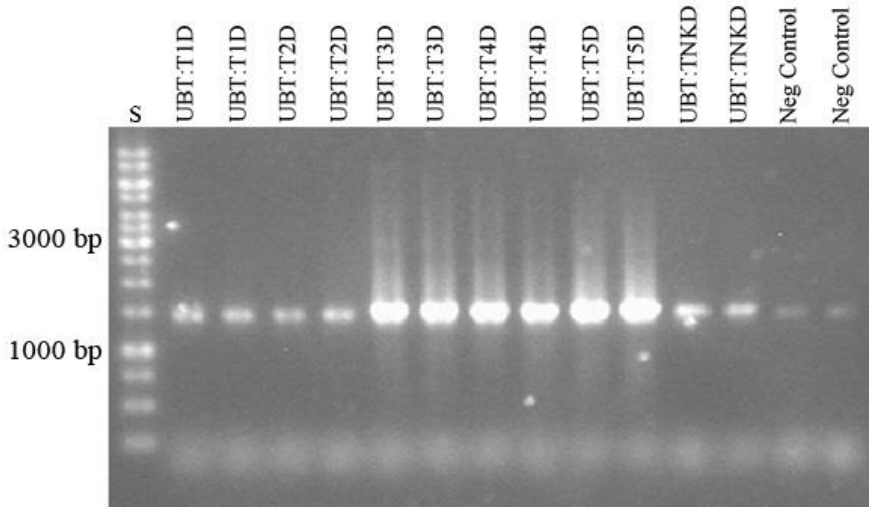

Figure 24: Gele electrophoresis image PCR products obtained using primer 27f/1492r primer set. Each sample was amplified in duplicate. Sample IDs are specified in the upper part of the image for each sample respectively. Abbreviations: S, GeneRuler 1kb DNA Ladder.

Colony PCR was used to exclude false positive results in the blue/white screening. Control agarose gel showed that not all picked colonies from the induction plate carried the amplified 16S rRNA gene fragment (Figure 25A-F). The length of PCR product containing insert should increase from 1465 bp to 1715 bp. The PCR products of approximate size of 1700 bp were chosen for sequencing. One negative control showed a false positive result and the second one failed to amplify.

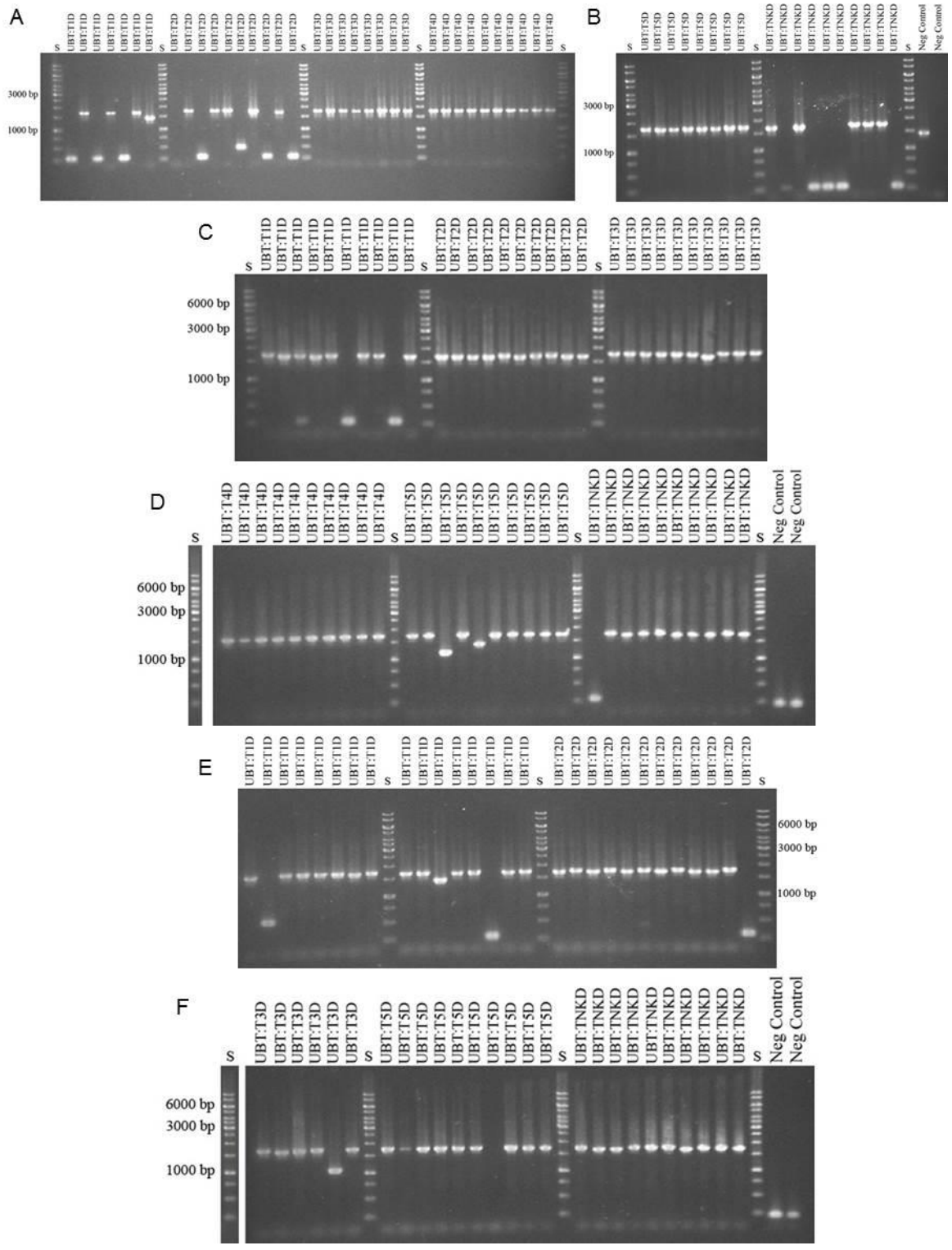

Figure 25: Control gel-electrophoresis image of a colony PCR of blue-white screening positive. The *E. coli* cells were screened for the 16S rRNA fragment using USP/RSP primer. IDs are specified in the upper part of the image for each sample respectively. A PCR products containing insert have approximate size of 1715 bp. Abbreviations: S, Standard GeneRuler DNA Ladder 1kb.

To reveal a microbiome composition of CVC-associated biofilms 120 clones (20 per sample) were sequenced and examined bioinformatically. Blast-search revealed the presence of microorganisms on catheters which were not detected via conventional cultivation based techniques. The abundances in the CVCs UBT:T1D and UBT:T2D were very similar (Figure 26A-B). The blast of the sequenced microbial 16S rRNA genes revealed that UBT:T1D and UBT:T2D were dominated by *Brucella* sp. with 40% and 35% relative abundance, respectively (98-99% sequence similarity). *Herbaspirillium seropaedicae* (20% of relative abundance in UBT:T1D and 25% in UBT:T2D, 98% of seq. similarity) was also detected in higher abundance compared to the other microorganisms. The number of sequences assigned to other microorganisms was low (5% to 10%). In sample UBT:T1D the minor microorganisms included following microorganism: *Bacteriodales* (84% seq. similarity), *Leptothrix* sp., *Clostridiales* (81%), *Sinorhizobium fredii* (96%), *Ralstonia pickettii* (99%), *Stenotrophomonas maltophilia* (99%) and *Rhodococcus erythropolis* (99%). 5% of the microbiome remained unclassified for UBT:T1D and UBT:T2D samples, respectively. In sample UBT:T2D the minor detected microorganism were: *Rubrivivax* sp. (94% seq. similarity), *Methylobacterium* sp. (94%), *Sphingobium* sp. (95% similarity), Sphingomonadaceae (93% similarity), Acinetobacter calcoaceticus (97%) and *Delftia acidovorans* (100%). 95% sequences of UBT:T3D belonged to *Enterococcus faecium* (99-100% of sequence seq. similarity) and 5% was assigned to *Brucella* sp. (94-100% seq. similarity). UBT:T4D was dominated by *Staphylococcus epidermidis* (90% abundance, 99% seq. similarity). *Stentrophomonas maltophilia* (96-98% seq. similarity) was a minor representative within the analysed community (10%) (Figure 26C-D). UBT:T5D was dominated by *Staphylococcus aureus* (100% of relative abundance, 99-100% seq. similarity). One sample (5%) of UBT:T5D subjected to sequencing failed. The microbiome of the negative control sample (UBT:TNKD) was dominated by *S. aureus* (50% of relative abundance, 99-100% seq. similarity) followed by *Brucella* sp (25% abundance, 98-99% seq. similarity). 10% of sequences were assigned to *Chlostridiales* (81-81% seq. similarity). *Achromobacter xylosoxisans* (99% seq. similarity) and *Herbaspirillium seropaedicae* (98%) and *Erythrobacteraceae* (93%) were represented by 5% of sequence in the sample (Figure 26E-F).

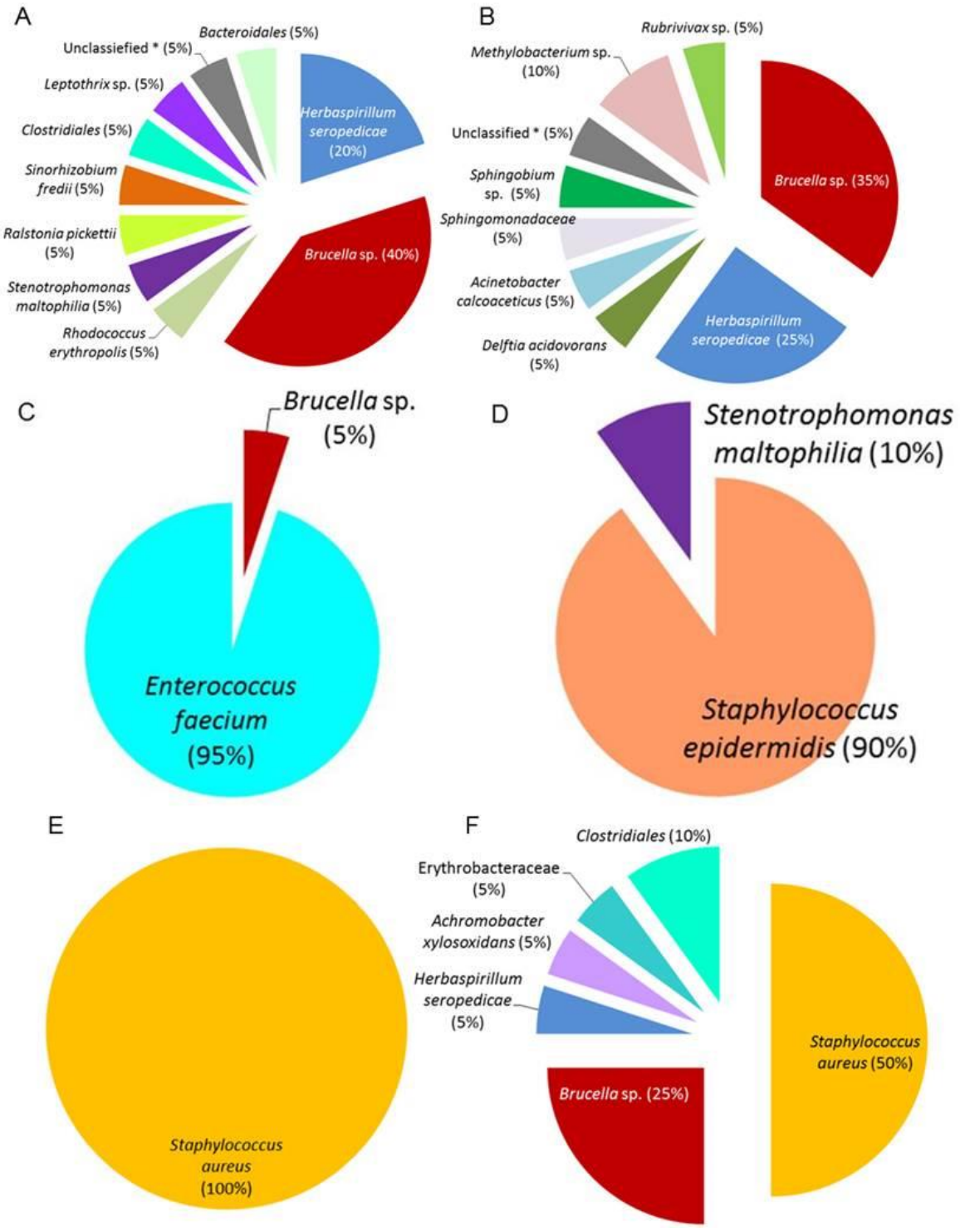

Figure 26: Pie charts showing the microbiome composition of catheter UBT:T1D (A), UBT:T2D (B), UBT:T3D (C), UBT:T4D (D), UBT:T5D (E) and UBT:TNKD (F). The charts are based on 20 clones that were sequenced and analyzed for each sample.

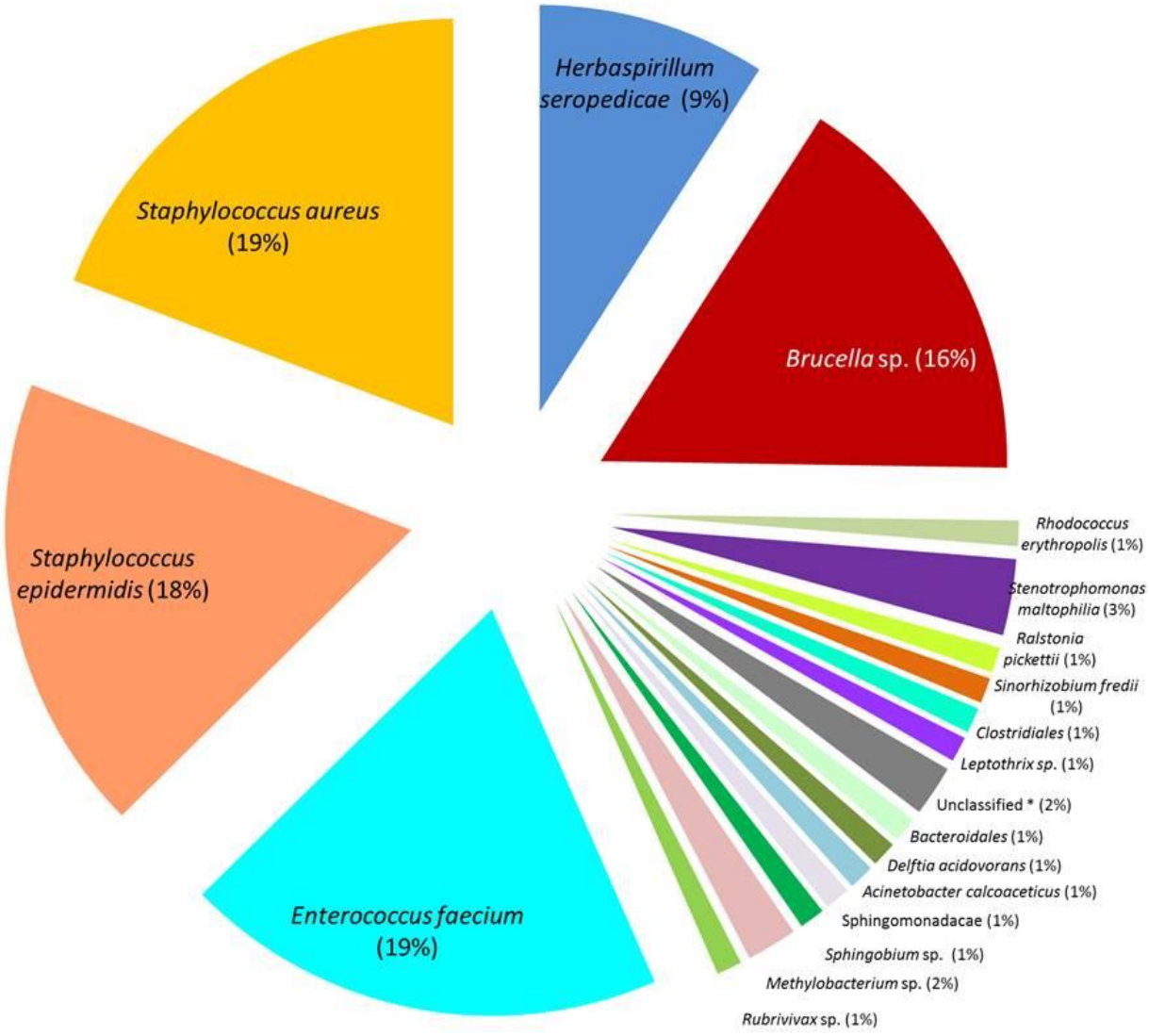

Figure 27: Pie-chart of microbiome composition overall catheter tips.

In conclusion, the community structure of the CVC-associated microbiome was case- and patient-specific.

Visualization of CVC-associated biofilms

Adapting fixation protocols to CVC-associated biofilms

Common fixation protocols use ethanol/PBS mixture to preserve the samples during freezing process. An application of ethanol for storage and preservation of CVC sections was not possible due to the biofilm destruction by ethanol. Glycerol, a widespread cryopreservative and cell protection agent, was used to overcome this problem.

FISH visualization of samples fixed with 4%PFA and stored in PBS/ethanol solution

UBT:T3D sample hybridized with NONEUB338-Cy3 showed false positive results (Figure 28). The detected red dots were a result unspecific binding of FISH probes or autofluorescent objects. UBT:T3D, which was incubated with EUB338-Cy3, showed no fluorescence signal (Figure 29). A positive result was expected as red cells. The experiment was repeated. The red particles, which were detected in the negative control in the first experiment, were detected also in the second experiment (Figure 30). In the second experiment, sample UBT:T3D was treated again with EUB338-Cy3. Thereby red particles were detected on the cutting surface of the CVC. The size of the detected particles ranged from 1 to 4.65 µm (Figure 31).

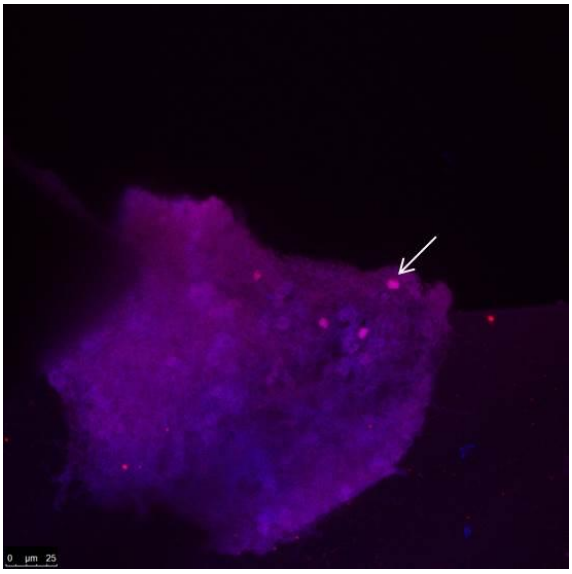

Figure 28: Cross-section of CVC sample (UBT:T3D) treated with non-specific FISH probe (NONEUB338-Cy3) and visualized using CLSM. Unspecific FISH-probe binding and/or autofluorescent object is marked with an arrow.

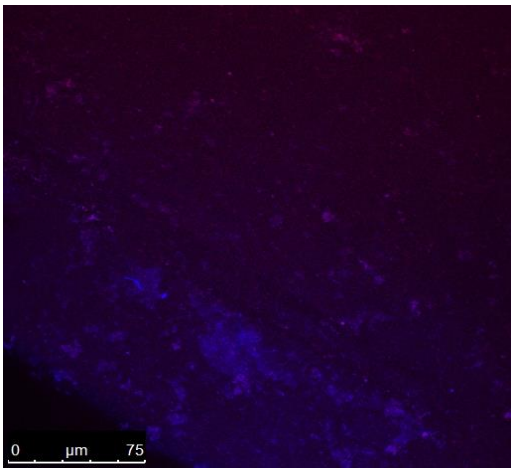

Figure 29: Detection of bacteria in CVC cross-section (sample ID, UBT:T3D) by FISH-CLSM visualization. CLSM image of a sample incubated with EUB338-Cy3 und DAPI.

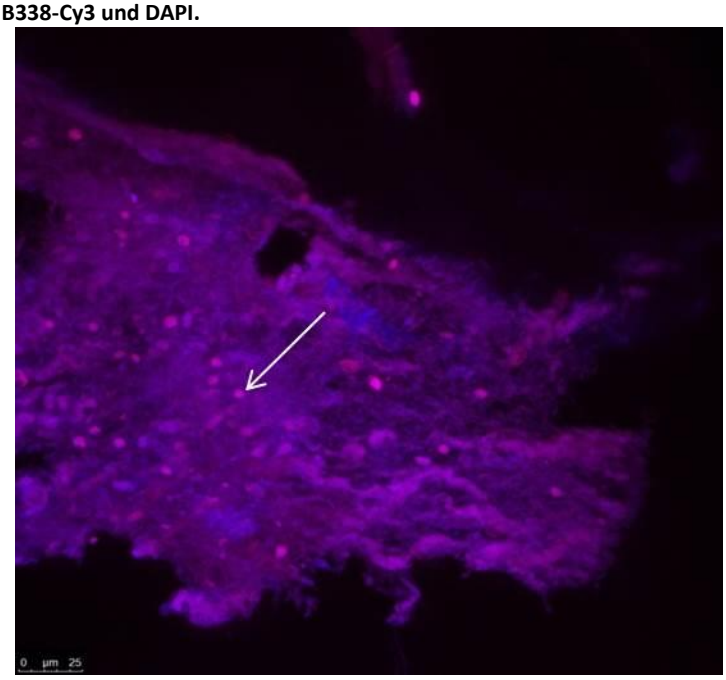

Figure 30: Negative control of CVC cross-section. The cross-section of sample UBT:T3D was treated with NONEUB338-Cy3 and visualized using CLSM. Different red/pink particles were located on the CVC section (marked with an arrow).

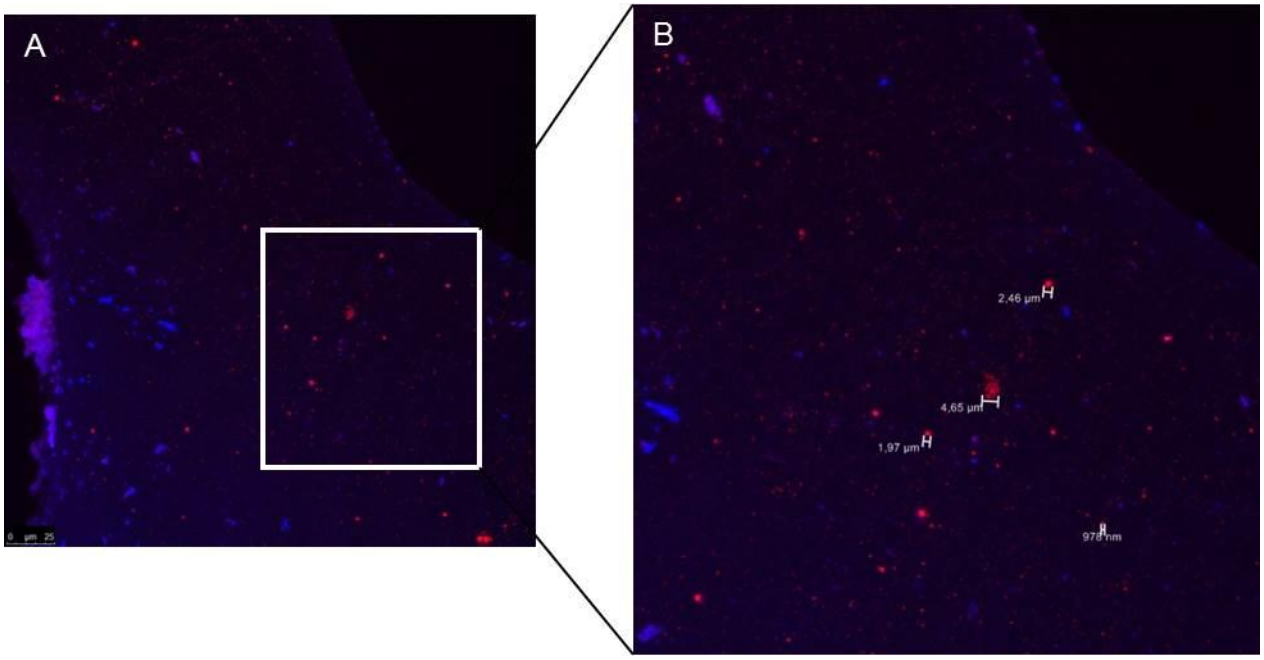

Figure 31: Detection of bacteria in UBT:T3D hybridized with EUB338-Cy3 and visualized using FISH-CLSM. A: Catheter cutting surface between two catheter lumen. B: Magnification of the cutting surface of the catheter. Red dots: unspecific binding of FISH-probe and/or autofluorescent objects.

Storage in ethanol had a negative effect on the stability of the biofilm. The detected particles cannot be considered as bacteria due to their unregularly size and shape.

Sample preparation and fixation of CVC sections in acrylamide

To test the robustness of the polyacrylamid after ethanolic series and the behavior of the CVC sections in the embedding material, the CVC was fixed in polyacrylamide (Figure 32).The polyacrylamide changed the color to white when treated with ethanol series. Moreover, ethanol treatment resulted in detachment of the polyacrylamide from the glass slide. After drying, polyacrylamide became firm and sticked hard to the glass surface.

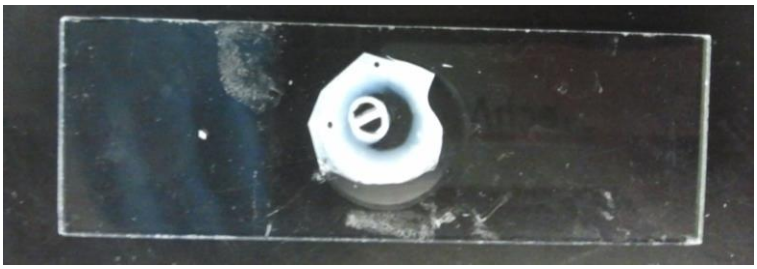

Figure 32: Polyacrylamide gel dehydrated with ethanol series. Catheter section placed in a microscope slide with cavity and embedded in polyacrylamide.

Detection of bacterial biofilms using DAPI-staining and CLSM succeeded only in the sample UBT:T3D. The polyacrylamide gel lead in sample UBT:T3D to strong background fluorescence (Figure 33A-B). The second image taken from this sample showed bacterial cells close to the wall of the catheter lumen (Figure 33C-D).

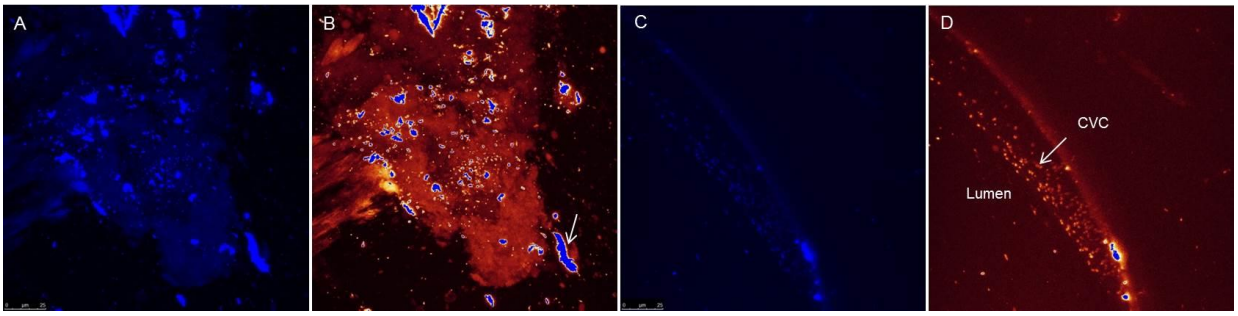

Figure 33: Bacterial biofilm on CVC UBT:T3D fixed in polyacrylamide gel and visualized using DAPI staining and CLSM. Samples are shown in overlay mode and saturation mode. The saturation mode images showed the objects with the strongest fluorescence in blue and with the lower fluorescence in red. A-B: Image showing the lumen of the CVC section in overlay mode (A) and saturation mode (B). C-D: Biofilm on the inner lumen of the CVC in overlay mode (C) and saturation mode (D). The location of the CVC and the lumen are described in the image.

Based on the handling challenges when using ethanol as dehydration agent and the high background signal, this method was considered as inappropriate to visualize an intact biofilm *in situ*.

Modified paraformaldehyde fixation method for CVC-sections

An intact biofilm was detected when CVC-section was fixed with the new method using glycol as cryopreservative instead of ethanol. With the new fixation method, the detection of a biofilm in sample UBT:T3D was possible (Figure 34A). An ethanolic series of increasing ethanol concentration is commonly used to dehydrate bacterial cells during the FISH procedure. To test the stability of the biofilm, sections were treated using ethanolic series. Figure 34B showed that the biofilm of UBT:T3D was stable after treatment using ethanolic series.

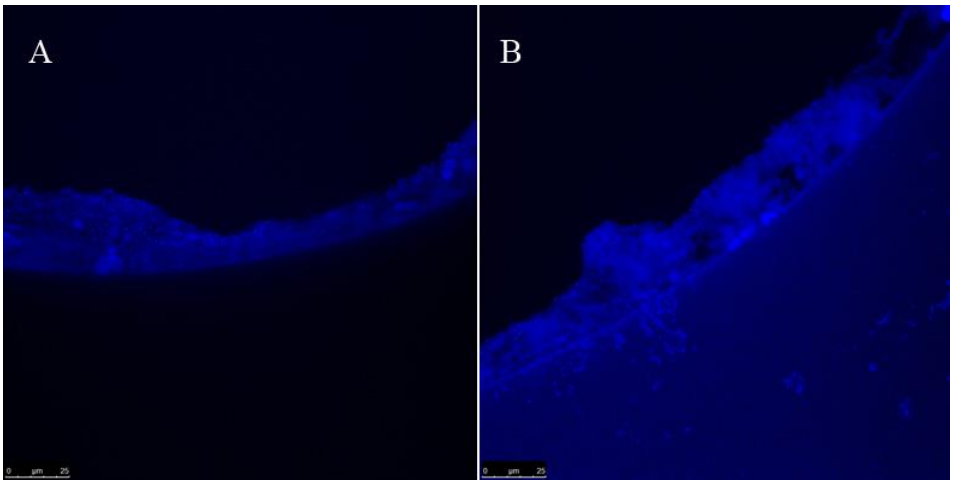

Figure 34: Bacterial biofilm on CVC section UBT:T3D with and without ethanol series treatment visualized using CLSM and DAPI staining. A: UBT:T3D without treatment of ethanol series. B: UBT:T3D treated with ethanol series.

Fluorescent *in situ* hybridization (FISH)

No fluorescence signal was detected in the negative control indicating an unspecific hybridization or unspecific binding to matrix component. Using taxon-specific FISH-probes, *Firmicutes* were detected in CVC samples UBT:T2D (Figure 35A) and *Gammaproteobacteria* in UBT:T4D (Figure 35B). Red blood cells embedded in the biofilm were detected in the sample UBT:T4D (Figure 35B). An image of sample UBT:T4D at the same position but with higher resolution showed more details, especially in the region around the red blood cells (Figure 35C).

Visualization of bacterial biofilm on sample UBT:T3D showed two different density layers. The biofilm layer, which was closer located to the wall of the catheter (yellow bar), appeared on the images more dense than the layer, which was located more in the lumen of the catheter (green bar). The visualised bacteria were of unidentified taxonomy. Bacterial cells were located in the biofilm in the soft layer or between the soft and the dense layers (Figure 36B-D).

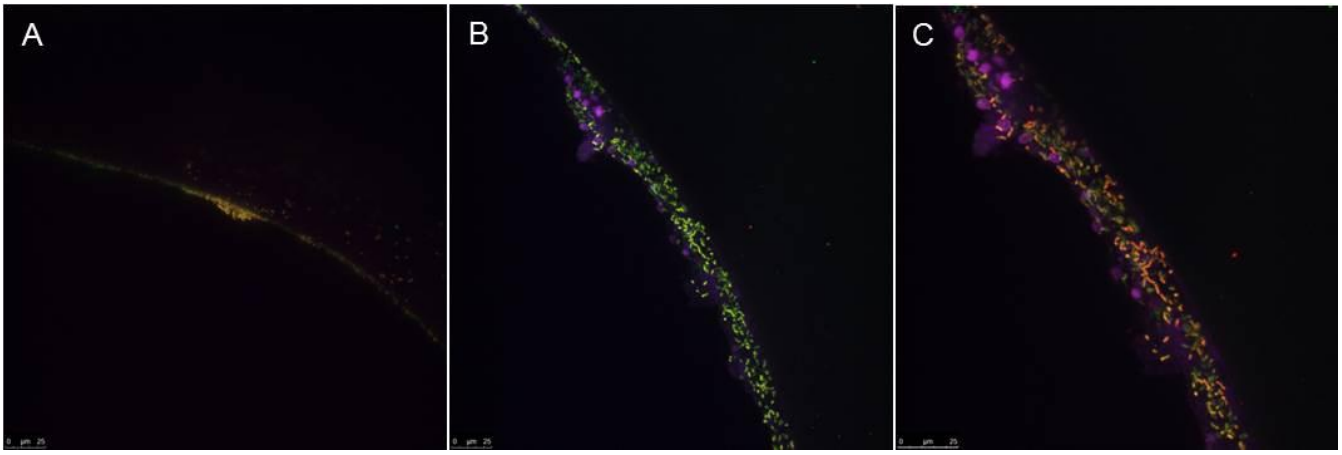

Figure 35: Colonization pattern of the CVC-sections visualized using FISH-CLSM. A: UBT:T2D. B: UBT:T4D. C:UBT:T4D higher resolution Yellow: *Firmicutes*; light-green (B): *Gammaproteobacteria;* orange (C): *Gammaproteobacteria*; violet: red blood cells.

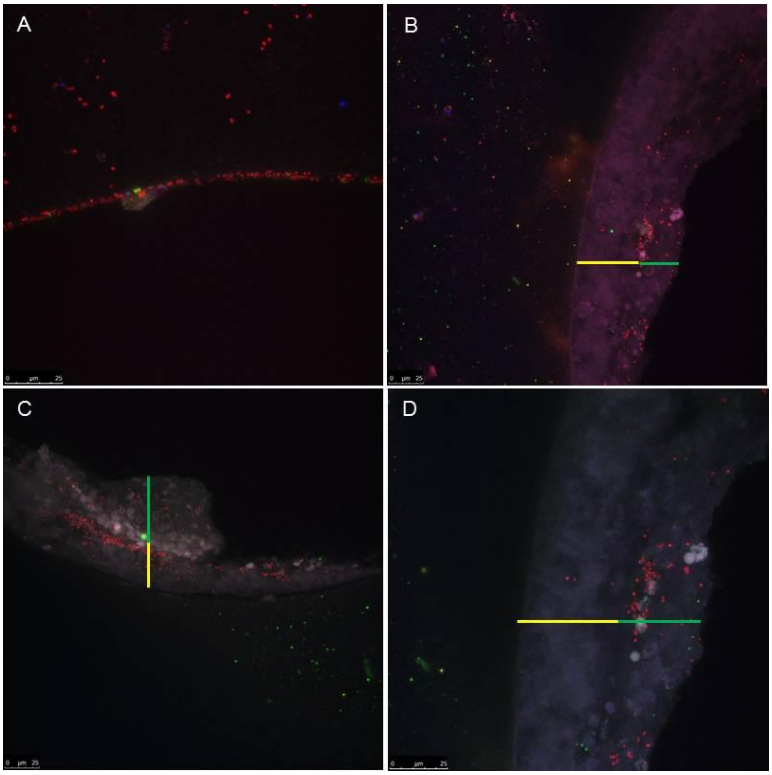

Figure 36: FISH-CLSM images of sectioned CVCs UBT:T2D and UBT:T3D. Microbial cells were hybridized using universal and low-GC –specific FISH probes. A: UBT:T2D, B, C, D: UBT:T3D; yellow bar: inner biofilm; green bar: outer biofilm. A-D: red: bacteria of unidentified taxa; yellow, green, blue: unspecific autofluorescent signals.

To investigate the biofilm *in-situ* FISH-CLSM experiments using catheter sections of explanted catheter CVC1-CVC3 were used. No bacterial biofilm was detected in the inner lumen or the external surface of the catheter.

In conclusion, biofilm visualization using FISH-CLSM strongly depends on the type of the catheter and the indwelling time. Storage of catheter sections using PBS/glycerol is a better biofilm preserving strategy than ethanol, which is a potential biofilm destruction agent. FISH-CLSM revealed *Firmicutes* in UBT:T2D and *Gammaproteobacteria* located in the biofilm of sample UBT:T4D. FISH-CLSM experiments showed a complex two-layers structure of the CVC-associated biofilms.

Investigation of biofilm formation on CVCs

Colonization of catheter sections by *S. aureus* and biofilm treatment using pyrazine

Different types of catheter sections were artificially colonized by single or multiple biofilm-forming strains and were treated by different concentrations of pyrazine. The treatment efficiency was tested by placing the sections in sterile medium, termed as treatment control. Treatment efficiency was determined by pyrazine concentration, which was necessary to remove bacteria from CVC section.

Sections of extracorporal and intracorporal parts of SplitCathIII and Softline were compared in the first experiments. The OD_{600} values of the negative control were between 0.736 and 0.914. CVC-sections treated by a pyrazine concentration of 0.2% showed a higher OD than the negative control. Softline-extravasal-extracorporal fragment showed an OD value of $1*10^{-3}$. Due to such low $OD_{600,}$ the sample Softline^{b} was not diluted and was treated as sample with OD_{600} of zero. In the treatment control no bacterial growth was detected in sections, which were treated by 0.4% and 1% pyrazine (Figure 37).

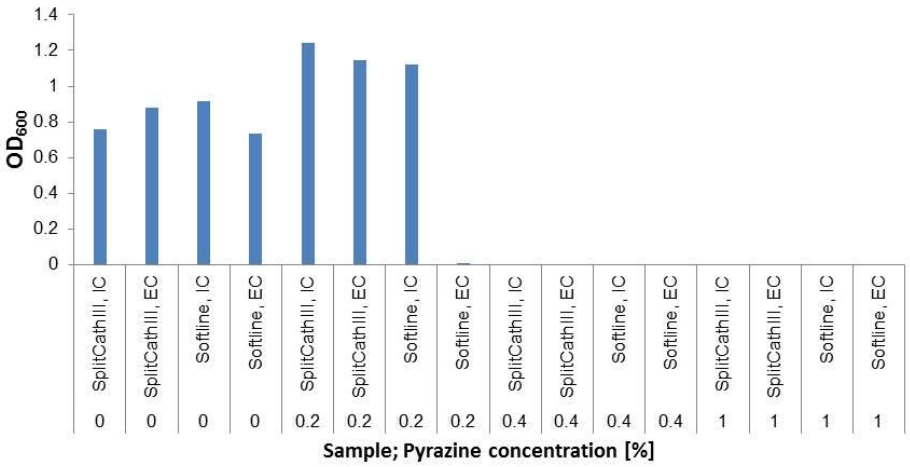

Figure 37: OD_{600} value of the LB medium used for testing treatment efficiency. Treatment control medium showed an increased OD_{600} only at a concentration of 0.2% and in the negative control. Abbreviations: IC: intracorporal part; EC: extracorporal part.

In the repeated experiment three sections of intracorporal and extracorporal part were colonized by *S. aureus* and treated by an increasing pyrazine concentration. In this experiment CFU ml^{-1} of negative control were determined only in one replicate of extracorporal part. In the other replicates of extracorporal part and intracoporal part no growth was detected on LB agar plates. A pyrazine concentration of 0.6% resulted in reduced bacterial concentration or no bacterial growth in treatment control. A total growth inhibition was reached when using a pyrazine concentration of 0.8% in PBS on sections of extracorporal and intracorporal part (Figure 38).

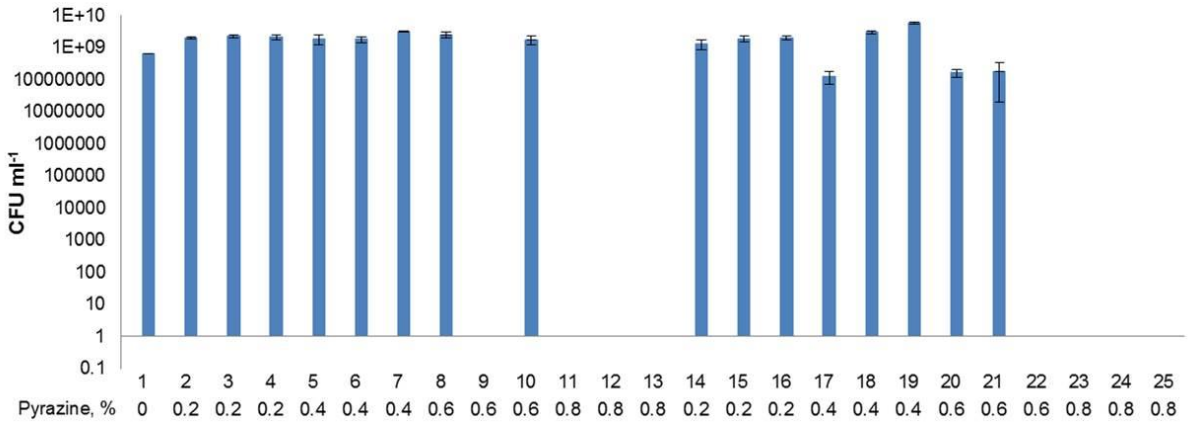

Figure 38: Treatment of extracorporal and intracorporal SplitCathIII catheter sections using increasing concentrations of pyrazine. Numbers on the x-axis: 1, negative control; 2-13 intracoporal catheter sections; 14-25 extracorporal catheter sections. The pyrazine concentrations are speicified below the x-axis.

Treatment of catheter sections, which were colonized by *S. aureus*, was effective when using low concentrations of pyrazine and the modified protocol. The standard protocol, which was applied first, did not work due to chemical reaction of pyrazine with the polystyrole. Bacteria eradication was observed when using the modified protocol. The effective treatment concentration of pyrazine, which was necessary to inhibit the growth of the biofilm-forming bacteria inactivate all bacteria on a catheter section was 0.8%.

Colonization of catheter sections by a mixture of *S. aureus, P. aeruginosa* and *S. maltophilia* and treatment using pyrazine

In the first experiment four replicates of intracorporal part of SplitCathIII and ARROW were colonized by a multispecies biofilm and were treated using 0.4% pyrazine. Treatment control medium of two SplitCathIII sections showed a reduced OD_{600} after treatment (2.25 and 3.06; negative control: 5.40). In the other two replicates neither an increased OD_{600} was measured nor were any colonies detected on LB agar plates. One treatment control of catheter ARROW showed an OD_{600} reduction to 0.008 (negative control: 4.79). In the other three replicates the OD_{600} did not increase and no growth was detected on agar plates.

Three replicates of intracorporal part of SplitCathIII and ARROW intracorporal part were included in the second experiment. In all three replicates of negative control the CFU ml^{-1} was higher than in the treated sections (Figure 39). Treatment of SplitCathIII catheter section using 0.2% and 0.4% pyrazine showed a reduction of bacterial growth compared to the negative control (Figure 39). A pyrazine concentration of 0.6% showed to be the at least minimal concentration necessary to combat all bacteria from CVC section. No bacterial growth was detected visually and via plating in the wells of ARROW (Figure 40).

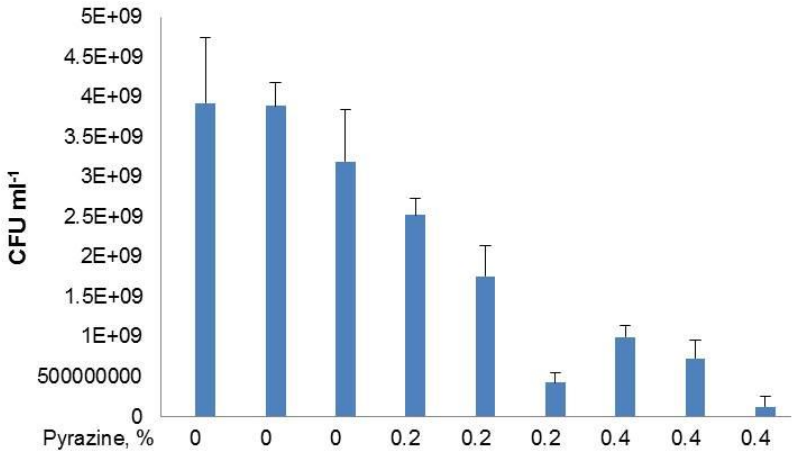

Figure 39: Bar chart showing the CFU ml^{-1} in the treatment control medium after CVC section treatment by different concentrations of pyrazine and negative control. The applied concentrations of pyrazine are shown in the x-axis.

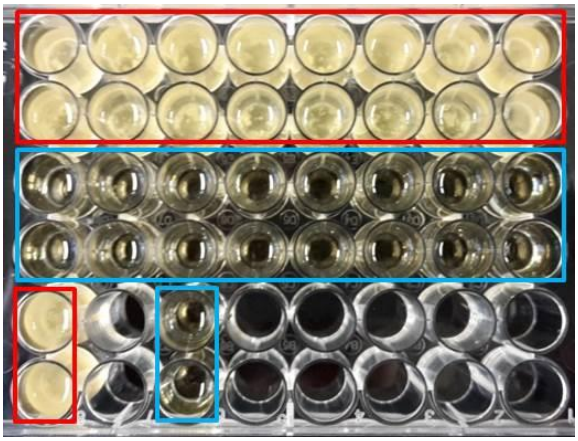

Figure 40: Photograph of the 48-well plate used for colonization of SplitCathIII and ARROW catheter sections. Red rectangle: colonization of SplitCathIII sections using *S. maltophilia*, *S. aureus* and *P. aeruginosa*. Blue rectangle: colonization of ARROW catheter sections by *S. maltophilia*, *S. aureus* and *P. aeruginosa*.

The minimal lethal concentration for multispecies biofilm comprising *S. aureus*, *P. aeruginosa* and *S. maltophilia* was 0.6%. A pyrazine concentration below 0.2% showed a reduction of bacterial growth and optical density compared to the negative control in all experiments.

A coated catheter was used beside the uncoated SplitCathIII to test whether it was possible to colonize a coated catheter *in-vitro* and to compare the antimicrobial activity of pyrazine and coating. Results showed that commercial available catheters protected

by silver coating are an effective way to prevent biofilm formation on CVC and even inactivate planktonic bacteria in the medium.

Sterilization of non-autoclavable biofilm formation plant and validation of sterilization method

Due to the use of different non-autoclavable parts in the first plant it was necessary to find a sterilization agent which is known for effective sterilization and do not chemically interact using the materials of tube system of the plant.

Prior to sterilization, water from the tube was plated on agar plates to check for bacterial contamination. Plating of the water from the tube showed low numbers of planktonic cells – which is 3 cells in the undiluted sample (Figure 41A). To check if a biofilm was formed in the system, the speed of the circulation was increased. Plating of a sample on agar plates after vigorous circulation showed high numbers of colonies – around 1262 bacterial colonies (Figure 41). Plating of a sample after sodium hypochlorite treatment showed no bacterial growth.

Colonization test using *S. aureus*, followed by sterilization with 6 % sodium hypochlorite showed no bacterial growth in the rinsing water of the tube system.

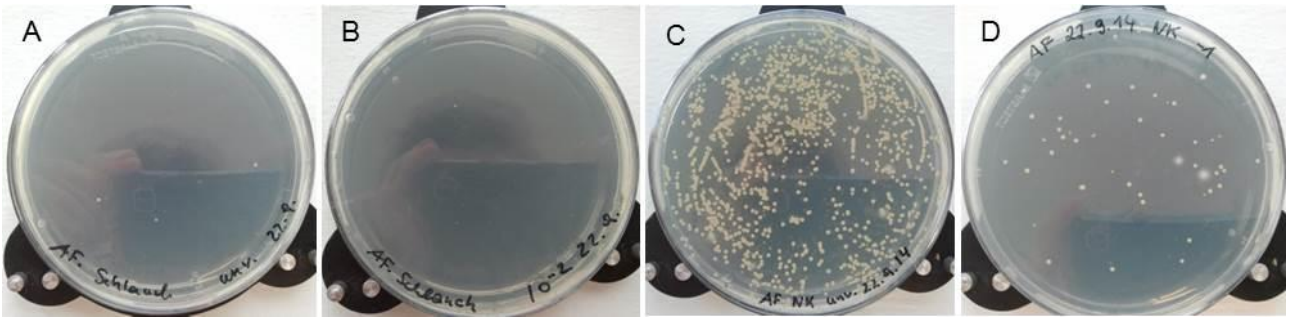

Figure 41: Colony growth on sample "Schlauch1". A: Undiluted sample of "Schlauch1"; B: Dilution 10^{-2} of sample "Schlauch1".

Usage of 6%sodium hypochlorite was effective method for initial sterilization and sterilization biofilm formation plant after experiments.

Re-isolation of bacteria from CVC by using modified vortexing method and by ultrasonication method

To test which method was the best for re-isolation of bacterial cells from colonized CVC, these two methods were compared via 1) plating of re-isolation solution on LB agar

plates (not statistical analyzed) and 2) statistical analysis of counted colonies. In the first experiments, which were not analyzed statistically, more bacteria were visually detected on the plates with re-isolation solution when using modified vortexing method (Table 13, Figure 42).

In a second experiment the catheter tubes were gently rinsed using PBS. After re-isolation a total reduction of bacteria was detected compared to the CVC directly applied for re-isolation. This experiment showed again a higher number of bacterial cells grown on LB agar plates (Table 13), on which re-isolation solution from the modified vortexing method was plated (Figure 43).

To test if there is a statistical difference, a catheter tube was colonized for five days using *S. aureus*. In this test, re-isolation using modified vortexing showed a statistical significantly higher number of CFU (modified vortexing method: $2.30\pm0.46*10^5$ CFU ml^{-1} to ultrasonication method: $7.73\pm0.61*10^4$ CFU ml^{-1}) compared to ultrasonication method.

Table 13: Counted colonies of re-isolated bacteria using different re-isolation methods.

	1st experiment		2nd experiment (including rinsing step)	
Dilution/re-isolation method	Vortexing	Ultrasonication	Vortexing	Ultrasonication
undiluted	nc	615	266	21
10^{-1}	nc	72	33	-
10^{-2}	468	6	3	1
10^{-3}	40	1	1	-

nc: not countable

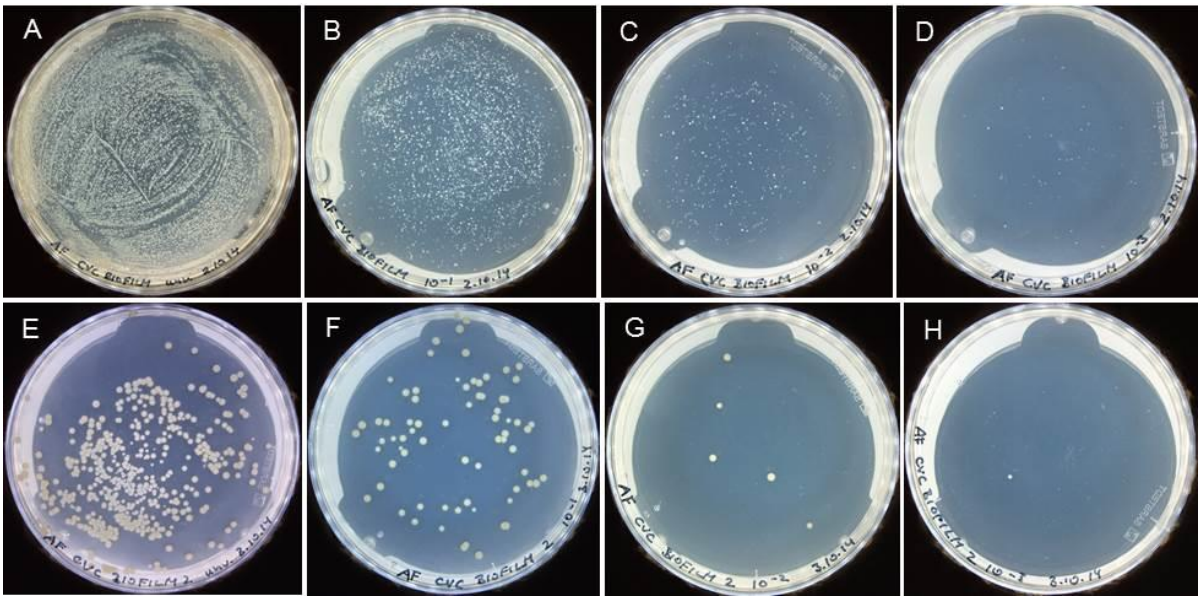

Figure 42: Bacterial re-isolation using the modified vortexing method and ultrasonication method. Plates with increasing dilutions (from the left to the right: undiluted to 10^{-3}) show the re-isolation capacity of the modiefied vortexing method (A-D) and ultrasonication method (E-H).

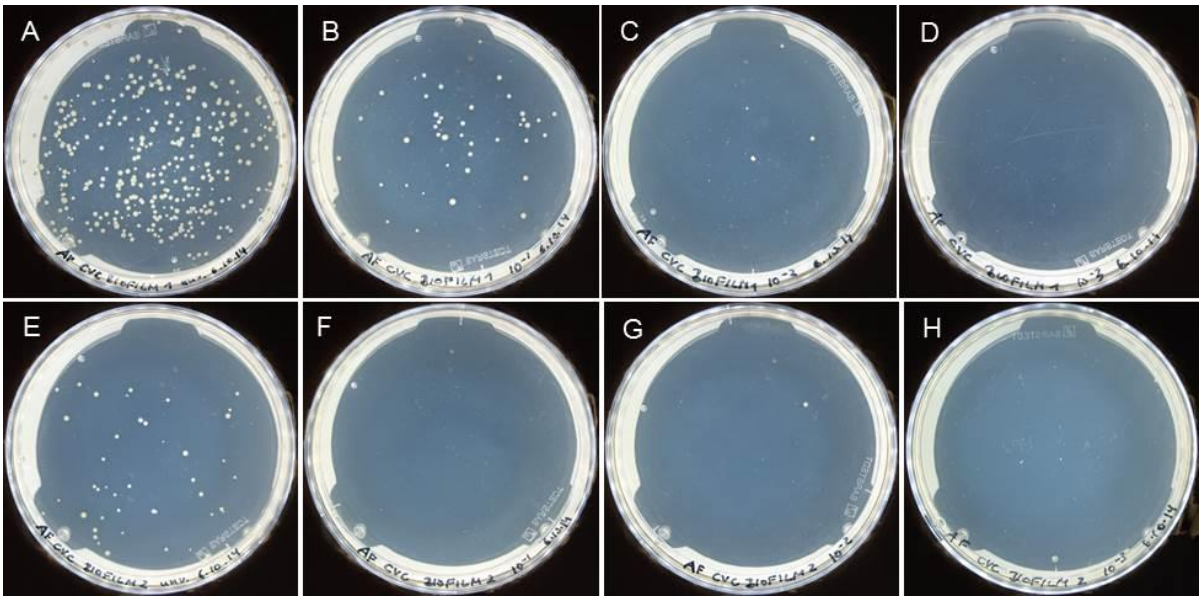

Figure 43: Bacterial re-isolation using the modified vortexing method and ultrasonication method. Plates with increasing dilutions (from the left to the right: undiluted to 10^{-3}) show the re-isolation capacity of the modiefied vortexing method (A-D) and ultrasonication method (E-H).

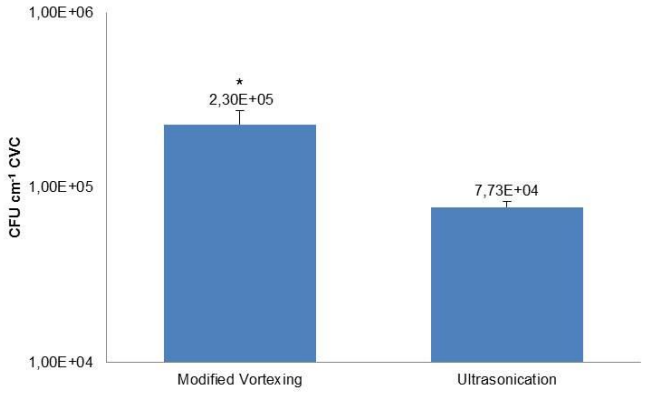

Figure 44: Colony forming units per cm of the CVC using the modified vortexing method and ultrasonication method for re-isolation of bacteria. Error bars show the standard deviation. Asterisks denote the significant differences (P < 0.05) between the re-isolation methods.

Bacteria re-isolation using the modified vortexing method was shown to be an effective strategy to re-isolate bacteria from the catheter surface. The modified vortexing methods showed a statistically significantly higher (*$P\text{-value}<0.05$) re-isolation capacity.

Pyrazine-treatment of a catheter colonized by a mixture of *S. aureus, P. aeruginosa* and *S. maltophilia* in the non-autoclavable pump system.

To test whether it is possible to treat a multispecies biofilm in the biofilm formation plant 0.6% pyrazine was injected directly into the system. The injection of the 0.5ml PBS carrying the pyrazine in the system led to a leakage, due the increased pressure in the system. Therefore, the total concentration of pyrazine in the system was reduced. Furthermore, a counting of the colonies was impossible due to the high contamination (Figure 45).

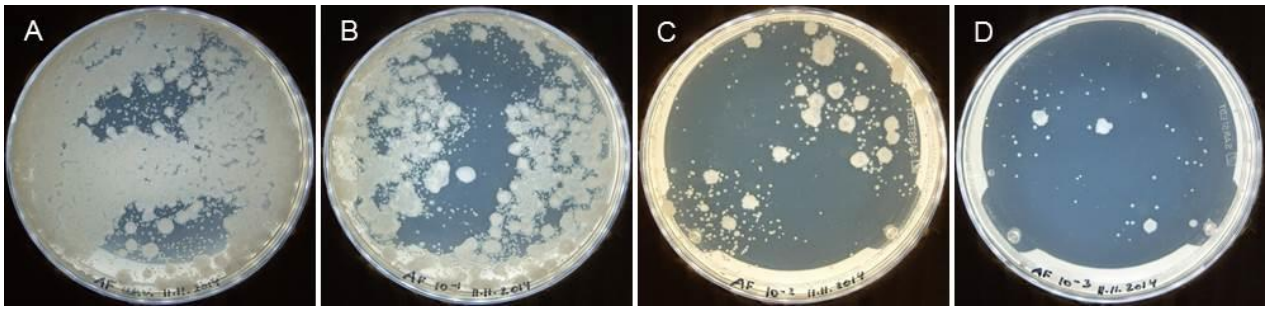

Figure 45: Treatment of a mixed biofilm (*S. aureus*, *P. aeruginosa* and *S. maltophilia*) using a pyrazine concentration of 0.6%. A-D showing different dilutions of the sample. Large round colonies were considered as a contamination.

For application of pyrazine in a circulating system the whole system has to be re-designed to avoid leaking due to the increased pressure.

Catheter colonization by clinical strains

Clinical strains such as *S. aureus* and *S. epidermidis* showed to have high potential to form biofilms *in-vitro*. To investigate whether these clinical strains form an extensive biofilm on catheter surfaces storage medium from the contaminated CVCs was used to prime a catheter within the biofilm formation plant.

Plating of the medium obtained from the pump system showed a high number of bacteria of different morphological forms and size. Statistical analysis of colonies, which grew on the plates, was not possible due to high number of the colonies per plate (Figure 46).

FISH-CLSM of CVC sections of the UBT:T4D sample, which was primed by clinical strains, showed a thin layer of bacteria in the inner lumen of the catheter (Figure 47A). Figure 47B showed a structure close to the edge of the inner lumen, in which different bacteria of undefined taxa were located. CLSM-FISH of the cutting surface of the catheter showed rod-shaped bacteria of undefined taxa (Figure 47C)

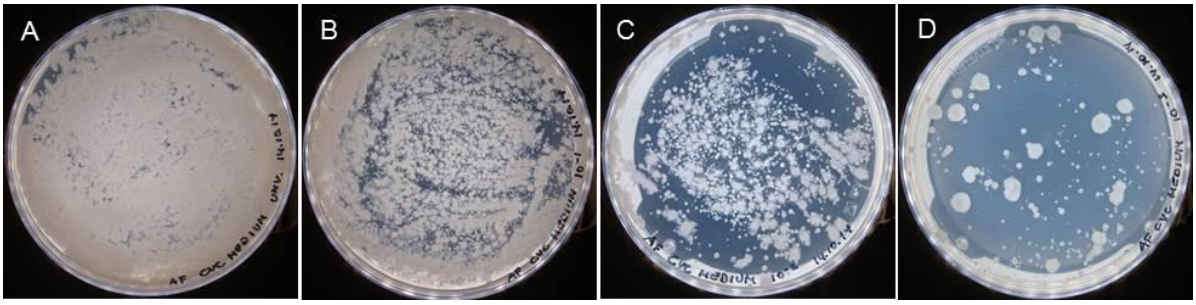

Figure 46: Agar plates showing different dilution steps of biofilm formation plant medium. The dilution increases gradually from A (undiluted) to (10^{-3}).

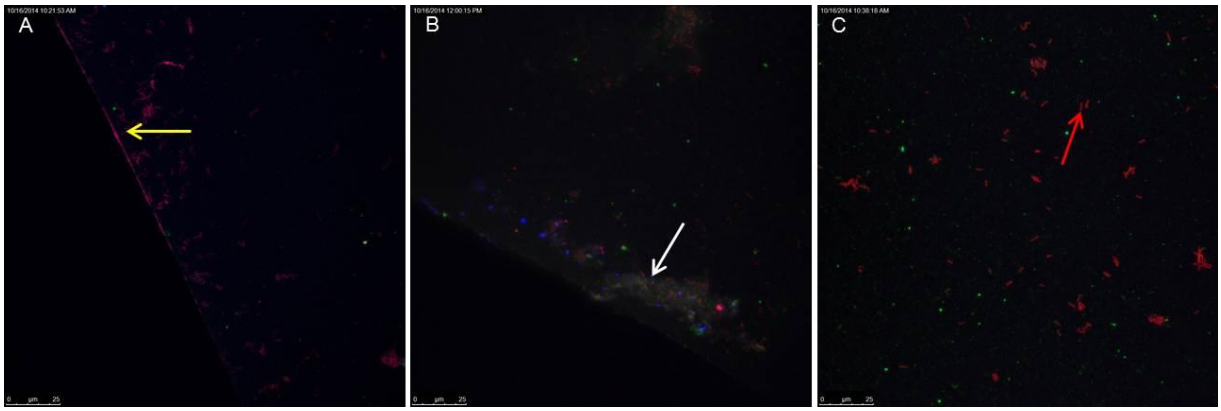

Figure 47: FISH-CLSM of the *in vitro* colonized catheter. A: thin biofilm layer on the inner side of the catheter (yellow arrow). B: cloudy structure surface close to the edge of the catheter showing different auto fluorescent particles and bacteria of universal taxa (red) (white arrow). C: Rod shaped bacteria on the cutting surface of the catheter hybridized by FISH probe for universal taxa (red arrow).

Using clinical strains to form a biofilm *in-vitro* resulted in a very thin but detectable biofilm.

Colonization of autoclavable *in vitro* biofilm formation plant using *S. aureus*

To get an insight into the kinetics of a biofilm formation by single strains a catheter was colonized by *S. aureus* and sampled every 24 hours for ten days.

Plating of re-isolation solution showed an increase of bacteria from CVC up to time point 5. On time point three (T3) first contaminations were detected on the plates, but did not overgrow *S. aureus* and counting of the colony was not impaired. The introduction of new sterile LB medium into the biofilm formation plant resulted in a decreased number of re-isolated bacteria from the CVC (Figure 48).

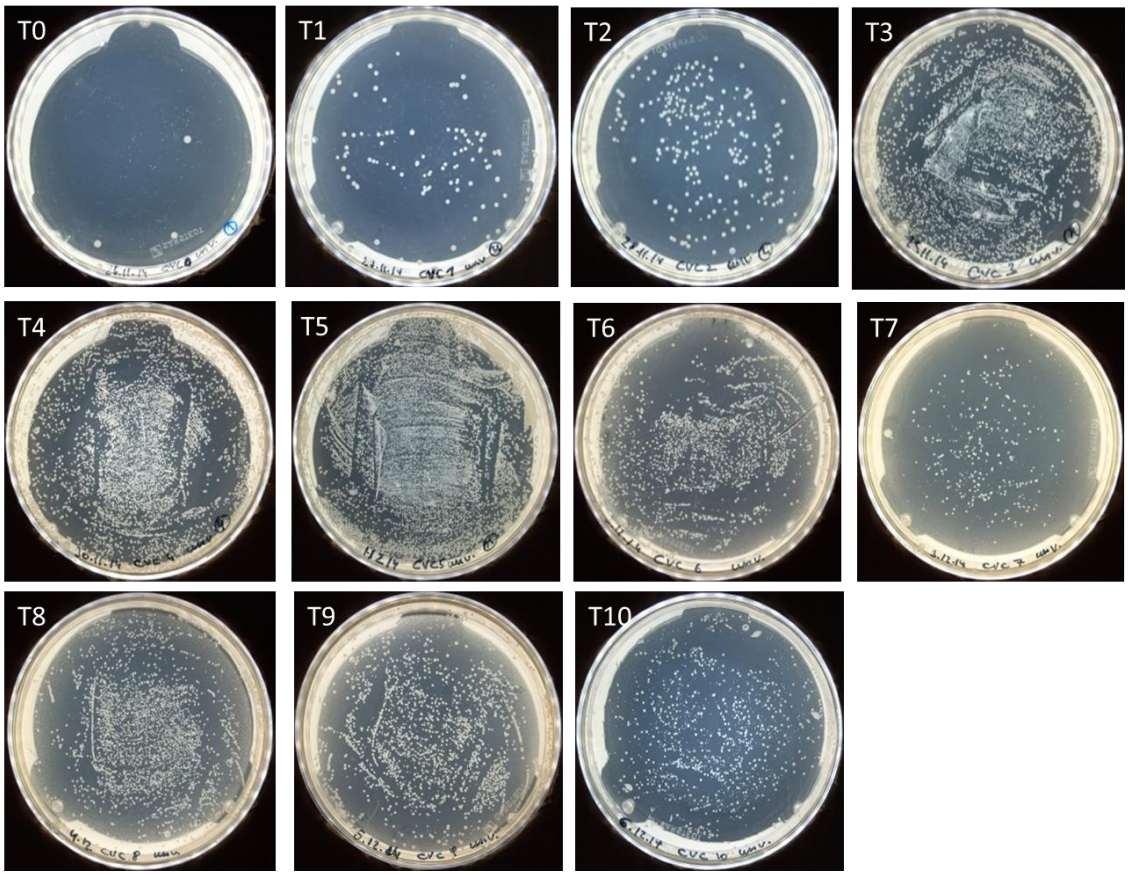

Figure 48: Viability test of cells isolated from 1 centimeter of CVC at different time points. Plating method on Luria/Miller (LB) agar plates showed a constant increase of bacteria up to day 5. Introduction of a fresh medium after sampling of day 5 showed a decreased number of attached bacteria.

Statistical analysis of the counted CFUs in the control and the medium of the biofilm formation plant showed the highest number of *S. aureus* colonies on time point 2 and time point 3. The number of CFUs ml^{-1} in the biofilm formation plant reduced faster than in the control. After exchanging the medium in the biofilm formation plant the bacterial concentration increased again. Graphical course showed the bacterial growth in the plant after exchanging the medium was similar to the control (Figure 49).

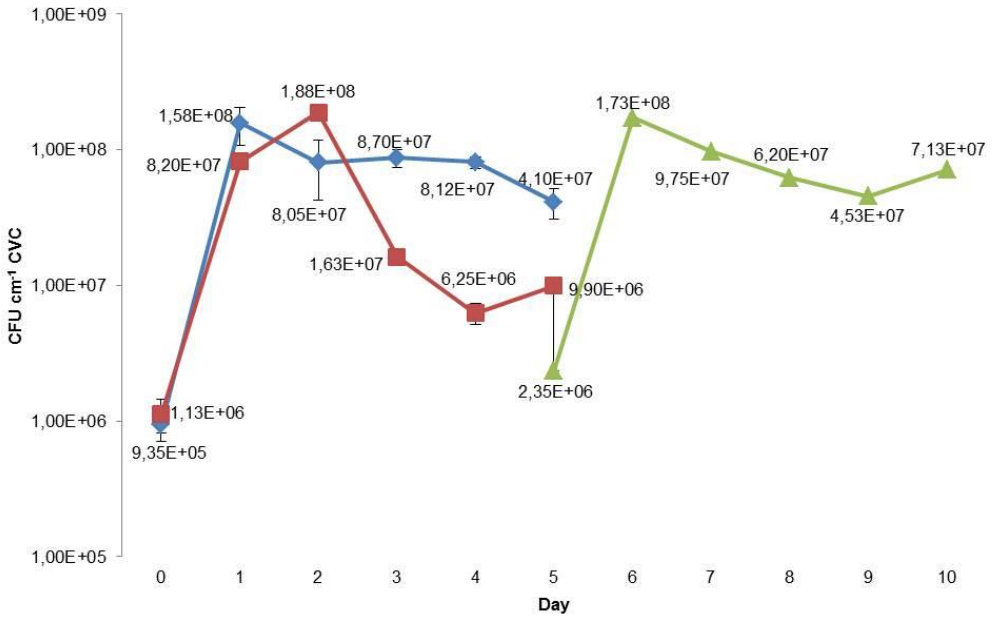

Figure 49: Growth of *S. aureus* in the plant system over 10 days experient. Abbreviations: PS, pump system; PSN, pump system new (medium from pump system after indroduction of new medium); C, control.

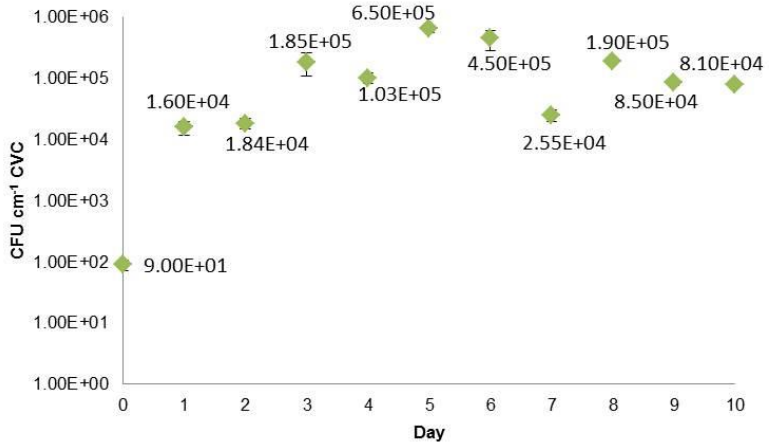

Figure 50: Scatter chart showing the CFU cm^{-1} CVC on different time points. The maximum of re-isolated bacteria was reached on day5 before introducing a new medium.

Based on these results the ideal colonization period for colonizing a catheter by *S. aureus* is five days.

Treatment of catheter tubes colonized with *S. aureus*

Antimicrobial lock technique and treatment in Eppendorf tubes

In clinical routine different methods are applied to get rid of the biofilm from CVCs. One of these methods is the antimicrobial lock technique. This experiment was aimed to find an experimental setup to apply pyrazine as an antimicrobial lock solution.

The negative control was mainly overgrown by the contaminating bacteria but single *S. aureus* colonies were visible (Figure 51D). Pyrazine concentration of 0.4% showed a reduction of *S. aureus* cells in the catheter tube compared to the negative control (Figure 51A). A complete removal of *S. aureus* was noted at both concentration, of 0.6% and 0.8% (Figure 51B-C).

Re-isolation of bacteria from the CVC after treatment in Eppendorf tubes showed a low number of re-isolated bacteria. On the plate, on which the negative control (Figure 51D) was applied, only nine colonies were grown. Catheter treated with 0.4% pyrazine showed contamination but no *S. aureus* (Figure 51A). From the CVC treated with 0.6% pyrazine no bacterial growth was detected (Figure 51B). 0.8% pyrazine showed a contaminations and one *S. aureus* colony (Figure 51C).

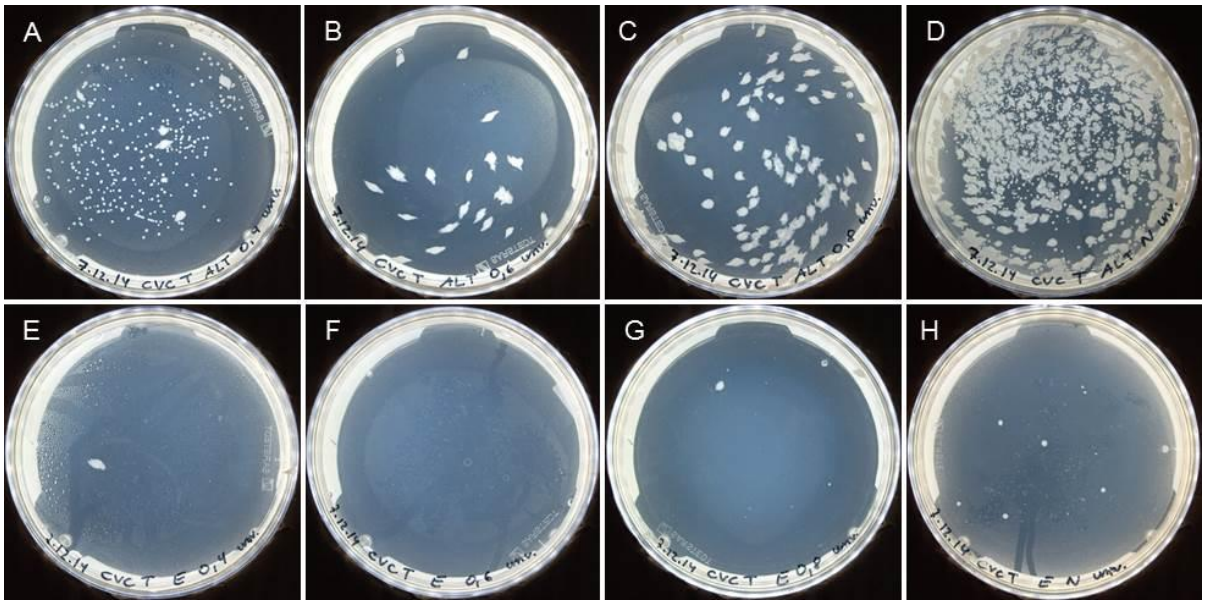

Figure 51: Treatment of the CVC colonized by *S. aureus* using different concentrations of pyrazine via the antimicrobial lock technique in Eppendorf tubes.

Plating of re-isolated bacteria from CVC treated via antimicrobial lock technique showed that using 0.6% pyrazine destroys *S. aureus* cells but has low influence on the contaminating bacteria. The contamination was detected on all plates when using different pyrazine concentrations. A comparison of treatment efficiency of the pyrazine by using Eppendorf tubes was difficult due to the low numbers of colonies on the negative control.

Discussion

Investigation of central venous catheter-associated microorganisms is of high importance due to the development of CRBSI causing higher mortality rates and prices for patients' treatment. Once after attaching to catheter surface bacteria start to produce complex exopolysaccharide matrix as a survival strategy. Recently Frasca *et al.* (2010) wrote that in short term catheters the route of colonization starts from the insertion side to tip by different microorganisms whereas in long term catheters (more than 15 days) the colonization route is located in the inner lumen of the catheter. In this thesis the working hypothesis was that the composition of bacteria species varies within the different sections of a central venous catheter. We assumed that the number of bacterial cells increases from the hub to the tip of the catheter and the bacterial diversity is specific to the catheter parts. In this study the catheters were removed by using a new approach - surgical explanation, which aimed to avoid biofilm destruction on the outer side of the catheter. To address this hypothesis a multi-faceted approach was developed. Colonization density of different CVC fragments was determined using qPCR with universal bacterial primers. Colonization pattern *in situ* was examined using FISH-CLSM technique. Taxonomic composition of the biofilm was addressed by using the clone libraries.

Quantification of bacteria in CVC-associated biofilms

Establishing the appropriate methods for CVC investigation using qPCR

Analysis of bacterial colonization in clinics is commonly done by using cultivation-independent methods. The reason for this is that only living bacterial cells play a role in infectious diseases. Using qPCR to determine bacterial abundance on CVC is rarely used and only applied for specific scientific questions. Zandri *et al.* (2012) used qPCR to enumerate unculturable *S. epidermidis* and *S. aureus* strains by primers targeting the 16S rRNA gene and strain specific regions. In this study the qPCR was used as tool to determine gene copy numbers cm^{-1} CVC on different types and parts of catheters. Correspondingly, in current discussion section the results are discussed in terms of different gene copy numbers cm^{-1} CVC on the different parts of the catheter.

Isolation of total-community DNA using the PowerBiofilm DNA Isolation Kit followed by qPCR using Unibac II-515f / Unibac II-927r primers showed to be a reliable method to determine the gene copy numbers cm^{-1} CVC. The qPCR revealed that the gene copy numbers cm^{-1} CVC were different within a single catheter and between different catheters. Based on qPCR an increase in gene copy numbers cm^{-1} CVC were detected from the medial to the proximal part of catheters CVC2 and CVC3. This result contradicts to a study of Boon Chai Koh *et al.* (2012). The authors analyzed the catheter tips of 48 peripheral arterial, 135 central venous and 106 peripherally inserted catheters using an Odds-ration based approach and showed that the number of bacteria decreased from the proximal site (closer to the blood vessel) to the distal site. As shown in Figure 6E, the middle part of the catheter was covered by a plastic foil, which belonged to the catheter. For DNA isolation the catheter and the foil was used. The increased surface of the analyzed CVC may be an explanation for the higher gene copy numbers cm^{-1} CVC on the medial part of the catheter. The variations in gene copy numbers cm^{-1} CVC may be linked to the catheter surface. As it is shown in the catheter images (Figure 5 & Figure 6A-I), the diameter and the altered surface of the tubes are different which may be a reason for variation in gene copy numbers cm^{-1}.

Despite a relatively simple setup, an improvement in experimental setup would help to generate more comparable data. Therefore one should find a method to measure the total CVC surface. To improve the sampling for further studies, one should determine the gene copy numbers cm^{-2} CVC or to normalize the numbers based on cm^2.

Bacterial community composition of CVC-associated biofilms

Biofilms represent a complex habitat for different bacterial species. Different studies addressed the composition of biofilms using high-throughput sequencing (Zhang *et al.* 2014) or clone libraries (Larsen *et al.* 2008). Clone libraries were used in these experiments because of several advantages. Compared to a next generations sequencing approach, the clone libraries have several advantages. This method gains relatively simple insight in bacterial community composition excluding extensive bioinformatics analysis and the taxonomic detection is more accurate due to longer gene fragments which allow a better taxonomic resolution. A disadvantage of the clone libraries is the low screening capacity. Dunbar *et al.* (2002) showed that 40.000 clones

are necessary to detect 50% of all bacteria in one gram soil. However, clone libraries were the method of choice due to the positive aspects.

Surprisingly, analysis of the clone libraries revealed a high species diversity within the CVC-associated biofilms of the samples UBT:T1D and UBT:T2D. The clone libraries of the other samples did not show such a high diversity of microorganisms. The results obtained from our clone libraries underline that CVC-associated biofilms are composed of multiple species. This was also shown by Larsen *et al.* (2008). The group detected 30 different bacterial species of six phyla and two uncultered species. Many bacteria are overseen when using the common semi-quantitative methods, which were not related to CVC infections but other infections such as *Acidovorax* sp., *Massilia* sp. This fact was proved by Zhang *et al.* 2010. It was shown that semi-quantitative “negative” catheters harbors high diversity of bacterial strains.

Based on literature research more information was obtained for the most abundant species found within the clone libraries: *Herbaspirilium seropaedicae*, *Staphylocoocus aureus*, *Staphylococcus epidermidis* and *Enerococcus faecalis* and bacteria of the genus *Brucella*. *Brucella* spp. cause an infection named as Brucellosis or Malta fever (Wyatt 2005). The incubation time after infection is 5-60 days. The incidence in Austria is very low (<0.2/100.000 population) (Pichler *et al.* 2008). In 2007, an infection by *Brucella* spp. was detected in an immigrant worker who might get the infection during a visit of his home-country (Pichler *et al.* 2008). Because of low infection rarity *Brucella* spp. were unexpected in our samples. When using cultivation-independent methods, for the first time *Brucella* sp. was detected in the CVC samples from the Medical University of Graz (Graz, Austria).

Herbspirillum seropaedicae is a typical plant-associated bacterium which can be found in maize (*Zea mays*) and other plants (Ye *et al.* 2012). Balsanelli *et al.* (2014) investigated the role of the *H. seropaedicae* gene *epsB* on biofilm formation by knocking out this gene. In contrast to the wild type, the knock-out strain showed a reduced production on exopolysaccaride and 45% reduction of biofilm formation on glass fiber. Species of *Herbaspirillum* are non-pathogenic for human (Spilker *et al.* 2008) but have shown to trigger infections in severe ill patients. This was shown in a case report, in

which a child with acute lymphoblastic suffered from a *Herbaspirillum seropaedicae* infection (Ziga *et al.* 2010). A recent case report of Suwantarat *et al.* (2015) reported *H. seropaedicae* in a patient with end-stage renal disease and multiple myeloma. These two reports suggested that the health status is crucial in outbreak of a *H. seropaedicae* bacteremia. The health status of patients, which harbored catheters UBT:T1D and UBT:T2D, was unknown. The patients may get colonized by this bacterium from the environment, by getting into contact with wild or indoor plants, but do not developed a bacteremia due to good physical condition.

Enterococcus faecium is a microorganism that inhabits the gut of humans and animals (Fisher & Phillips 2009). Bacteria of the genus *Enterococcus* are known as opportunistic pathogens that are resistant to high concentrations of vancomycin (Gold 2001). Compared to *E. faecalis*, the overall infection rate is lower but in hospital surroundings it causes main proportion of infections (Gold 2001).

Gram positive microorganisms, such as *S. epidermidis* and *S. aureus*, play an important role in CRBSI. Due to their clinical relevance, their detection and treatment is highly essential. When comparing these two species, *S. aureus* causes CRBSI more often (Consensus Statement, OIG 2011). The source for contamination of CVC by *S. epidermids* can either be the health care personnel during wound care or patient itself (Sadfar & Maki 2004). *S. aureus* can be found in the nasal passage and axillae (Foster 1996). This bacterium is a major agent of nosocomial infections and in combination with *S. epidermidis* causes biofilm formation (Foster 1996).

The colonization pattern of UBT:T1D and UBT:T2D were similar between each other but outstanding in comparison to the other samples. The bacterial diversity of UBT:T3D, UBT:T4D and UBT:T5D was low, comprising one or two different species, compared to UBT:T1D and UBT:T2D. The results of the clone libraries revealed an interesting colonization pattern and the importance of using cultivation-independent methods for catheter investigation. Despite these results, the sampling procedure has to be improved. An improvement compromises the separation of outer side of the catheter and inner side of the catheter and a combination of both. In this study, 20 clones per CVC were sequenced. Based on the high diversity of detected bacterial species in samples

UBT:T1D and UBT:T2D, the number of clones per sample has to be increased, which enables a higher coverage depth per sample.

Adapting visualization protocols and investigation of colonization pattern of CVC-associated biofilms

Using the adapted biofilm fixation protocol we were able to visualize CVC-associated biofilms *in situ*. The FISH-CLSM images demonstrated the complex structure of biofilms and the interaction between bacteria and blood components.

Using the FISH-CLSM approach intact biofilms were visualized in different catheters of set 1. The bacteria in the biofilm of the catheter UBT:T3D were embedded into the biofilm matrix and were in close contact with red blood cells. Different review papers have suggested that blood components, for instance fibrinogen, increase the biofilm formation on medical indwelling devices (Esposito *et al.* 2013). Betran *et al.* (2013) showed that the biofilm formation of *Staphylococcus mutans* is significantly increased in the presence of fibrin compared to other blood plasma proteins on endothelial cells. A study by Murga *et al.* (2001) showed the importance of human blood for biofilm formation. The authors investigated the influence of human blood for the biofilm formation of *Enterobacter cloacae in vitro* on needleless connectors. The group found that the biofilm formation is significantly higher after conditioning using human blood.

A complex structure biofilm consisting of different polysaccharides layers and role of blood proteins and red blood cells for biofilm formation was especially underlined by the results that were obtained for the sample UBT:T3D. A biofilm image from UBT:T3D showed that the bacteria were concentrated in specific regions of the biofilm. This may be a result of bacterial migration towards regions with higher nutrient concentration or the bacteria initially formed the biofilm closer to the surface and this biofilm then trapped the blood cells.

Visualization of intact biofilms on CVC1-CVC3 using FISH-CLSM showed to be a critical procedure and strongly depend on factors such as biofilm thickness and biofilm attachment to catheter surface. In contrast to the catheters of set 1, the catheters of set 2 showed less blood residues on the catheter surface. The reduced blood residues may

had an influence on the visualization approach due to reduction of bacteria beneath the detection limit. The limitations for biofilm visualization were discussed in study of Larsen *et al.* (2008). The authors were only capable to detect bacteria using FISH-CLSM in one out of 18 investigated catheter sample. By applying this method the authors did not discriminate colonization of the outer and inner surfaces. The bacteria were detached from the catheter by using centrifugation followed by hybridization using specific probes on microscope slide. In this study they concluded that cells abundance was too low for FISH detection. The results for the CVC1-CVC3, which were explanted from the patient´s bodies, may be explained by the same reason.

The handling of the catheters might have a negative influence on the visualization of biofilms *in situ*. The handling and storage conditions of CVC1 were not known. CVC2-CVC3 were stored at -20°C without cryopreservative. This handling procedure causes cell destruction due to formation of water crystals. The freezing procedure may lead to biofilms' detachment from the catheter, due to the high water content in biofilms. It may be possible that the bacteria detached from the surface during catheterization process and therefore not detectable. It was unclear whether the catheters were rinsed using higher amounts of different substances. This procedure may led to detaching of biofilms in the catheter.

Investigation of in vitro biofilm formation on CVCs and its treatment by pyrazine

Understanding of biofilm formation on medical devices, especially CVCs, it is important to develop an effective treatment strategy. For biofilm formation different materials are available on the market including relatively small and cheap drip flow reactors to complex biofilm reactors (Coenye & Nelis 2005). In this study, CVC sections were colonized by a single strain (*S. aureus*) or multiple strains (*S. aureus*, *P. aeruginosa* and *S. malophilia*). Furthermore, a biofilm formation plant was developed to get an insight in kinetics of biofilm formation.

The catheter sections were infected by single or multiple bacterial strains and were treated by pyrazine to analyze the antibacterial effect of pyrazine on bacteria attached catheter surfaces. In the treatment control medium, we noticed that the CFU ml^{-1} in the

catheter sections treated by 0.2% pyrazine were slightly higher as in the negative control. This result was observed in both experiments using *S. aureus* for catheter colonization. This result may be an effect of the experimental setup. A possible explanation to this effect could be that living bacteria were carried over via the residual treatment solution in the treatment control medium. Improvements are necessary for further experiments with CVC sections. These improvements include comparison of catheter sections that were unrinsed and rinsed using sterile buffer and removal of all residual treatment solution by pipetting from the CVC sections.

Different methods for bacteria isolation from CVCs have been developed in the past years. Many of these methods are a combination of vortexing and ultrasociation steps (Donlan *et al.* 2001; Murga *et al.* 2001). A modified vortexing method and an ultrasonication method described by Murga *et al.* (2001) were compared to find the gentlest method for re-isolation of bacteria from the CVCs. In the first experiments, re-isolation solution was plated on LB-agar plates to check re-isolation efficiency. The plating was done without replicates. Therefore the data were not analyzed statistically. Based on visually discrimination of the plates the modified vortexing method is more efficient than the ultrasonication method. Based on these results the data of the following experiment was designed using a statistical approach.

Again, visually and statistically the highest number of re-isolate cells was reached using the modified vortexing method. Only the data from one colonization and re-isolation experiment were used for statistical test and, therefore, more repetitions are necessary to prove the obtained results. In these experiments the real number of bacterial cells that remained on the catheter sections after pyrazine treatment was unknown. qPCR could be a reliable method to determine the detachment efficiency of bacterial cells from the surface.

A biofilm formation device for CVCs was developed to analyze the biofilm formation on CVCs. The whole system underwent several improvement steps. After the last optimizing step of the biofilm formation device the whole system was made appropriate for autoclaving. Working with the improved system reduced the possibility of contamination. The most critical point was the catheter sampling. Surface disinfection

using ethanol seemed to have no or only little effect in avoiding contamination. The manual connection and the parafilm sealing were the most critical steps to avoid contamination. Despite these problems the biofilm formation device is now operable and is a novel method to form a biofilm on catheter surface.

The aim of the next experiment was to determine the ideal bacterial concentration to inoculate the system. In the first colonization experiments *S. aureus* and *S. maltophilia* were used in equal volumes of 1ml. The OD was approximately 2 times higher in *S. maltophilia* than in *S. aureus* culture that was used to infect the CVCs. This difference may have a negative influence on the biofilm formation due to imbalance.

To generate reproducible data and to overcome the imbalance it was necessary to set a specific value. Based on a protocol for biofilm formation in a 96-well plate (Haggag & Timmusk 2007) the system was inoculated by bacterial cultures with OD of 0.002. Based on literature research it was difficult to find comparable studies. A similar experiment was done by Murga *et al.* 2001. The setup in this study consisted of a biofilm forming reactor similar to the one we used in these experiments but was different in important experimental setups. In contrast to our study the authors colonized a needleless connector instead of a CVC and *Enterococcus cloacae* was used. Based on these differences a direct comparison of the data is not reasonable but interestingly the *E. cloacae* plateau was reached at the 5^{th} day.

Prior to this study it has been shown that pyrazine is an effective antimicrobial agent against bacteria attached on textiles (Liebminger *et al.* 2012). To test the antimicrobial effect of pyrazine on CVC-associated biofilms, catheter sections were treated using increasing concentrations of pyrazine and it was used as an antimicrobial lock solution. The experiments revealed that pyrazine applied on colonized CVC section and as antimicrobial lock solution is an effective antimicrobial agent at a concentration of 0.6% or higher. The advantages of pyrazine as antimicrobial agent are that relative low doses can be used to efficiently treat biofilms on CVC surfaces. The disadvantage of pyrazine is its hydrophobicity, the low vapor pressure and interaction with different materials. Pyrazine may be used as new ALT solution if its' hydrophobicity could be reduced and the substance could be integrated in a gel matrix.

In conclusion, the biofilm formation plant for CVCs is a novel device to investigate biofilm formation *in-vitro* under flow conditions. Different commercial products are on the market, which meet the same requirements that we needed in our experiments. The advantages using commercial available products are the standardized setup, the sterile sampling.

Outlook

Study of CVC-associated biofilms and different aspects of infection development are of high importance for fighting against CVC related death. Biofilm formation is a complex process which depends on factors compromising the environmental factors, materials of CVC, health status of a patient and many others. An understanding of biofilm formation on different CVC materials can help in the decision whether a catheter should be removed or can stay in the patient.

This study underlines the importance of biofilm analysis beneath the conventional cultivation-dependent methods. The results showed that cultivation techniques alone are not enough to give a deeper insight into bacterial colonization of catheters. Keeping the possibility in mind that not only nosocomial bacteria can lead to an infection, especially in immunosuppressed patients, more effective target-oriented patient treatment is possible.

The upcoming experiments should include a clone library of CVC1-CVC3 with an increased number of clones to get a deeper insight in bacteria colonization pattern. Using an Amplification Ribosomal DNA Restriction Analysis (ARDRA) approach would reduce the number of clones for sequencing due to clustering of similar strains.

Further experiments should treatment of colonized catheters by pyrazine in combination with ALT technique and statistical data analysis. In this process the re-isolation method has to be re-evaluated. To my opinion, it is necessary to colonize a CVC using different biofilm forming strains in one mixture and to monitor an alteration of cell concentrations for each strain over the time to understand bacterial interactions. The purchase of a commercially available biofilm formation device would have different advantages, especially a standardized setup.

References

Amann RI, Binder BJ, Olson RJ, Chisholm SW, Devereux R, Stahl DA. (1990). Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Appl Environ Microbiol* **56**: 1919–1925.

Balsanelli E, Antonia de Baura V, De Oliveira Pedrosa F, Maltempi de Souza E, Monteiro RA. (2014). Exopolysaccharide Biosynthesis Enables Mature Biofilm Formation on Abiotic Surfaces by *Herbaspirillum seropedicae*. *PloS ONE* **9**: e110392. doi:10.1371/journal.pone.0110392.

B. Braun Melsungen AG. (2002). Guidance for Ventral Venous Catheterization. cvc-partner. 1B.03.03.05/1 Nr. 6061686.

Bedran TBL, Azelmat J, Spolidorio DP, Grenier D. (2013). Fibrinogen-Induced Streptococcus mutans Biofilm Formation and Adherence to Endothelial Cells. *Biomed Res Int* **2013**. http://dx.doi.org/10.1155/2013/431465.

Beutlhauser T. (2012). Der zentrale Venenkatheter. *Intensivmedizin up2date* **8**: 93104. DOI http://dx.doi.org/10.1055/s-0032-1306778.

Blot F, Schmidt E, Nitenberg G, Tancrède C, Leclercq B, Laplanche A, Andremont A. (1998). Earlier Positivity of Central-Venous- versus Peripheral-Blood Cultures Is Highly Predictive of Catheter Related Sepsis. *J Clin Microbiol* **36**: 105–109.

Boon Chai Koh D, Robertson IK, Watts M, Davies AM. (2012). Density of Microbial Colonization on External and Internal Surfaces of Concurrently Placed Intravascular Devices. *Am J Crit Care* **21**: 162–171.

Bouza E, Avarado N, Alcalá L, Pérez MJ, Rincón C, Munoz P. (2007). A Randomized and Prospective Study of 3 Procedures for the Diagnosis of Catheter-Related Bloodstream Infections without Catheter Withdrawal. *Clin Infect Dis* **44**: 820–6.

Brileya KA, Camilleri LB, Fields M. (2014). *Engineering and Analyzing Multicellular Systems: Methods and Protocols. Methods in Molecular Biology.* Sun L, Shou W (eds.). Heidelberg (Germany): Springer-Verlag GmbH.

Brun-Buisson C, Abrouk F, Legrand P, Huet Y, Larabi S, Rapin M. (1987). Diagnosis of Central Venous Catheter-Related Sepsis. *Arch Intern Med* **147**: 873–877.

Chen YM, Dai AP, Shi Y, Liu ZJ, Gong MF, Yin XB. (2014). Effectiveness of silver-impregnated central venous catheters for preventing catheter-related blood stream infections: a meta-analysis. *Int J Infect Dis* **29**: 279–286.

Coenye T, Nelis HJ. (2005). *In vitro* and *in vivo* model systems to study microbial biofilm formation. *J Microbiol Meth* **83**: 89–105.

Consensus Statement, OGI, in Österreichische Ärztezeitung (ÖÄZ), Supplementum April 2011.

Costerton JW, Stewart PS, Greenberg EP. (1999). Bacterial Biofilms: A Common Cause of Persistent Infections. *Science* **284**: 1381–1322.

Daims H, Brühl A, Amann R, Schleifer KH. Wagner M. (1999). The domain-specific probe EUB338 is insufficient for the detection of all Bacteria: Development and evaluation of a more comprehensive probe set. *Syst Appl Microbiol* **22**: 434–444.

Davey ME, O´Toole GA. (2000). Microbial Biofilms: from Ecology to Molecular Genetics. *Microbiol Mol Biol Rev* **64**: 847–867.

De Carvalho CCCR. (2007). Biofilms: Recent developments on an Old Battle. *Recent Pat Biotechnol* **1**: 49–57.

Donlan RM. (2001). Biofilm Formation: A Clinically Relevant Microbial Process. *Clin Infect Dis* **33**: 1387–92.

Donlan RM, Murga R, Bell M, Toscano CM, Carr JH, Novicki TJ. *et al.* (2001). Protocol for Detection of biofilms on Needleless Connectors Attached to Central Venous Catheters. *J. Clin Microbiol* **39**: 750–753.

Donlan RM. (2002). Biofilms: Microbial Life on Surfaces. *Emerg Infect Dis* **8**: 881–890.

Donlan RM. (2011). Biofilm Elimination on Intravascular Catheters: Important Considerations for the Infectious Disease Practitioner. *Clin Infect Dis* **52**: 1038–1045.

Dunbar J, Barns SM, Ticknor LO, Kuske CR. (2002). Empirical and theoretical bacterial diversity in four Arizona soils. *Appl Environ Microbiol* **68**: 3035–3045.

Elliott TSJ. (2005). Can antimicrobial Central Venous Catheters prevent associated Infections?. *Brit J Haematol* **107**: 235–241.

Esposito S, Purrello SM, Bonnet E, Novelli E, Tripodi F, Pascale R *et al.* (2013). Central venous catheter-related biofilm infections: An up-to-date focus on methicillin-resistant *Staphylococcus aureus*. *JGAR* **1**:71–78.

Fisher K, Phillips C. (2009). The ecology, epidemiology and virulence Enterococcus. *Microbiology* **155**: 1749–1757.

Flecher S. (2005). Catheter related blood stream infections. *Continuing Education in Anaesthesia, Critical Care & Pain* 5: 49–51. doi 10.1093/bjaceaccp/mki011.

Foster T. 1996. Staphylococcus. In: Baron, S (ed.). Medical Microbiology. 4th edition. Galveston (TX): University of Texas Medical Branch at Galveston. http://www.ncbi.nlm.nih.gov/books/NBK8448/

Frasca D, Dahyot-Fizelier C, Mimoz O. (2010). Prevention of central venous catheter-related infections in the intensive care unit. *Crit Care* **14**: 212. doi:10.1186/cc8853.

Gold HS. (2001). Vancomycin-Resistant Enterococci: Mechanisms and Clinical Observations. *Clin Infect Dis* **33**: 211–219.

Grube M, Cardinale M, Vieira de Castro Jr J, Müller H, Berg G. (2009). Species-specific structural and functional diversity of bacterial communities in lichen symbioses. *ISME J* **3**: 1105–1115.

Haggag WM, Timmusk S. (2007). Colonization of peanut roots by biofilm-forming *Paenibacillus polymyxa* initiates biocontrol against crown rot disease. *J Appl Microbiol* **104**: 961–969.

Hall-Stoodley L, Stoodley P, Kathju S, Høiby N, Moser C, Costerton JW *et al.* (2012). Towards diagnostic guidelines for biofilm-associated infections. *FEMS Immunol Med Microbiol* **65**: 127–145.

Helewa, BSc RM, Embil JM MD, FRCPC. (2007). What to do with Coagulase Negative-Staphylococci. *The Canadian journal of CME* **February**: 19-20.

Jefferson KK. (2004). What drives bacteria to produce a biofilm. *FEMS Biotech Lett* **236**: 163–173.

Jiang H, Dong H, Zhang G, Yu B, Chapman LR, Fields MW. (2006). Microbial Diversity in Water and Sediment of Lake Chaka, an Athalassohaline Lake in Northwestern China. *Appl Environ Microb* **72**: 3832–3845.

Kite P, Dobbins BM, Wilcox MH, Fawley WN, Kindon AJL, Thomas D, Tighe MJ. *et al.* (1997). Evaluation of a novel endoluminal brush method for in situ diagnosis of catheter related sepsis. *J Clin Pathol* **50**: 278–282.

Kite P, Dobbins BM, Wilcox MH, MacMahone MJ. (1999). Rapid diagnosis of central-venous-catheter-related blood stream infections without catheter removal. *The Lancet* **354**: 1504–1507.

Lai NM, Chaiyakunapruk N, Lai NA, O'Riordan E, Pau WSC, Saint S. (2013). Catheter impregnation, coating or bonding for reducing central venous catheter-related infections in adults (Review). *Cochrane Database of Systematic Reviews* Issue 6. Art. No.: CD007878. DOI: 10.1002/14651858.CD007878.pub2.

Larsen MKS, Thomsen TR, Moser c, Høiby N, Nielsen PH. (2008). Use of cultivation-dependent and –independent techniques to assess contamination of central venous catheter: a pilot study. *BMC Clin Path* **8**: 10, doi: 10.1186/1472-6890-8-10.

Li YH, Tian X. (2012). Quorum Sensing and Bacterial Social Interactions in Biofilms. *Sensors* **12**: 2519–2538.

Lieber A, Kiesel B, Babel W. (2003). Microbial diversity of soil by SSCP fingerprinting technique using TGGE Maxi System. In: Merbach W, Hütsch BW, Augustin J (ed). Ökophysiologie des Wurzelraumes. Stuttgart: Teubner Verlag.

Liebminger S, Aichner M, Oberauner L, Fürnkranz M, Cardinale M, Berg G. (2012). A new Textile-Based Approach to Assess the Antimicrobial Activity of Volatiles. *Text Res J* **82**:484–491.

Longmate AG, Ellis KS, Boyle L, Maher S, Chairns CJS, Lloyd S, Lang C. (2011). Elimination of central-venous-catheter-related bloodstream infections from the intensive care unit. *BMJ Quality and Safety* **20**: 174–180.

MacKintosh EE, Patel JD, Marchant RE, Anderson JM. (2005). Effects of biomaterial surface chemistry on the adhesion and biofilm formation of *Staphylococcus* eidermidis *in vitro*. *J Biomed Mater Res Part A*: 836-842. DOI 10.1002/jbm.a

Maki DG, Weise CE, Sarafin HW. (1977). A semiquantitative culture method for identifying intravenous-catheter-related infection. *New Eng J Med* **296**: 1305–1309.

Meier H, Amann R, Ludwig W, Schleifer KH. (1999) Specific oligonucleotide probes for in situ detection of a major group of gram-positive bacteria with low DNA G+C content. *Syst Appl Microbiol* **22**: 186–196.

Manz W, Amann R, Ludwig W, Wagner M, Schleifer KH. (1992). Phylogenetic oligodeoxynucleotide probes for the major subclasses of Proteobacteria: problems and solutions. *Syst Appl Microbiol* **15**: 593–600.

Mallick EM, Bennett RJ. (2013). Sensing of the Microbial Neighborhood by *Candida albicans*. *PLoS Pathog* **9**: 1–4. doi:10.1371/journal.ppat.1003661

Mickley V. (2002). Central venous catheters: many questions, few answers. *Nephrol Dial Transplant* **17**: 1368–1373.

Moter A, Göbel UB. (2000). Fluorescence in situ hybridization (FISH) for direct visualization of microorganisms. *J Microbiol Meth* **41**: 85–112.

Murga R, Miller JM, Donlan RM. (2001). Biofilm Formation by Gram-Negative Bacteria on Central Venous Catheter Connectors: Effect of Conditioning Films in a Laboratory model. *J Clin Microbiol* **39**: 2294-2297.

Neef A. (1997). Anwendung der *in situ* Einzelzell-Identifizierung von Bakterien zur Populationsanalyse in komplexen mikrobiellen Biozönosen. Doctoral thesis (Technische Universität München).

Pilchler J, Kasper SS, Much P. (2008). Report on Zoonoses and Zoonotic Agents in Austria 2007. AGES – Austrian Agency for Health and Food Safety.

Raad I, Hanna HA, Alakech B, Chatzinikolaou I, Johnson MM, Tarrand J. (2004). Differential Time to Positivity: A Useful Method for Diagnosing Catheter-Related Bloodstream Infections. *Ann Intern Med* **140**: 18–25.

Rivera AM, Strauss KW, Van Zundert A, Mortier E. (2001). The history of peripheral intravenous catheters: How little plastic tubes revolutionized medicine. *Acta Anaesthesiol Belg* **56**: 271–282.

Sabatier C, Garacía X, Ferrer R, Duarte M, Colomina M, Alcaráz D. *et al.* (2013). Blood culture differential time to positivity enables safe catheter retention in suspected catheter-related bloodstream infections: a randomized controlled trial. *Med Intensiva* **39**: 135–41. *doi:* http://dx.doi.org/10.1016/j.medin.2013.12.012

Sadfar N, Maki DG. (2004). The pathogenesis of catheter-related blood stream infection with non-cuffed short term central venous catheters. *Intens Care Med* **30**: 62–67.

Spilker T, Uluer AZ, Marty FM, Yeh WW, Levison JH, Vandamme P. *et al.* (2008). Recovery of *Herbaspirillum* Species from Persons with Cystic Fibrosis. *J Clin Microbiol* **46**: 2774–2777.

Sutherland IW. (2001). Biofilm exopolysaccharides: a strong and stick framework. *Microbiology* **147**: 3–9.

Suwantarat N, Adams LTL, Romagnoli M, Carroll KC. (2015). Fatal case of *Herbaspirillum seropaedicae* bacteremia secondary to pneumonia in an end-stage renal disease patient with multiple myeloma. *Diagn Micr Infect Dis* **82**: 331–333.

Schwetz I, Hinrichs G, Reisinger EC, Krejs GJ, Olschewski H, Krause R. (2007). Delayed Processing of Blood Samples Influences Time to Positivity of Blood Cultures and Results of Gram Stain-Acridine Orange Leukocyte Cytopin Test. *J Clin Microbiol* **45**: 2691–2694.

Reischl U, Rabenau HF. (2015). *Molekulare Diagnostik*. Thiemann F, Cullen PM, Klein HG (eds.). Weinheim (Germany): Wiley-VCH.

Sager C. (2015). *Molekulare Diagnostik*. Thiemann F, Cullen PM, Klein HG (eds.). Weinheim (Germany): Wiley-VCH.

Treter J, Macedo AJ. (2011). Catheters: a suitable surface for biofilm formation. In: Méndez-Vilas, A (ed.). Science against microbial pathogens: communicating current research and technological advances. Badajoz (Spain): Formatex Research Center 2011.

Sousa C, Henriques M, Oliveira R. (2011). Mini-review: Antimicrobial central venous catheters – recent advantages and strategies. *Biofouling* **27**: 609–620.

Stoodley P, Sauer K, Davies DG, Costerton JW. (2002). Biofilms as Complex Differentiated Communities. *Annu Rev Microbiol* **56**: 187–209.

Wagner J, Schilcher G, Zollner-Schwetz I, Hoenigl M, Valentin T, Ribitsch W. *et al.* (2013). Microbiological screening for earlier detection of central venous catheter-related blood stream infections. *Eur J Clin Invest* **43**: 964–9.

Wallner G, Amann R, Beisker W. (1993). Optimizing fluorescent in situ hybridization with rRNA-targeted oligonucleotide probes for flow cytometric identification of microorganisms. *Cytometry* **14**: 136–143.

Waters CM, Bassler BL. (2005). Quorum Sensing: Cell-to-Cell Communication in Bacteria. *Annu Rev Cell Dev Biol* **21**: 319–46.

Wyatt HV. (2005). How Themistocles Zammit found Malta Fever (brucellosis) to be transmitted by the milk of goats. *J R Soc Med* **98**: 451–454.

Ye W, Ye S, Liu J, Chang S, Chen M, Zhu B, Guo L, An Q. (2012). Genome Sequence of the Pathogenic Herbaspirillum seropaedicae Strain Os34, Isolated from Rice Roots. *J Bacteriol* **194**: 6903–6904.

Zandri G, Pasquaroli S, Vignaroli C, Talevi S, Manso E, Donelli G. *et al.* (2012). Dedection of viable but non culturable staphylococci in biofilms from central venous catheters negative on standard microbial assay. *Clin Microbiol Infect* **18**: E259–E261.

Zhang L, Sriprakash KS, McMillan D, Gowardman JR, Patel B, Rickard CM. (2010). Microbial pattern of arterial catheters in the intensive care unit. *BMC Microbiol* **10**: 266.

Zhang L, Gowardman J, Morrison M, Krause L, Playford EG, Rickard CM. (2014). Molecular investigation of bacterial communities on intravascular catheters: no longer just *Staphylococcus*. *Eur J Clin Microbiol Infect Dis* **33**: 1189–1198.

Ziga ED, Druley T, Burnham CAD. (2010). Herbaspirillum Species Bacteremia in a Pediatric Oncology Patient. *J Clin Microbiol* **28**: 4320–4321.

Zitterkopf NL. (2008). The Advance of Molecular Diagnostics in the Surveillance of Health Care-Associated Infections. *Labmedicine* **39**: 623–625, doi: 10.1309/LMS6D81LGCNPNWWX.

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