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TITLE:

An *In vitro* Model of a Parallel-Plate Perfusion System to Study Bacterial Adherence to Graft Tissues

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KEYWORDS:

Staphylococcus aureus; infective endocarditis; adhesion; subendothelial matrix; shear stress; flow chamber; valvular graft tissues; RVOT;

SUMMARY:

We describe an in-house designed *in vitro* flow chamber model, which allows the investigation of bacterial adherence to graft tissues.

ABSTRACT:

Various valved conduits and stent-mounted valves are used for right ventricular outflow tract (RVOT) valve replacement in patients with congenital heart disease. When using prosthetic materials however, these grafts are susceptible to bacterial infections and various host responses.

Identification of bacterial and host factors that play a vital role in endovascular adherence of microorganisms is of importance to better understand the pathophysiology of the onset of infections such as infective endocarditis (IE) and to develop preventive strategies. Therefore, the development of competent models to investigate bacterial adhesion under physiological shear conditions is necessary. Here, we describe the use of a newly designed *in vitro* perfusion chamber based on parallel plates that allows the study of bacterial adherence to different components of graft tissues such as exposed extracellular matrix, endothelial cells and inert areas. This method combined with colony-forming unit (CFU) counting is adequate to evaluate the propensity of graft materials towards bacterial adhesion under flow. Further on, the flow chamber system might be used to investigate the role of blood components in bacterial adhesion under shear conditions. We demonstrated that the source of tissue, their surface morphology and bacterial species specificity are not the major determining factors in bacterial adherence to graft tissues by using our in-house designed *in vitro* perfusion model.

INTRODUCTION:

Staphylococcus aureus (*S. aureus*) employs a variety of virulence strategies to circumvent the host immune defense system colonizing biological or non-biological surfaces implanted in the human circulation, which leads to severe intravascular infections such as sepsis and IE[1-5]. IE remains an important treatment associated complication in patients after implantation of prosthetic heart valves while individual factors contributing to the onset of IE are not yet fully understood[6,7]. Under flow conditions, bacteria encounter shear forces, which they need to overcome in order to adhere to the vessel wall[8]. Models, which allow studying the interplay between bacteria and prosthetic valve tissue or endothelium under flow, are of interest as they reflect the *in vivo* situation more.

Several specific mechanisms facilitate bacterial adherence to endothelial cells (ECs) and to the exposed subendothelial matrix (ECM) leading to tissue colonization and maturation of vegetations, being essential early steps in IE[9]. Various staphylococcal surface proteins or MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) have been described as mediators of adhesion to host cells and to ECM proteins by interacting with molecules such as fibronectin, fibrinogen, collagen and von Willebrand factor (VWF)[8,10,11]. However, in view of intra-molecular folding of some virulence factors, mostly studied in static conditions, many of these interactions may have different relevance in endovascular infections in circulating blood.

Therefore, we present an in-house designed *in vitro* parallel-plate flow chamber model, which allows the assessment of bacterial adherence to different components of ECM and ECs in the context of tissue grafts implanted in the RVOT position. The overall purpose of the method described in this work is to study mechanisms of interaction between bacteria and underlying

endovascular tissues in flow conditions, which are closely related to the *in vivo* environment of bloodstream pathogens such as *S. aureus*. This novel approach focuses on the susceptibility of graft tissue surfaces to bacterial adherence to identify potential risk factors for the development of IE.

PROTOCOL:

1. Preparing Graft Tissues for *In Vitro* Studies

Note: Three types of tissues were used: Bovine Pericardium patch (BP), Cryopreserved Homograft (CH) and Bovine Jugular Vein grafts (BJV). In case of BJV conduit and CH (tissue processed by the European Homograft Bank (EHB) and stored in liquid nitrogen prior to use), both the wall and valvular leaflets were used. BP patch and BJV conduit were purchased from the manufacturers. Prior to use, thaw the CH following the EHB instructions[12].

1.1. Rinse all tissues with 0.9% NaCl prior to use.

1.2. Prepare tissue biopsies using a disposable skin biopsy punch to cut circular tissue pieces (10 mm in diameter).

1.3. Cut all tissue patches to the same height using disposable sterile scalpels.

1.4. For tissues fixed with glutaraldehyde (*for example* BJV conduit), incubate graft pieces overnight at 4 °C with 200 g/L of human albumin to neutralize the fixative.

1.5. Wash out residues of glutaraldehyde with 0.9% NaCl.

2. Preparing Bacteria for Perfusion Experiments

Note: Three bacterial isolates were used: *S. aureus* Cowan (ATCC 12598), *S. epidermidis* ATCC 149900 and *S. sanguinis* NCTC 7864. *S. aureus* and *S. epidermidis* were grown at 37 °C in tryptic soy broth (TSB) and *S. sanguinis* was grown at 37 °C with 5% CO_2 in brain heart infusion broth (BHI).

2.1. Prepare overnight culture of bacteria on a solid blood agar plate.

2.1.1. Use a sterile loop to scrape the frozen bacteria off and inoculate onto a Mueller-Hinton blood agar plate for overnight culture at 37 °C.

2.2. Use a sterile inoculation loop to pick up a single colony from the overnight blood agar culture and inoculate into 10 mL of TSB or BHI in a 15 mL tube and culture overnight at 37 °C.

2.3. Centrifuge overnight cultures (3000 x g, 4 °C, 10 min) and resuspend the pellets in 10 mL of phosphate buffered saline (PBS). Place 15 mL tubes on ice.

2.4. Prepare an aliquot of 3.7 mg/mL solution of 5(6)-carboxy-fluorescein N-hydroxy-succinimidyl ester (CF) in ethanol and store at -20 °C. Further dilute the stock of CF to 150 µg/mL using 'ultrapure' water.

Note: Protect tubes from light using aluminum foil and store at -20 °C.

2.5. Centrifuge the bacteria (3000 x g, 4 °C, 10 min) and resuspend the pellets in 800 µL of PBS and add 200 µL of the 150 µg/mL CF solution (final concentration of 30 µg/mL used for perfusion experiments). Protect tubes from light with aluminum foil and incubate for 30 min using an orbital shaker.

2.6. After labeling, block with 2% of bovine serum albumin (BSA) solution in PBS and spin (3000 x g, 4 °C, 10 min). Follow with a wash step using 10 mL of PBS and pellet bacteria by centrifugation (3000 x g, 4 °C, 10 min).

2.7. Dilute bacteria with PBS to obtain 10^7 colony-forming units (CFU)/mL (verified by CFU counting on Mueller-Hinton blood agar plates), which corresponds to an OD_{600} (optical density) of 0.65. Keep the tubes in the dark on ice prior to perfusion experiments.

Note. Keep in mind that OD_{600} measurements reflect the approximate number of bacteria. To count the effective inoculation dose, the serial dilution method is an additional necessary step to verify the OD based numbers as described in section 3.8.

3. *In vitro* Perfusion Experiments using a Parallel-Plate Flow Chamber

3.1. Mount tissue biopsies of 10 mm in diameter and the same thickness (prepared in the steps 1.1 - 1.5) into a flow chamber system with the inner surface facing up to get in contact with the bacterial suspension.

Note: The same tissue thickness across various grafts ensures that the same tissue height is reached in the channel allowing laminar flow in all conditions. All elements of the flow chamber are presented and described in **Figure 1**.

3.1.1. Place the round tissue piece between a microscope slide with an 8 mm circular perforation and a rubber gasket.

Note: The microscope slide possesses the ultra-thin bottom film to allow the generation of the 8 mm hole. Together with the rubber sheet, it fixes the tissue to enable the direct contact between the specimen and the flowing medium and also prevents the dislocation of the biopsy during the experiment. The surface of the investigated tissue, which is exposed to the flow (smaller diameter) cannot be manipulated by the forceps.

3.1.2. Insert the holder with the tissue into the gasket sheet that is embedded in the bottom metal frame of the chamber. Subsequently, to bridge the upper and bottom part of the chamber, place an additional rectangular flexible ring gasket sheet of 1 mm thickness onto the perimeter of the metal frame.

3.1.3. Put the upper metal frame with the corresponding gasket sheet onto the bottom part of the chamber with the previously inserted tissue holder. Subsequently mount the entire chamber with four screws and screw nuts. Make sure that the chamber height is always the same across grafts.

Note: The chamber height should be determined always upon tightening the screws.

3.2. Connect the flow chamber with a peristaltic pump and the fluid reservoir with the tubes.

3.3. Perfuse tissues with suspensions of 10^7 CFU/mL (verified by CFU counting and related to OD_{600} measurements) fluorescently-labeled bacteria in PBS with a shear stress of 3 dyne/cm^2 (dyne per square centimeter pressure unit) by means of the peristaltic pump (flow rate 4 mL/min) for 1 h using a bacterial 400 mL reservoir (**Table of Materials**) conditioned at 37 °C using a plate thermostat (**Table of Materials**).

3.4. Recirculate the bacterial suspension of 100 mL using another collection reservoir.

3.5. After perfusion, dismantle the chamber to release the graft and wash the tissue piece twice with 10 mL of PBS for 5 min in a 24-well plate using the laboratory orbital shaker. Subsequently cut the inner part of the graft using a punch of a smaller diameter.

3.6. Place each tissue biopsy into a separate 15 mL tube containing 1 mL of sterile 0.9% NaCl. Label the tube as #1.

3.7. Detach bacteria from the tissue using a sonication bath for 10 min (amplitude = 100% and frequency = 45 kHz).

Note: Full detachment of bacteria from the tissue grafts should be evaluated upon incubation of patches overnight at 37 °C in TSB liquid medium followed by OD_{600} measurements compared to control patches treated with a bacteria free solution.

3.8. Use a serial dilution method on Mueller-Hinton blood agar plates to count CFUs.

3.8.1. Prepare a single 15 mL tube with 10 mL of sterile saline for serial dilutions of bacterial suspension obtained after sonication. Label this tube as #2.

Note: For each tissue experiment one tube with 10 mL of 0.9% NaCl is necessary.

3.8.2. Prepare three 15 mL tubes with 10 mL of sterile 0.9% NaCl for serial dilutions of initial bacterial suspension from step 2.7. Label the tubes as follows #3, #4, #5.

Note: This step is necessary to know the real CFU number in bacterial suspension used for the perfusion experiment.

3.8.3. Vortex mix each tube for 15 s. Vortex the tubes with the tissue biopsy as well as the starting the bacterial suspension to make serial dilutions.

3.8.4. Prepare three agar plates, two for the tissue experiment (perfusion of bacteria and control perfusion of PBS) and the third one for the initial bacterial suspension used for perfusions.

3.8.5. Label three sectors per plate for the tissue experiment in the following manner 10^{-1}, 10^{-3} and 10^{-4}. To count the number of CFUs in the starting bacterial suspension, label the third plate as follows: 10^{-1}, 10^{-3}, 10^{-5} and 10^{-7}.

Note: All indications on agar plates such as 10^{-1}, 10^{-3} and so on refer to the final number of CFU/mL calculated on the next day. Before use, blood agar plates should be placed under the laminar hood and opened to remove excess moisture.

3.8.6. To prepare serial dilutions, transfer 100 µL of tube #1 to tube #2 and mix well by vortex.

3.8.7. Spread 100 µL of the contents of tube #1 and #2 onto the corresponding sectors 10^{-1} and 10^{-3} of the agar plate. Likewise, spread 10 µL of tube #2 on the sector 10^{-4}, repeat this step 4 times to obtain 4 separate growths from each volume of 10 µL.

Note: Due to the small volume used for plating onto the sector 10^{-4}, it is advised to have multiple number of droplets to make an average number of grown CFUs.

3.8.8. To prepare serial dilutions of the overnight culture transfer 100 µL of bacterial suspension from step 2.7 to tube #3 and mix vigorously. Add 100 µL of tube #3 to tube #4 and mix well, repeat the procedure for subsequent tube #5.

3.8.9. Spread 100 µL of the contents of tubes #3, #4, #5 and the overnight culture, respectively, onto sectors 10^{-3}, 10^{-5}, 10^{-7} and 10^{-1} of the blood agar plate.

3.8.10. Leave the blood agar plates under the laminar hood to air dry the bacterial spreads. Afterwards, place the plates at 37 °C for overnight incubation.

3.8.11. After overnight incubation, count the bacterial colonies to obtain the number of CFUs resulting from the adhesion to the tissue biopsies as well as from the CFU/mL in the starting bacterial suspension used for the perfusion. Express results as CFU/cm^2.

4. Fluorescence Microscopy of Adhered Bacteria to Graft Tissues upon Perfusion

4.1. After perfusion, wash tissue pieces with PBS and cut the inner part of a graft using a punch of a smaller diameter.

4.2. Prepare a 6-well plate and place droplets of mounting medium (**Table of Materials**).

4.3. Place each piece of tissue with its perfused surface downward on a single drop of mounting medium.

4.4. Read a plate using a fluorescence scanner. Set parameters of excitation and emission wavelengths according to a fluorophore used for bacterial labeling.

REPRESENTATIVE RESULTS:

To better understand the mechanisms behind IE development, this model enables the evaluation of bacterial and tissue associated factors present in the *in vivo* situation of infection onset.

In detail, the novel *in vitro* approach allows to quantify bacterial adhesion in flow conditions to different graft tissues by perfusing fluorescently labeled bacteria over the tissues exerting the shear stresses in the physiological range of 3 – 10 dyne/cm^2. In this work, we used a flow rate of 4 mL/min that corresponded to 3 dyne/cm^2. Taking into consideration the channel height of 0.3 mm across all tissue patches, the distance between the mounted graft and the medium inlet of about 39 mm, the perfusion chamber (shown in **Figure 1**) guarantees fully developed laminar flow (Re = 3.89 is significantly lower than 2000; the entrance length = 0.05 mm is significantly smaller than the distance `inlet-graft`, parameters necessary for assuming appropriate flow pattern).

Under shear stress conditions, a similar bacterial attachment across the various graft tissues was observed for both *S. aureus* and *S. epidermidis* infection (**Figures 2 and 3**). Although not significant, a trend towards higher adhesion of *S. aureus* to the CH leaflets was noticeable.

For *S. sanguinis* a significant reduction of adherence to the BJV wall was found when compared to the BP patch (**Figure 4**; $P < 0.05$). When comparing the 3 species of bacteria, *S. sanguinis* presents significantly lower adhesion to the BJV wall ($P < 0.05$) in relation to *S. aureus* and *S. epidermidis*. In general, we observed a similar bacterial adhesion to all tissues investigated under shear stress.

Our data from CFU counting (**Figures 2-4**) are supported by fluorescence microscopy using a high throughput scanner (**Figures 5-7**). Images are presenting pronounced foci of labeled bacteria adhering to graft tissues. Due to this approach, we were able to directly visualize tissues upon perfusion without any processing for illustration purposes.

Results demonstrate that the source of a graft tissue, surface morphological differences as well as bacterial adhesins are not major determinants of bacterial adherence to these biological materials.

FIGURE AND TABLE LEGENDS:

Figure 1. Image of a newly developed flow chamber system (in-house design by the Department of Biohybrid & Medical Textiles, AME – Helmholtz Institute for Biomedical Engineering, Aachen, Germany). **A. The flow chamber (1)** mounted flow set of dimensions LxWxH: 125 mm x 55 mm x 18 mm (screws in combination with screw-nuts hold the chamber`s parts together and put pressure *via* the metal frame on the gaskets to prevent leakage); **(2)** the upper part of the column; **(3)** the upper gasket sheet with two holes to fix tubing connectors, which connect the flow chamber with the pump and the fluid reservoir by means of the tubing system; **(4)** distance between the medium inlet and the tissue (the entrance length); **(5)** thin foil slide (with an 8 mm circular perforation to allow the exposure of the tissue to the bacterial suspension) (in a recess of the slide there is the space for a rubber gasket **B9**, to immobilize the tissue piece during the perfusion); **(6)** the bottom gasket sheet with a dedicated recess to place the tissue graft mounted between the microscope slide and the rubber gasket**; (7)** the bottom part of the chamber (the metal frame with an additional rectangular ring gasket sheet **B11** used to bridge the two parts of the chamber and prevent leakage); **B. The full set-up (8)** the thin foil slide; **(9)** the rubber gasket; **(10)** four screws with four screw-nuts; **(11)** the rectangular ring gasket to prevent leakage; **(12)** the fluid reservoir (400 mL); **(13)** tubing system; **C. Perfusion unit (14)** the peristaltic pump; **(15)** dedicated tubing that withstands the rigors of peristaltic pumping action.

Figure 2. Adhesion of *S. aureus* Cowan to graft tissues under flow conditions. Fluorescently labeled bacteria were perfused over 5 graft tissues (conduit walls or valvular leaflets) in PBS. Bacteria were detached from infected tissue pieces by sonication. Bacterial adhesion was evaluated by serial dilutions using the CFU counting method and indicated as CFU/cm^2. All results are expressed as mean ± SEM (n > 3 for valvular leaflets due to limitation of material; n > 5 for conduit walls). *CFU:* colony-forming unit; *BP:* bovine pericardium; *BJV:* bovine jugular vein; *CH:* cryopreserved homograft. This figure has been modified from Veloso *et al.* (*Journal of Thoracic and Cardiovascular Surgery* **155** (1), 325-332 (2018)).

Figure 3. Adhesion of *S. epidermidis* to graft tissues under flow conditions. Fluorescently labeled bacteria were perfused over 5 graft tissues (conduit walls or valvular leaflets) in PBS. Bacteria were detached from infected tissue pieces by sonication. Bacterial adhesion was evaluated by serial dilutions using the CFU counting method and indicated as CFU/cm^2. All results are expressed as mean ± SEM (n > 3 for valvular leaflets due to limitation of material; n > 5 for conduit walls). *CFU:* colony-forming unit; *BP:* bovine pericardium; *BJV:* bovine jugular vein; *CH:* cryopreserved homograft. This figure has been modified from Veloso *et al.* (*Journal of Thoracic and Cardiovascular Surgery* **155** (1), 325-332 (2018)).

Figure 4. Adhesion of *S. sanguinis* to graft tissues under flow conditions. Fluorescently labeled bacteria were perfused over 5 graft tissues (conduit walls or valvular leaflets) in PBS. Bacteria were detached from infected tissue pieces by sonication. Bacterial adhesion was evaluated by serial dilutions using the CFU counting method and indicated as CFU/cm^2. All results are expressed as mean ± SEM (n = 3 for valvular leaflets due to limitation of material; n > 5 for conduit walls). *CFU:* colony-forming unit; *BP:* bovine pericardium; *BJV:* bovine jugular vein; *CH:* cryopreserved homograft. This figure has been modified from Veloso *et al.* (*Journal of Thoracic and Cardiovascular Surgery* **155** (1), 325-332 (2018)). *P < 0.05.

Figure 5. Visualization of *S. aureus* Cowan adherence to graft tissues by means of fluorescence microscopy. *Left to right*: BJV conduit wall, BJV leaflet, CH wall and CH leaflet. This figure has been modified from Veloso *et al.* (*Journal of Thoracic and Cardiovascular Surgery* **155** (1), 325-332 (2018)).

Figure 6. Visualization of *S. epidermidis* adherence to graft tissues using fluorescence microscopy. *Left to right*: BJV conduit wall, BJV leaflet, CH wall and CH leaflet. This figure has been modified from Veloso *et al.* (*Journal of Thoracic and Cardiovascular Surgery* **155** (1), 325-332 (2018)).

Figure 7. Visualization of *S. sanguinis* adherence to graft tissues using fluorescence microscopy. *Left to right*: BJV conduit wall, BJV leaflet, CH wall and CH leaflet. This figure has been modified from Veloso *et al.* (*Journal of Thoracic and Cardiovascular Surgery* **155** (1), 325-332 (2018)).

DISCUSSION:

Recent clinical observations give special awareness to IE as a complication in patients having undergone valve replacement of the RVOT[6,13]. Dysfunction of the implanted valve in IE is the result of bacterial interaction with the endovascular graft leading to extensive inflammatory and procoagulant reactions[1,14]. The presented novel *in vitro* model allowed us to investigate if differences in tissue structures and bacterial factors are likely to modulate the susceptibility to infections of *in vivo* used grafts[15]. BJV and CH graft tissue showed similar propensity towards bacterial recruitment in flow conditions. Therefore, data suggest that in general the source of the tissue and its surface structure as well as specific bacterial adhesive proteins *per se* are not the major determinant factors in initial bacterial adherence.

In general, pathways evoking inflammation, tissue damage, platelet and fibrin deposition at the infected endovascular site are activated by multiple players[1,16]. A major advantage of the developed *in vitro* model is the opportunity to analyze stepwise the contribution of involved players. Single bacterial factors can be investigated by using bacterial mutant strains or genetically modified bacteria expressing single adhesion proteins on their surface[14]. By choosing different perfusion media, plasma or blood, the involvement of plasma proteins and blood cells can be evaluated. Further studies will focus on tissue related factors for which tissues will be pre-incubated with *for example* plasma proteins before mounted in the flow chamber for subsequent perfusion. Since players contributing to the onset of prosthetic valve IE

remain unclear, future studies might unravel the potential factors by building up to a more complex experimental setup. Furthermore, this experimental setup inherits the possibility that tissues can be seeded with an EC layer to analyze shear-dependent EC gene expression. The parallel-plate flow chamber also allows perfusion over EC-covered microscope slides due to a flexible inner height of the perfusion chamber. Different coatings of cover slips using various extracellular matrix proteins are also a possible option to assess important interactions with the subendothelial matrix. In addition, pharmacologic inhibitors or functional antibodies can be investigated for their effect in the respective condition in our flow chamber. In summary, various conditions can be studied by increasing complexity.

Inflammatory activation at the infected area of the graft is a crucial, shear-controlled step favoring deposition of activated platelets and monocytes. The impact of shear forces on bacterial adherence to tissue surfaces are of major concern. To address this issue, the novelty of the presented *in vitro* system focuses on the possibility to mount tissues in a flow chamber. This reinforces the significance of the method beyond existing alternatives, in which usually static interactions between bacteria and underlying tissues have been investigated. Even though shear stress was submitted by shaking or other external forces, it has not been standardized to the same level as we can gain from our uniform flow model.

In vivo, a non-physiologic flow pattern can favor bacterial adhesion as the onset of IE at the valve level of implanted conduits. Shear stress was found to up-regulate endothelial inflammatory parameters such as cytokine secretion and to increase tissue factor mediated coagulation[17]. The interaction of the underlying tissue used for valve prostheses with bacteria and their influence on EC gene expression under shear stress is important to construct a valve less capable to bacterial adhesion and chronic inflammation.

The basal technical issues of the constructed chamber allow investigations under standardized conditions in laminar flow[18]. To ensure the fully developed laminar flow at the site of the investigated tissue the chamber is constructed to mount the graft in a certain distance from the medium inlet (significantly longer than the entrance length, see Results and **Figure 1**). Using different pumps in the system would allow performing experiments under pulsatile or turbulent flow conditions in the future.

The flexible frame of the chamber prevents the chamber effectively from leaking and the internal height of the frame allows adapting for tissue thickness. The construction of the whole system enables a circulating flow, which is of importance to perform long lasting perfusions with using a respective amount of medium. Based on previous studies our adhesion protocol assumed a bacterial inoculation dose of 10^7 CFU/mL for a 1 h incubation[4,19]. By using these settings, adhesion levels were detectable, albeit low enough to be able to observe significant enhancement of bacterial adherence without saturation of the tissue graft surface. Moreover, in this period of time, it was feasible to notice potential differences in binding across strains taken in this study. Shear parameters addressed here were in the physiological range and optimized rather for big blood vessels, which were our target in respect to the RVOT.

Further modifications of the method will focus on more efficient consumption of medium during the procedure as well as on simplification of mounting the setup. In addition, a new design including multiple slots for tissue assembly would ease an entire experiment in aspects such as efficiency.

At this stage our method is focused on the end-point results and was not tested for real time applications such as the time course of dynamic events occurring on the tissue surface. Thus, this broader application remains under consideration; however, issues such as tissue autofluorescence, optimization of an appropriate fluorescence microscope protocol as well as adaptations of the chamber need to be addressed. Further on, the method in its current state may be adapted to real-time monitoring of bacterial binding to EC layers on microscope slides by upright fluorescence microscope. Currently, we are able to visualize bacteria and other blood components/cells bound to tissues by confocal microscopy without a need for post experimental tissue processing, which is predisposing for the real-time visualization under flow by inverted fluorescence microscopes.

In this study, the quantification of bacterial adhesion was provided by CFU counting while fluorescence microscopy was a supportive, non-quantitative tool. Due to resolution issues resulting from the lack of an adequate microscope lens, fluorescence imaging turned out to be less reproducible in our hands than serial dilutions. Nevertheless, it is possible to use fluorescence scanning for quantification when suitable objective lens could illuminate the entire graft size of 8 mm in diameter for reliable foci quantification. Using an image processing program (such as ImageJ), absolute fluorescence units might be quantified for investigated tissue specimens and the bacterial adhesion might be expressed for example as a relative signal to the internal control (grafts perfused with non-labelled bacteria).

The major limitation of this experimental setting are the issues associated with *in vitro* studies in general. Results reached by using this *in vitro* flow chamber model could be transferred to an animal model for *in vivo* confirmation.

In conclusion, this *in vitro* model allows investigation of single bacterial, tissue and shear-based factors contributing to the onset of bacterial adhesion to tissues in a stepwise manner. The hereby enabled knowledge could contribute to the development of more effective prevention and treatment of IE.

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DISCLOSURES:
None.

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Fig. 2

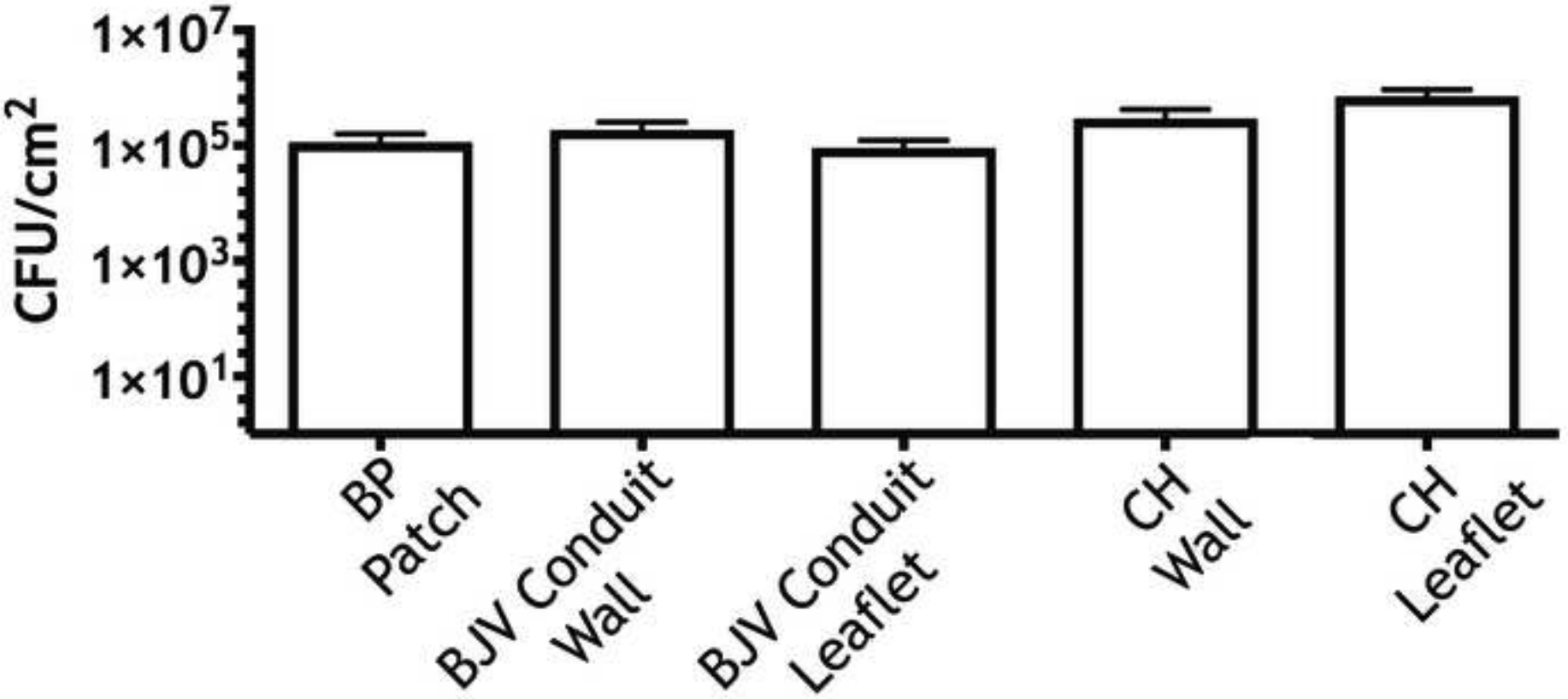


Fig.3

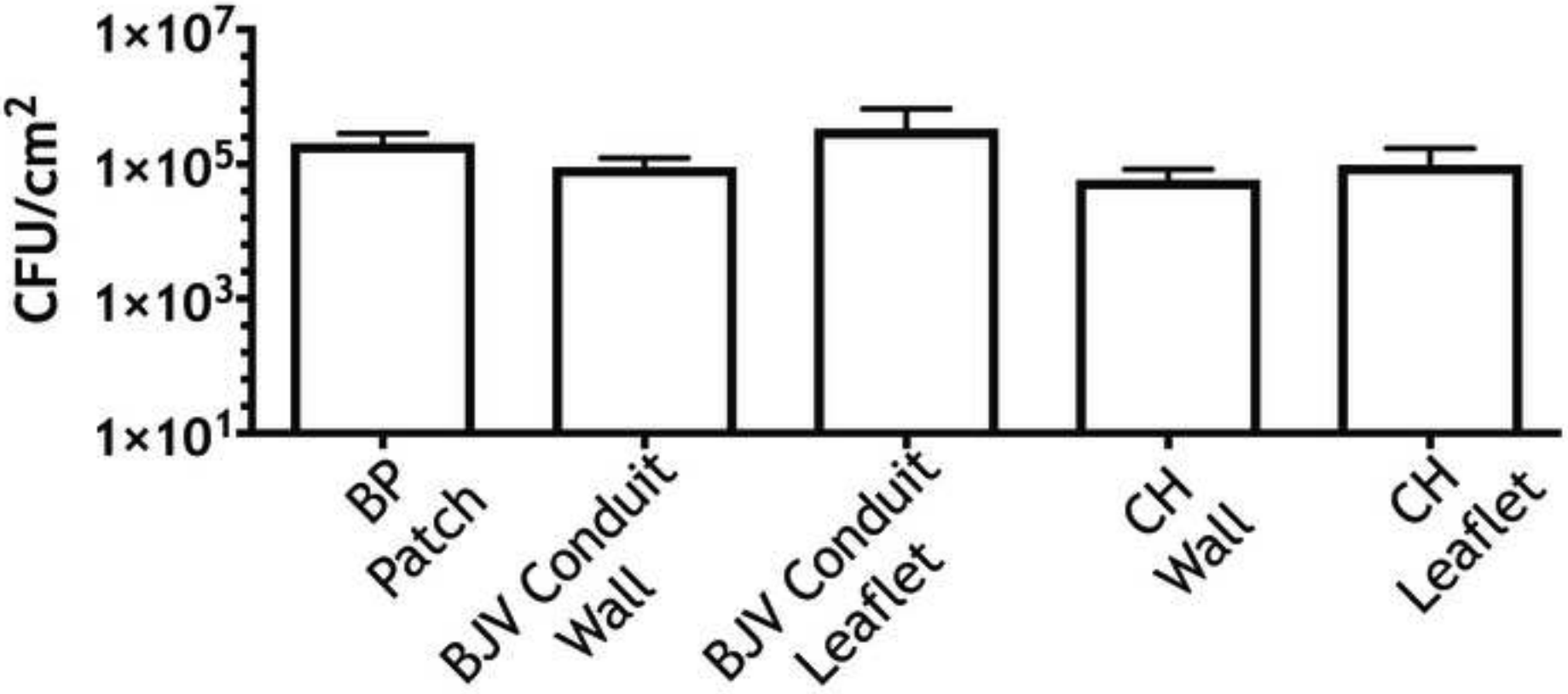


Fig. 4

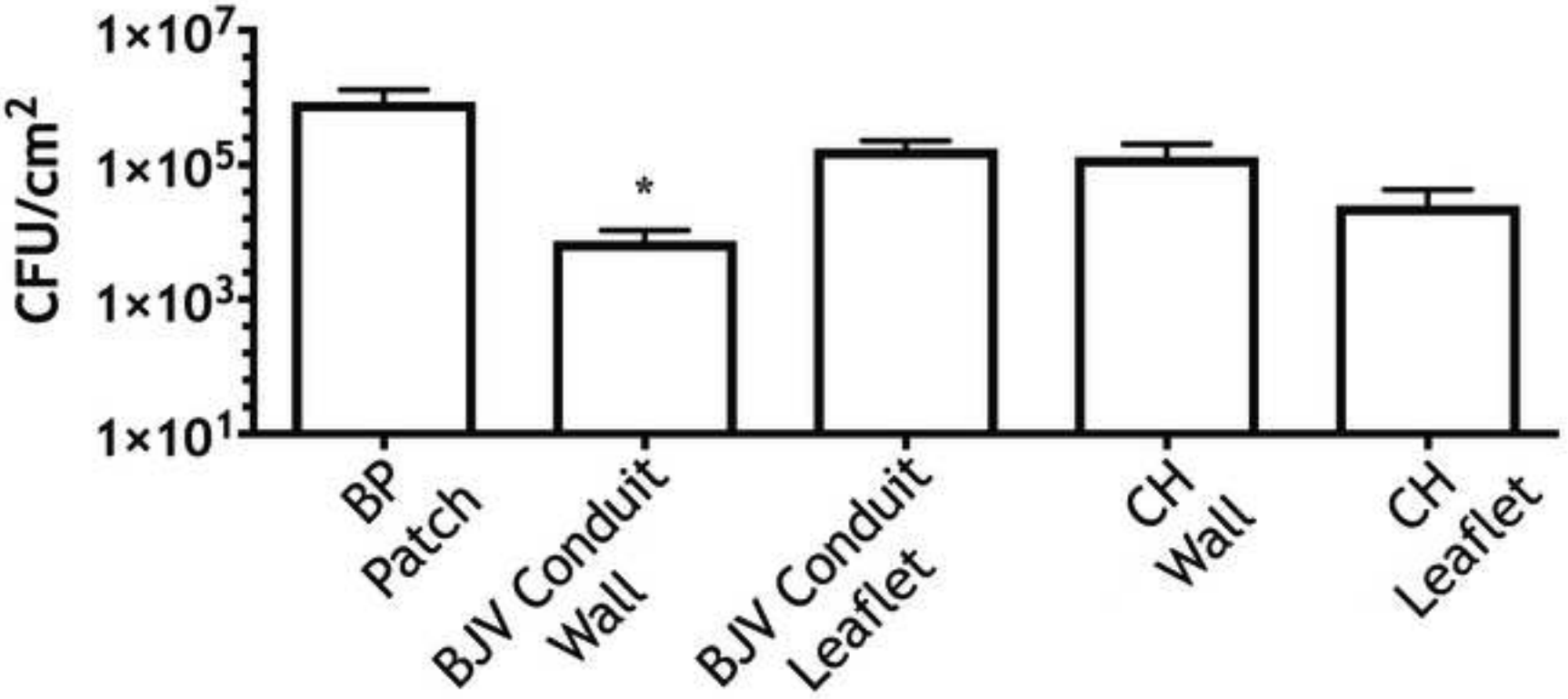
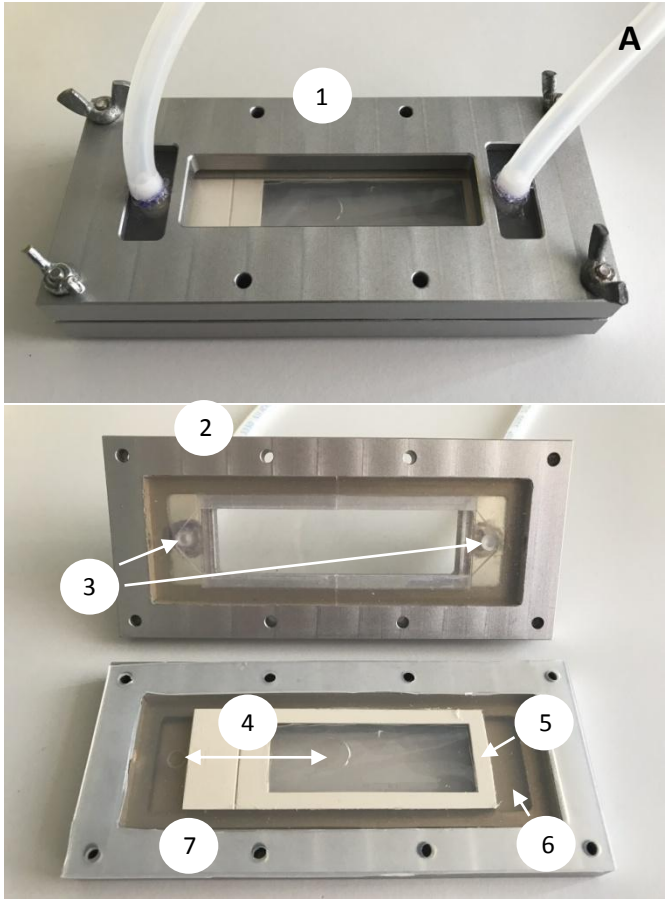
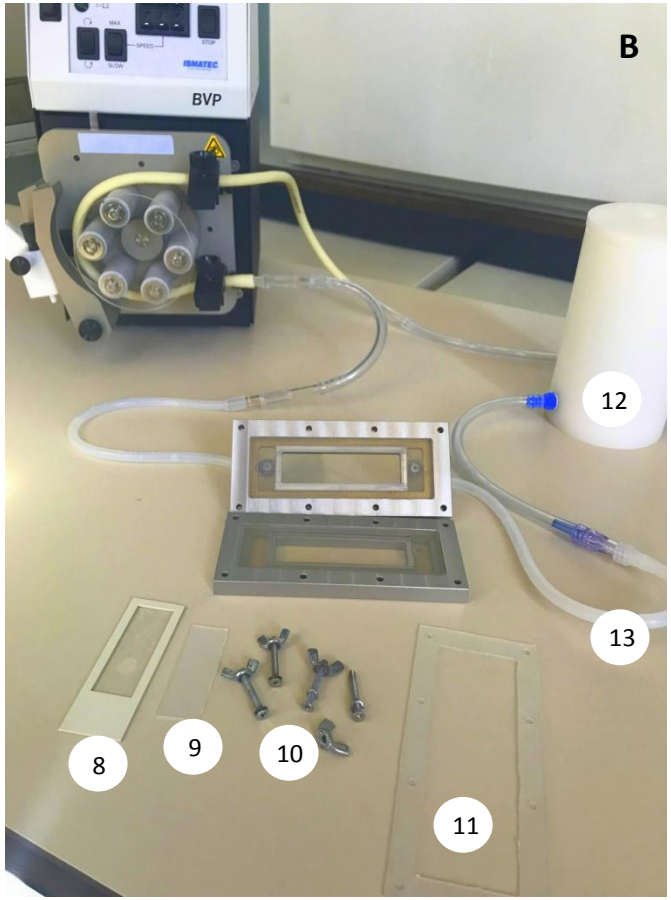


Fig.1





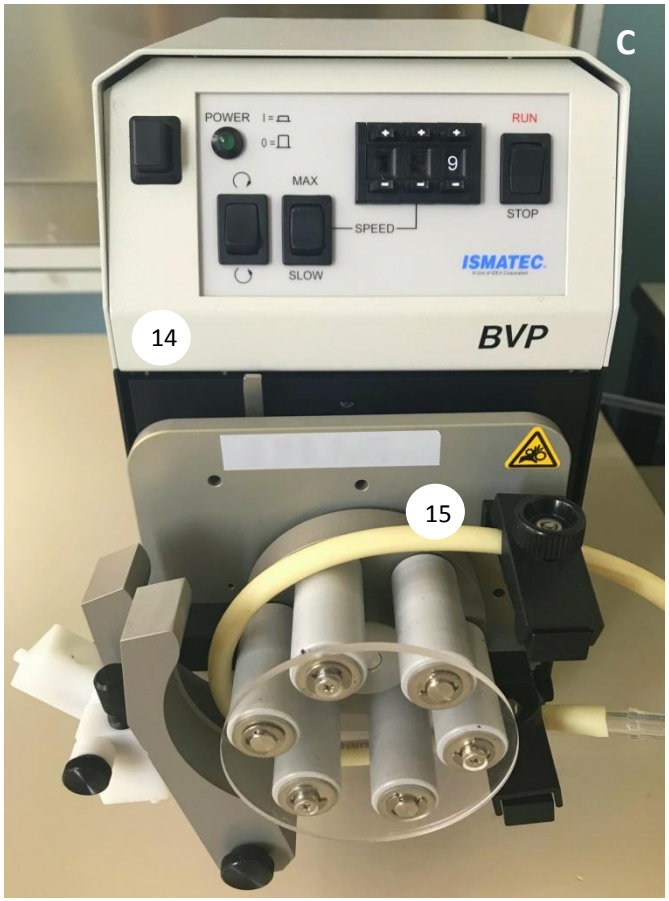


Fig. 5

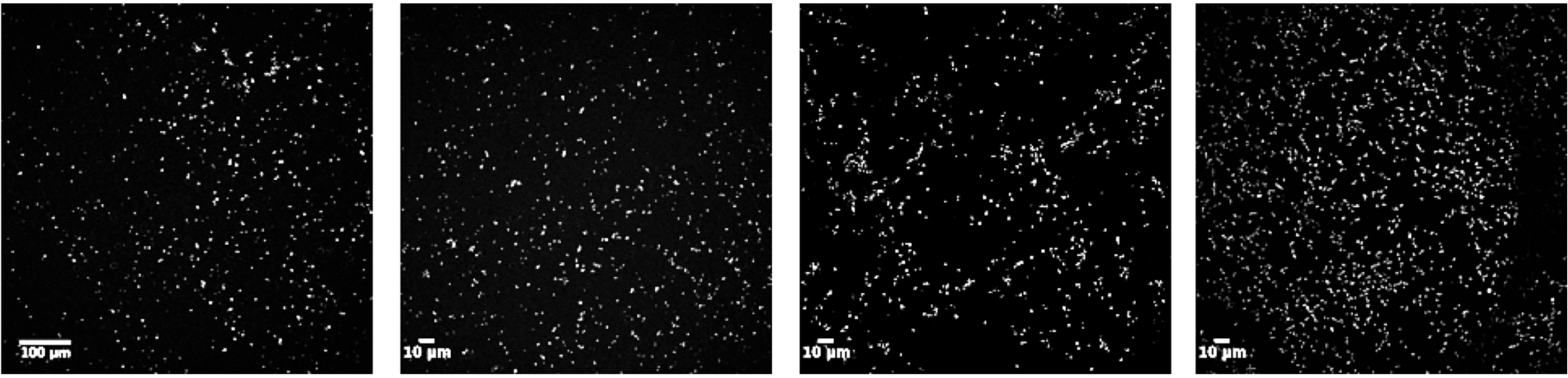


Fig. 6

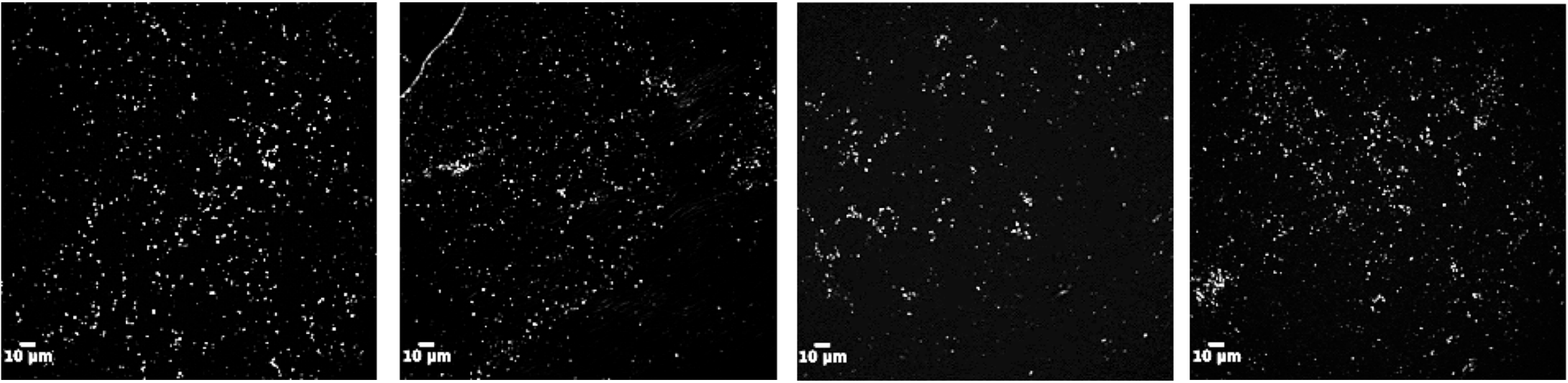


Fig. 7

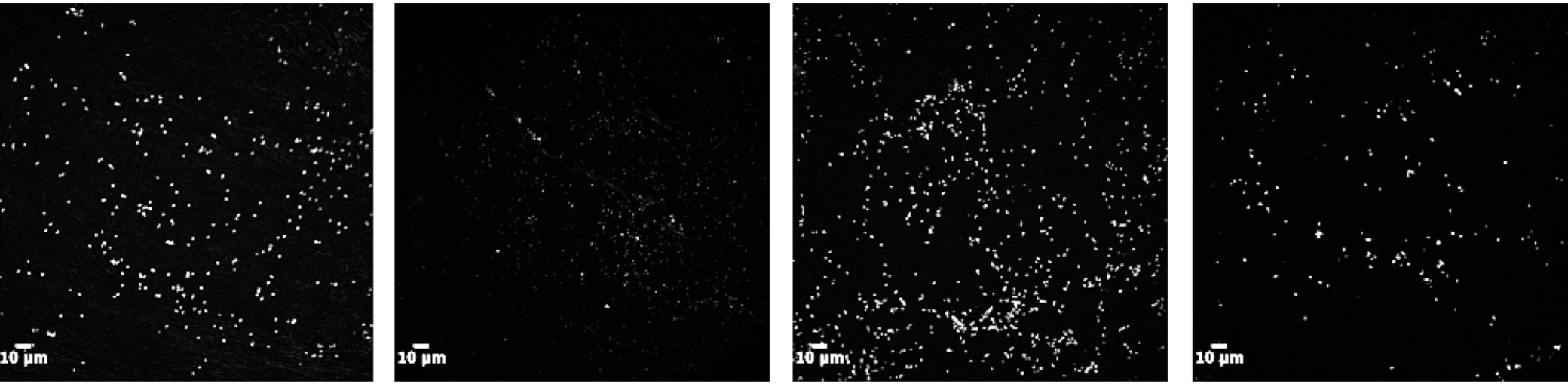


Table of Materials

Click here to download Table of Materials 1.xlsx

Name of Material/ Equipment	Company
Bovine Pericardium (BP) patch, Supple Peri-Guard Pericardium	Synovis Surgical Innovations, USA
Bovine Jugular Vein conduits (BJV)	Contegra conduit; Medtronic Inc, USA
CH cryopreserved homograft	European Homograft Bank (EHB)
Acu-Punch	Acuderm Inc, USA
human Albumin	Flexbumin; Baxter, Belgium
Tryptic soy broth (TSB)	Fluka, Steinheim, Germany
Heart infusion broth (BHI)	Fluka
Phosphate buffered saline (PBS).	Gibco
5(6)-Carboxyfluorescein N-hydroxysuccinimide ester (CF)	Sigma-Aldrich, Germany
Peristaltic pump (MODEL ISM444B)	Ismatec BVP-Z Standard; Cole Parmer, Wertheim, Germany
Sonication bath	VWR Ultrasonic Cleaner; VWR, Radnor, Pa
ProLong Gold Antifade Mountant	Invitrogen by ThermoFisher
InCell Analyzer 2000 (fluorescence scanner)	GE Healthcare Life Sciences, Pittsburgh, Pa
Arium Pro VF - ultrapure water - H_2O MilliQ	Millipore
Microscopic slides - Tissue Culture Chambers (1-well)	Sarstedt
1-well on Lumox detachable	Sarstedt
Stainless Steel - surgical Blades	Swann-Morton
Tygon Silicone Tubing, 1/8"ID x 1/4"OD	Cole-Parmer
PharMed BPT Tubing	Saint-Gobain
Tygon LMT-55 Tubing	Saint Gobain Performance Plastics™
Thermostat	BMG BIOMEDIZINTECHNIK

Catalog Number	Comments/Description
PC-0404SN	
M333105D001	
-	
P850 (8 mm); P1050 (10 mm)	
BE171464 LOT:16G12C	
22092-500G	
53286-500G	
14190-094	
21878-100MG-F	
631942-2	
142-6044	230V/50 -60Hz 60VA; HF45kHz, 30W
P36930	
29027886	
87206462	
94.6140.102	
94.6150.101	
311	
EW-95702-06	Temperature range: –80 to 200°C Sterilize: With ethylene oxide, gamma irradiation, or autoclave for 30 min, 15 psi of pressure
AY242012	Autoclavable 30 min at 121°C
15312022	
300-0042	230V, 90VA, 50Hz

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Title of Article: In vitro model of a parallel-plate perfusion system to study bacterial adherence to graft tissues

Author(s): Ditkowski B, Veloso T, Ditkowska M, Lubig A, Mela P, Jockenhövel S, Jashari R, Gewillig M, Meyns B, Hoylaerts M, Heying R.

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Article Title: ~~In vitro model of a parallel-plate perfusion system to study bacterial adherence to gut tissues~~

Signature: Ditkowski Bartosz

Date: 15.05.2015

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Leuven, July 26th, 2018

The Journal of Visualized Experiments

Review Editor

Vineeta Bajaj

Dear Dr. Bajaj,

We would like to thank the Editor and the reviewers for their thorough review and insightful comments on ms JoVE58476, entitled "*In vitro* model of a parallel-plate perfusion system to study bacterial adherence to graft tissues".

All the suggested changes have been addressed and implicated in the ms.
We hope that the ms is now suitable for publication in *JoVE*.

Please find below our *point to point* answers.

Sincerely,

Bartosz Ditkowski

Point to point answers:

Editorial comments:

1.
„Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version."

We have revised the manuscript to avoid any typos and grammar issues.

2.
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We have obtained the copyright permission from the editor of JTCVS in the form of an e-mail statement. This information is attached as a separate document to the submission document. Also figures are cited in an appropriate way in the figure legend.

3.

„Figures 2-4: Please note that there is no "ON" in the figure. So please remove "ON, overnight" from the figure legend."

We have deleted ON, overnight from the figure legend.

4.
"Please rephrase the Introduction to include a clear statement of the overall goal of this method."

We have substantially rephrased the *Introduction* section in a more structured manner, particularly stressing more the general aim of our method. Some sentences less relevant to the methodology were either rewritten or deleted.

5.
"Please define all abbreviations before use."

All possible abbreviations were defined before use in the text.

6.
"Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, dashes, or indentations."

We have checked carefully the numbering and improved it in the *Protocol* section according to the *JoVE* guidelines.

7.
„Please revise the protocol to contain only action items that direct the reader to do something. The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." Please revise lines 104-109, 123-126 accordingly."

We have revised the protocol and made sure to include only action items formulated in the imperative tense. Also, we introduced several phrases following the indication *Note*.

8.
"Line 116: Please spell out O/N."

The change has been adapted throughout the manuscript.

9.
„Line 141: What is mQ?"

We have more precisely defined this term as well as added an extra information of the producer of the water purifier and referred to the *Material Table*.

10.
"Lines 143, 147-148, 156: Please point out the specific step that is repeated."

We have included the specific information that was missing.

11.
"Line 165: What volume of PBS is used to wash? Wash for how many times? Please specify throughout."

This issue in now specified in the protocol. The tissue piece was washed two times with 10 mL of PBS for 5 min in a 24-well plate using a laboratory orbital shaker.

12.
"Line 168: Please specify the volume of the tube."

We have defined the volume of tubes throughout the manuscript.

13.
"Line 172: Please add more details to this step. This step does not have enough detail to replicate as currently written. Alternatively, add references to published material specifying how to perform the protocol action."

This action item has been expanded more explicitly by adding more details. We described the entire procedure of performing serial dilutions and spreading appropriate bacterial suspensions onto blood agar plates. The protocol is followed by counting colony-forming units on the day after plating. Results are expressed as CFU/mL or CFU/cm^2 of the tissue surface.

14.
"Line 179: Please provide the composition of antifade mounting medium. If it is purchased, please cite the Material Table."

Yes, the antifade mounting medium was purchased and therefore the information on its producer and article number are included in the *Material Table*, which is cited in the text.

15.
"References: Please do not abbreviate journal titles."

All journal titles have been spelled out according to the requirements of JOVE.

Comments Referee 1:

1.
Page 1, line 70. The authors should make judicious use of abbreviations throughout the manuscript, IE first appeared on line 70 instead of line73. Page2, line 109. Please add the full name of EHB.

We took care to define all possible abbreviations before their use in the text.

2.
Please add catalogue numbers alongside manufacturers for all reagents.

The *Material Table* has been updated with all (bio)chemicals and their catalogue numbers. Also devices or non-reagent articles were supplemented with either their catalogue or serial numbers.

3.
For both S. aureus and S. epidermidis infection, there was no difference between different graft tissues, but for S. sanguinis a significant reduction of adherence to the BJV wall was found when compared to BP patch, what is the possible explanation for this?

At this stage of our studies we have no proven explanation yet for the observation that *S. sanguinis* displayed a significant reduction of adherence to the BJV wall. In principal little information on pathogenesis of *S. sanguinis* adhesion to tissues is available. We can speculate that different bacteria employ repertoires of various receptors to bind underlying tissues especially under shear conditions. Or BJV conduit is submitted to the chemical cross-linking process that may affect ECM accessibility for bacterial surface molecules. Currently our studies are focusing on investigating the role of additional factors such as plasma proteins in bacterial adhesion under flow. This will deepen our knowledge and bring us new insights into mechanisms of adherence across different pathogenic strains. Plasma proteins might also be differentially involved in the bacterial adherence of *S. sanguinis* to tissues.
From work of Jalal *et. al.* (Int J Cardiol. 2015; 198: 201-5) we know that the source of bacterial isolates indeed matters and that strains may present different pro-adhesive features and subsequent pathogenicity.

4.
Figure legends need to be more clearly described so the reader can understand the intended explanation of the information contained in the Figures. As an example, it would be helpful for the reader to point out the name and purpose of each component by the authors in Figure 1.

We have defined each component of the chamber presented in the *Figure* 1 and included this information in the figure legend and the *Material list*. We also restructured necessary information in the figure legend to better guide the reader through.

5.
In Figure 6, why are the images of CH wall group and CH leaflet group the same?

We agree with the reviewer that indeed images are the same for CH wall and CH leaflet groups. We appologize for that mistake and corrected it by replacing the proper image in the group of CH wall.

Comments Referee 2:

Major Concerns:

1.
The critical step of mounting the graft patch onto the chamber is poorly described in the written protocol. The authors assume laminar flow for different patches without specifying patch height that could alter flow pattern within the chamber (or at least on the surface of the graft). Differences in flow profiles could significantly alter binding conditions, making the protocol poorly reproducible. It isn't clear from the article that the same geometry is achieved for patches of different grafts within the chamber. It is important that the authors clarify this point. Are the different patches of the same height or do they reach the same height in the chamber? If not, how can the same flow pattern be guaranteed across grafts in order to make

comparisons? Addressing this point is also important since the article suggests the use of different pumps to allow performing experiments under pulsatile or turbulent flow conditions in the future, however it isn't clear that laminar flow conditions were used in this protocol.

Yes, we agree with the reviewer that the step of graft mounting has not been described enough in detail to replicate it in an appropriate manner. We have substantially extended this section by adding more details.

Indeed, all used grafts have the same geometry when mounted in the chamber. By that, the same height of the different grafts is reached allowing reproduction of laminar flow conditions for all grafts. This information is included in the protocol.
Additionally, an explicit description of all elements of the entire chamber including physical parameters necessary to guarantee laminar flow conditions in our protocol has been added to the legend of Figure 1 and Result section.

2.
The results obtained with the protocol do not exhibit (except in one condition) significant variation of binding across 3 Staphylococcus species and 3 graft materials. Without running negative controls it is difficult to establish whether that is a genuine result or a limitation of the method. In order to establish the effectiveness of the method to assess binding differences, it is necessary to show results obtained with at least one bacteria species that shows reduced adhesion to BP, BJV and CH. In addition, a non-adherent material as negative control should be used to test binding of the Staphylococcus species.

We agree with the reviewer that negative controls would show differences in results and therefore ideally support the method. In our opinion, there is not a biologically relevant non-adherent material in our experimental conditions, which could serve as a valuable negative control. Fibrin matrices or collagen matrices would also show bacterial adhesion and would not give any further information in relation to our results. Nonetheless, in this study we primarily intended to compare different tissues and to investigate whether morphological tissue differences, bacterial species specificity or exposure to extracellular substrates determine bacterial adhesiveness to the graft tissues. After studying this basic approach, we included several blood components in the experimental set up where we could show significant differences in results. To support the reviewer's question of being able to identify differences in results we provide here some preliminary data of ongoing experiments. First, we have investigated the contribution of plasma proteins in bacterial adhesion to grafts using our flow model. These results reveal that bovine grafts show a significant higher Fg binding (Fig. 1) and subsequent *S. aureus* adhesion (Fig. 2).

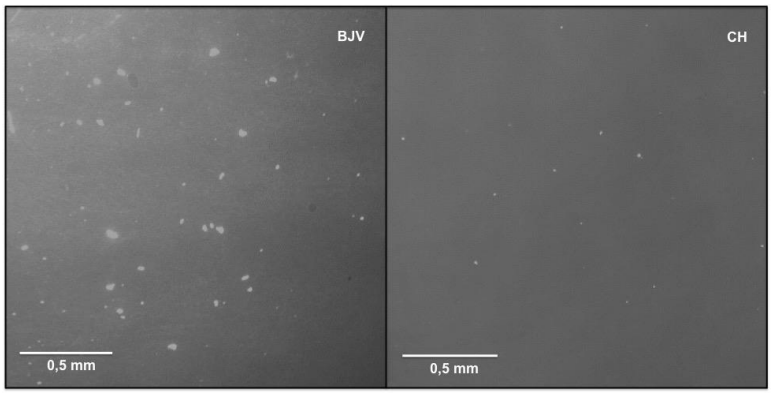

Fig. 1. Deposition of fluorescently labelled fibrinogen (white foci) on BJV and CH. Visualization by fluorescence scanning.

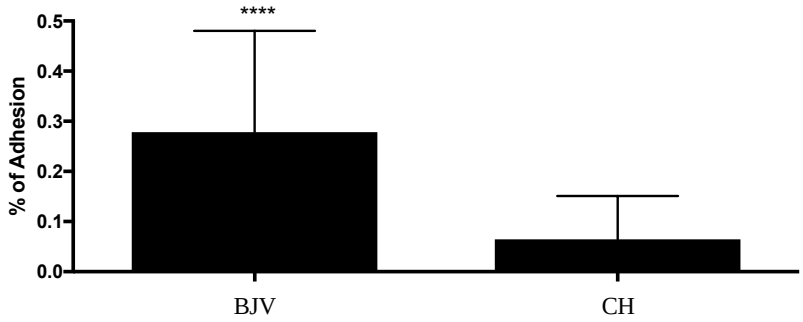

Fig. 2. *S. aureus* adheres significantly more to BJV than to CH under flow conditions. Tissues were pre-coated with Fg-carrying human plasma prior to perfusion. **** P< 0,0001.

3.

Similarly, the authors claim that this protocol can be used to analyze stepwise contribution of different factors (media, plasma, blood, plasma proteins, etc) in bacterial binding to endovascular surface; however this advantage is not illustrated in the article. It would be useful that the authors exemplify this versatility they envisage for their protocol with at least one condition.

In the article we state that our flow model will also be applicable for investigating a more `in vivo` like environment of bacterial binding to the endovascular surface. Since this has been meant as a general comment concerning future research possibilities and not been part of this article we removed that comment from the *Introduction* and discussed it in the *Discussion* section. To do so, we plan to investigate various factors, which may contribute to bacterial adherence, as fibrinogen and other plasma proteins. In these ongoing experiments, we analyze the influence of blood pre-perfusion on bacterial adhesion in PBS to bovine pericardium patch (BP patch). We are currently in the process of proceeding with this issue but have unfortunately only preliminary results, not suitable for publication in this article. In response to the reviewer`s comment we are presenting our preliminary results (Fig 3 below), which show significantly lower adherence of *S. aureus* to the BP upon treating the tissues with blood.

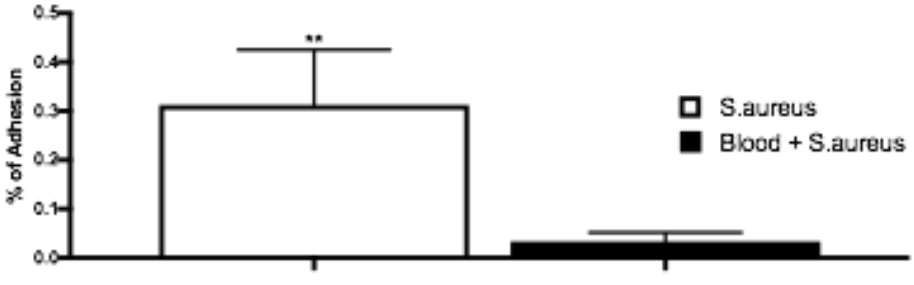

Fig. 3. *S aureus* adherence to BP patch is significantly diminished upon tissue treatment with blood. Bacteria were perfused in PBS. ** P< 0,01.

4.

The method assumes fully detachment of bacteria by sonication prior quantification. Please discuss how detachment was assessed (for example by incubating discs in broth after

sonication, measuring bacterial growth by OD and comparing with discs exposed to PBS alone).

Yes, indeed, full detachment of bacteria from the tissue grafts was evaluated upon incubation of patches overnight at 37°C in TSB liquid medium followed by OD_{600} measurements compared to control patches treated with a bacteria free solution. This information is added in the manuscript.

5.

Binding measurements are provided only by one method: CFU counting. Fluorescent images are only used for illustration purposes and not as an additional quantitative tool that confirms the results achieved by CFU determination. It would be useful that the authors provide (or explain why they do not do so) quantitative data obtained from fluorescence units measured with the fluorescence scanner, including description of use of no-binding controls (absolute fluorescence values obtained after perfusing grafts with bacteria free solutions). Unless the same result is achieved by at least to quantitative methods, the use of expressions such as "robust" or "satisfactory" should be avoided. The abstract is also misleading as it implies that confocal fluorescence was used to quantify binding.

The two methods, CFU counting and fluorescence imaging were both used to evaluate bacterial binding in our first approaches. However, it turned out that CFU counting was more reliable and resulted in reproducible data. Moreover, fluorescence visualization of the entire patch in one frame did not bring us satisfactory resolution of bacterial foci. Therefore, in this configuration we made use of the visualization method rather as a non-quantitative tool.
We rephrased slightly the abstract to avoid highlighting the methods, which were in fact not used to quantify the results in this article and additionally explained this issue in the *Discussion*.

6.

One-hour perfusion with recirculating media seems a long time to maintain endothelial cells viability if nutrients, oxygen and growth factors are not supplied and waste products (critically, CO2) are not continuously eliminated. For experiments performed on cryopreserved homograft tissue: how did the authors ensured endothelial cell viability or constant endothelialisation extent after 1-hour perfusion?

At this stage of our experiments, the tissue grafts were not covered with endothelial cells. This is also the case for the cryopreserved homograft. Koolbergen *et. al*. (J Thorac Cardiovasc Surg. 2002; 124:689-97) reported total or partial absence of endothelial cells on the surface of cryopreserved human allografts after storage and thawing. In our SEM images we also observed that the major surface area consists of exposed ECM what is in line with the absence of ECs.
Therefore, in the presented study we neglect the contribution of endothelial cells to the process of bacterial adherence.

7.

Include in discussion critical steps of the method and troubleshooting (perfusion time, bacterial suspension concentration and perfusion rate). Discuss significance of the method compared with existing alternatives? Being an end-point technique, this protocol presents disadvantages compared with real-time methods that can assess the effect of a drug when added half way during the perfusion step. How could this method be adapted to include timelapse binding data and dynamic information such as binding reversibility as shown by Garciarena et al Crit Care.

2017 Sep 26;21(1):246? Also discuss time consuming nature of bacterial culturing and CFU counting, and the possibility of measuring bacterial adhesion by fluorescence (as stated in 5).

According to the reviewer`s suggestion we have now expanded the *Discussion* section by including the troubleshooting to highlight potential optimization of the parameters used for our perfusion experiments. We also discussed that in this article we had presented endpoint readouts, albeit the method has a potential to be used for real-time analyses also in its current shape. Obviously, the method is under further development to provide the user with more advanced solutions.
The issue of fluorescence quantification was also risen to explain this alternative option of binding measurement to the reader. We also mentioned, as already stated in comment 5, that in our hands the fluorescence microscopy was less satisfactory due to some technical limitations.

Minor Concerns:

1
The most important contribution of the technique is the use of a perfusion chamber that allows for -grafts tissues to be mounted within; however specifications of chamber design and assembly are not provided in the article.

We have included the necessary information concerning the specifications of the chamber and its assembly in the protocol description and Fig 1.

2
The abstract states the use electron microscopy (EM) in this method; however EM is not described neither in the protocol nor the results.

Information on the use of EM has been deleted from the abstract. Its potential application together with fluorescence microscopy is now included in the discussion.

3
Please state the flow rate (in ml/min) used to achieve dyn/cm2

In order to achieve 3 dyne/cm^2 we used the flow rate of 4 mL/min. This information is now stated in the protocol.

4
Please provide intensity settings used in sonication bath

Parameters of the sonication bath used to detach bacteria from the grafts were as follows: amplitude = 100% and frequency = 45 kHz. These settings are included in the protocol and in the *Material Table* in the remark section.

5
Could the authors discuss the troubleshooting to end up using 107CFU/ml and 1-hour perfusion?

Our adhesion protocol was inspired from previous studies on Staphylococci of Veltrop *et al.* (Infection and Immunity, Nov. 1999, p. 6130–6138) and Jalal *et al*. (International Journal of Cardiology 198 (2015) 201–205). We employed 10^7 CFU/mL since from our and other`s

experiments adhesion levels were detectable and low enough to be able to obtain significant differences such as potential increased adherence without saturation of the tissue surface. Additionally, the 1-hour incubation period was the optimized range of time to observe possible differences in binding across strains employed.

6

Add to the materials list: cryopreserved homograft tissue, perfusion tubing and other relevant materials needed to assemble the perfusion chamber.

We have included all missing materials such as grafts, tubing, microscope slides, peristaltic pump to the *Material Table*. Also, all components of our chamber have been now explicitly indicated and described in Fig. 1 as suggested by the reviewer #1 in the comment 4.

7
Brightness of fluorescence images in figures 5-7 should be enhanced to better appreciate the presence of bacteria.

All fluorescence images have been carefully checked and brightness was enhanced where needed to better present bacterial foci.

Comments Referee 3:

Minor Concerns:

1
Please provide the dimensions of the parallel plate device used in the current work.

The dimensions of the flow chamber were included in the Fig. 1.

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