New quantitative automated model to simulate bacterial dissemination in human tissue during irrigation of contaminated wounds

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<td>Brochhausen, Christoph; University Hospital Regensburg, Pathology Froschermeier, Franziska; University Hospital Regensburg, Department of Trauma Surgery Alt, Volker; University Hospital Regensburg, Department of Trauma Surgery Pfeifer, Christian; University Hospital Regensburg, Department of Trauma Surgery Mayr, Agnes; University Hospital Regensburg, Department of Trauma Surgery Weiss, Isabella; University Hospital Regensburg, Department of Trauma Surgery Babel, Maximilian; University Hospital Regensburg, Pathology Siegmund, Heiko; University Hospital Regensburg, Pathology Kerschbaum, Maximilian; University Hospital Regensburg, Department of Trauma Surgery</td>
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New quantitative automated model to simulate bacterial dissemination in human tissue during irrigation of contaminated wounds

C. Brochhausen¹, F. Froschermeier², V. Alt², C. Pfeifer², A. Mayr², I. Weiss², M. Babel¹, H. Siegmund¹, M. Kerschbaum²

¹Institute of Pathology, University Regensburg, Regensburg, Germany.

²Department of Trauma Surgery, University Medical Centre Regensburg, Regensburg, Germany.

*Both authors contributed equally to this work = Contributed equally to first authorship

Corresponding author:

Priv.- Doz. Dr. med. Maximilian Kerschbaum

Department of Trauma Surgery

University Medical Centre Regensburg

Franz-Josef-Strauss Allee 11

93053 Regensburg, Germany

phone: +49 (0)941 944-16515

e-mail: maximilian.kerschbaum@ukr.de
Conflict of Interests Statement

The authors whose names are listed on the title page certify that they have NO affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers’ bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

Abstract

This study presents a simple and cost-effective model using microparticles to simulate the bacterial distribution pattern in soft tissue after low- and high-pressure irrigation. Silica coated iron microparticles (comparable diameter (1 µm) and weight (0.8333 pg) to S. aureus) were applied to the surface of twenty fresh human muscle tissue samples in two amputated lower legs. Particle dissemination into deep tissue layers as an undesired side effect was investigated in four measuring fields as positive control (PC) as well as after performing pulsatile high-pressure (HP, 8 measuring fields) and low-pressure flushing (LP, 8 measuring fields). Five biopsies were taken out of each measuring field to get a total number of 100 biopsies. After histological and digital image processing, the specimens were analysed and all incomplete sections were excluded. A special detection algorithm was parameterized using the open source bioimage analysis software QuPath. The application of this detection algorithm enabled automated counting and detection of the
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While both high- and low-pressure flushing techniques are able to reduce the amount of bacteria, a higher effectiveness is shown for high-pressure irrigation. Nevertheless, a challenge for the validity of the study is the use of avital tissue and therefore a possible negative influence of high-pressure irrigation on tissue healing and further dispersion of particles cannot be evaluated.

Keywords

Soft tissue contamination, Microparticles, Automated particle count, Digital whole slide image processing, Jet lavage

Introduction

Bacterial soft tissue infections are moving into the focus of clinical research (Gottlieb et al. 2019). This research area concentrates on investigating interactions of pathogens with surrounding tissue and the immune system (Sattler and Kennedy-Lydon 2017). Wound healing represents a multistep process including inflammation, proliferation and remodelling (Rahim et al. 2017). Infection significantly disturbs this well orchestrated wound healing system (Zhao et al. 2016). Effective cleaning as well as eradication of infectious agents is crucial to initiate wound healing processes. An essential step in anti-infectious treatment is the surgical reduction of bacterial load with debridement and careful irrigation (Fry 2017). When treating and irrigating wounds, the goal is to remove bacteria from the wound and minimize the spread to deeper tissue layers. Therefore, an appropriate method of wound irrigation would result in less contamination and lower particle concentration in the tissue depth. However, little is known about the influence of these physical methods on elimination and dissemination of bacteria to deeper soft tissue levels. To date, there are experimental and clinical studies investigating high- and low-pressure irrigation in human bone tissue. These studies point to greater tissue damage and intramedullar propagation of bacteria caused by high-pressure irrigation (Bhandari et al.
Negative effects on new bone formation have also been reported (Dirschl et al. 1998). So far, the influence of these methods on the transfer of bacteria to deeper soft tissue levels has only been investigated in animal tissue studies. Some authors concluded that high-pressure irrigation is more effective in removing bacteria from contaminated wounds (Brown et al. 1978, Tabor et al. 1998). Other authors showed that high- compared to low-pressure irrigation causes increased bacterial penetration depth, a higher level of retained bacteria in the wound and additionally an increase in cellular death and macroscopic tissue damage (Bhandari et al. 1999, Boyd et al. 2004, Hassinger et al. 2005).

In fact, there is a lack of experimental data on the impact of physical forces, such as high-pressure or low-pressure irrigation, on wound contamination in human tissue samples. This deficiency is potentially caused by the lack of models simulating the particle spread in soft tissue infections. We present an innovative approach to simulate particle dissemination caused by physical forces in wound infections. This is a novel method that uses microparticles as objects of comparison for *S. aureus* in an experimental setup. Utilization of microparticles, that mimick the physical behaviour of bacteria, enables a simple automated and quantitative analysis of particle distribution after physical wound treatment using high-pressure or low-pressure irrigation.

**Methods**

*Specimens and Particles*

Two fresh human amputated lower legs were dissected to create measurement fields of 4 cm² (2 cm x 2 cm) in each amputated lower extremity. Epidermis, subcutaneous adipose tissue, and muscle fascia were dissected off to expose the muscle tissue (tibialis anterior muscle; Fig. 1A). Further experiments were performed only on those ten measurement fields in every amputate, where exactly the anterior tibialis muscle was exposed in order to ensure a high reproducibility of the tests. 50 µl of an iron particle containing solution (SiMAG-Silanol, 50 mg/ml, 1.8 x 1012 particles/g, chemicell GmbH, Berlin, Germany) with 10-fold dilution were pipetted to the surface of each test area (Fig. 1B). Accordingly, approximately 450 million particles were applied within each test field. The diameter of the particles corresponded to that of *S. aureus*, which is about 1 µm (Lorian et al. 2016). SiMAG-Silanol particles consist of an iron core and a silica coat with a mean total diameter of 1 µm (Häfeli et al. 2016). One of the particles weighs about 0.8333 pg according to manufacturer's specifications. In comparison, a bacterial cell scales about 1 pg (Davis et al. 2016).

The samples were left untreated for an exposure time of five minutes. Eight of the measuring fields (four in each amputated lower leg) were treated with high-pressure pulsatile lavage (HP; Level 2, Jet Lavage Ocean Jet 200, Bluerock Medical GmbH, Feldafing, Germany) with a pressure of under 15 psi. Low-pressure irrigation (LP) with a 50-ml syringe was performed in eight measurement fields (four in each amputated lower leg). 0.9% NaCl solution was used as cleaning solution for both procedures. Each measuring field was cleaned for 30 seconds at a distance of 5 cm from the irrigation device.
to the tissue. An angle of 90° to the surface was thereby maintained. The remaining four fields (two in each amputate) were left untreated to obtain a positive control group. Both amputated lower legs were subsequently frozen to -4°C. Five biopsies were taken from every measuring area using biopsy punches with a length of 2 cm and a diameter of 2 mm (Fig. 1C/D). Thus, a total of 5 biopsies were obtained from each of 10 measurement fields from two amputated lower extremities, resulting in a total number of 100 tissue samples. The study has been approved by the Ethics Committee of the University of Regensburg in accordance with the Helsinki Declaration (ref.-number 18-1160-101).

**Histological and electron microscopical evaluation**

Punch biopsies from each measuring field were fixated in buffered formaldehyde (3.5%) by dehydration and paraffin embedding according to standardized and automated methods (Leica ASP 300S dehydration system, Leica Biosystems, Wetzlar, Germany; Thermo HistoStar™, Thermo Scientific, Waltham, USA). Paraffin sections of 4 µm thickness were cut (Microm HM 355S, Thermo Scientific™, Waltham, USA) and stained with Hematoxylin and Eosin (H&E), Elastica-van-Gieson (EvG) and Berlin Blue in a standardized and automated manner (Sakura Tissue-Tek Prisma®, Sakura Finetek, Alphen aan den Rijn, The Netherlands). Slides were digitalized (Pannoramic Digital Slide Scanner, 3DHISTECH, Budapest, Hungary) using a 40-fold objective. Resulting whole slide images were reviewed using a digital light microscope (CaseViewer, 3DHISTECH, Budapest, Hungary). Those specimens that were incompletely sectioned were excluded from further analysis (analysed samples: HP: n=17; LP: n=10; CP: n=7).

For electron microscopic analysis formalin fixed tissue samples were post-fixed with buffered glutaraldehyde (Karnovsky fixative) for at least 72h and then cut into smaller samples by use of a scalpel (2mm³). For the embedding process (post-fixation with osmium tetroxide, dehydration, infiltration with EPON) the LYNX microscopy tissue processor (Reichert-Jung, Wetzlar, Germany) was used. Semi-thin-sections and ultra-thin sections (80nm) were cut using the Reichert Ultracut S Microtome (Leica-Reichert, Wetzlar, Germany). Ultra-thin-sections were stained using aqueous 2%-uranyl-acetate and 2%-lead-citrate solution for 10 minutes each. Electron-microscopic analysis was performed using the EFTEM LEO 912AB electron-microscope (Zeiss, Oberkochen, Germany). Iron nanoparticles were detected by Electron Energy Loss Spectroscopy (EELS) and the distribution was shown by ESI (electron spectroscopic imaging).

**Quantitative Evaluation of Particle Distribution**

An algorithm for automated quantitative evaluation of the scans was parametrized from the bioimage analysis software QuPath (developed at the University of Edinburgh, UK). This open-source program enables automated digital analysis of whole slide images (Bankhead et al. 2016). In the bioimage analysis program QuPath, there is a preset function for automated detection and counting of cells. Depending on which cells are to be recognized by the program, the detection criteria can be adjusted according to special characteristics such as size and staining of the target cells. We have modified the detection parameters so that the used particles can be recognized by the algorithm. For this purpose,
we parameterized a script that detects the different coloration of the iron microparticles compared to the background. In addition, the small size of the particles compared to the large cells in the tissue background is an important distinguishing feature that was taken into account when designing the script. The script was used to automatically analyze the areas previously assigned manually to specific tissue types. The quality of this automated particle detection was validated. 30 high-power fields from the middle of the preparations were randomly and computer-assisted selected for this purpose. Automated and manual particle counts were performed on these 30 high-power fields. Sensitivity of the automated quantitative evaluation was calculated in comparison to manual counts. To reduce the risk of mismeasurement due to dissemination of particles from the surface to the depth during biopsy collection, the margins of the histological samples were excluded for quantitative analysis. The superficial particle layer and connective tissue below the fascia were also excluded from quantitative analysis, because only particles in muscle tissue should be analysed. In addition, overlapping, blurred or non-contiguous areas were excluded. The resulting particle distribution data were exported. The number of detections was offset against the area. Resulting data were evaluated using SPSS. We conducted a one-way ANOVA to assess the effects of irrigation methods on the particle concentration (measured as particle number per mm²). Additionally, statistical analysis was conducted for three groups HP, LP and PC resulting in Games-Howell post-hoc analysis.

Results

Histological Detection of Particles
Particles were detected in every staining type in conventional light microscopy due to their original brown colour. The contrast between particles and background was best in Berlin Blue staining compared to H&E and EvG stainings (Fig. 2). In all analysed biopsies, particles were detected on the surface of the specimen and in deeper tissue levels. The particles were visualized in both conventional microscopy and after whole slide imaging. Furthermore, particles were also identified by electron microscopy. In this context, the iron core of the particles was clearly demonstrated by electron energy loss spectroscopy (Fig. 3).

Quantitative Evaluation of Particle Distribution:
Our script allowed the detection of iron particles in Berlin Blue stained slices due to their different size and coloration compared to the background. The distribution pattern of particles could only be assessed in at least 20-fold magnification by conventional microscopy due to the small particle size. After applying the detection script, particles were marked by circles with a diameter of 100 µm (Fig. 4). The particles and even clusters could thereby be displayed in the whole slide image overview for inspection and comparison by conventional histological analyses (Fig. 5). We included examplary whole slide images with particles marked in red from each of the irrigation groups and the control group (Fig. 6). From these images it would be difficult to detect any difference in
particle concentration or distribution pattern between the different irrigation groups by visual analysis. Automated counting enabled quantitative analysis despite this challenge. Validation of the automated particle count was pursued by comparing to manual count in 30 predefined high-power fields. The mean count in these 30 areas was 43.5 ± 13.2 particles in manual evaluation compared to 44.8 ± 13.2 particles in automated analysis. This results in a sensitivity of 95% comparing the automated algorithm count to the manual procedure. We conducted a one-way ANOVA to assess the effects of irrigation methods on the particle concentration in the tissue depth (measured as particle number per mm²). We had three different sample groups: HP (M = 1608, SD = 302), LP (M = 2176, SD = 609) and PC (M = 4011, SD = 686). There were no outliers, according to inspection with a box-plot. Data were normally distributed for each group (Shapiro-Wilk test, p > .05). Homogeneity of variance was not given (Levene’s test, p < .05). The level of depression differed statistically significant for the different levels of physical activity, Welch’s F (2, 31) = 588.5; p < .001. Games-Howell post-hoc analysis revealed a significant difference (p < .05) between particle concentration in all groups. Mean level of particle concentration increased from HP to LP (+567.9, 95%-CI [16,323; 1119,5]), from HP to PC (+2403, 95%-CI [1607,9; 3198]), and from LP to PC (+1835, 95%-CI [972,8; 2697,2]).

Discussion
The present study demonstrates a simple and cost-effective model simulating bacterial dissemination in muscle tissue following high- and low-pressure flushing using iron particles. These microparticles were detectable in light microscopy as well as electron microscopy. Especially in Berlin Blue stainings the particles were well distinguishable, whereas the visualization of bacteria would need special complex histological stainings. Automated counting enables precise evaluation of particle distribution in high sample numbers. Since the present model is based on freshly amputated human lower legs the tissue characteristics represent a physiological situation. Nevertheless, a major challenge for the validity of the study samples is that they were taken from avital tissue. This means that drawing conclusions about dissemination through blood vessels and other tissue structures in vital tissue remains difficult.

Applying iron microparticles for simulation of bacteria offers new possibilities in medical research and costs are amenable (about 40$ (30€) per 1 ml containing 50 mg of SiMAG Silanol particles). Another advantage of this procedure is that experiments such as this can be carried out in a usual laboratory without special precautions e.g. biohazard qualifications for use of (inactivated) bacteria.

Automated analysis enables objective and unbiased evaluation in histopathological questions (Colling et al. 2016; Huss and Coupland 2020). The results can be objectified, applying a predefined script on each section. Counting the number of particles in a conglomerate by conventional light microscopy remains challenging. The algorithm is even capable of detecting single particles in clusters by splitting their shape. The automated particle count showed a high sensitivity for particle detection. This automated procedure may also be useful for evaluation of large scale analysis of samples. Moreover, the present study is the first study on this topic to use fresh human muscle tissue samples.
(amputated lower legs), whereas previous studies were performed on animal tissue (Boyd et al. 2004, Hassinger et al. 2005). Due to the use of human tissue, the validity of the results is significantly higher than in the previous studies and can therefore be more easily transferred to clinical questions. Previous studies looking at the effectiveness of different flushing methods on bacterial clearance have provided contradictory results. While some authors concluded that high-pressure irrigation is more effective in removing bacteria from contaminated wounds (Brown et al. 1978, Tabor et al. 1998), other studies showed that the amount of retained bacteria is higher after high-pressure irrigation compared to low-pressure irrigation methods (Bhandari et al. 1999, Boyd et al. 2004, Hassinger et al. 2005). We show that in our sample set, lower particle concentrations were detected in deeper tissue layers after performing high-pressure lavage than after low-pressure lavage. The results of the present study on bacterial clearance in human tissue suggest that pulsatile high-pressure irrigation is more effective compared to the low-pressure method. Nevertheless, the present results also indicate that both methods are able to significantly reduce the bacterial load (compared to the positive controls). This fact is consistent with the limited clinical data on this topic.

In a large-scale randomized controlled clinical trial, it was found that there were no significant differences between high-pressure and low-pressure irrigation for contaminated wounds in terms of reoperation rate outcomes (FLOW investigators 2015). The methodological heterogeneity of experimental work on this topic makes it difficult to draw valid conclusions in this area. The present simulation model can help to investigate novel irrigation methods in a cost-effective and simple way with respect to cleaning efficacy and bacterial dissemination within human tissue.

Nevertheless, some limitations of the presented model need to be discussed. Bacteria have bioadhesive properties to tissue composites and use quorum sensing communicating to each other (Kai 2018). These properties may influence the kinematic characteristics of bacteria and cannot be imitated by lifeless particles. Bacterial cells can even reply to environmental influences (e.g. chemical, thermal, mechanical). One example is the ability of bacteria to use a flagellum for a certain degree of mobility in tissues responding to mechanical stimuli (Persat et al. 2016). This type of self-mobility cannot be represented by the present particle model. The microbiome in the wound itself influences contaminating bacterial species and thus also has an impact on wound healing, which cannot be simulated (Scales and Huffnagle 2013). Furthermore, the present model cannot simulate any tissue changes caused by an inflammatory process. At this point, further studies are needed to compare particle behavior with the behavior of vital bacteria in tissue. Results might be influenced by the fact that only biopsies and not the whole measuring area can be investigated in this model. In addition, dissemination of particles through punch biopsy sampling is possible. An additional limitation of the model is the fact that no information is available on whether and to what extent the iron microparticles cause damage when penetrating the tissue. To limit this source of error, the biopsies were taken from the frozen amputated lower legs and, in addition, the marginal areas of the punch cylinders were excluded from the quantitative analysis.

Conclusion
In summary, the presented method is simple and cost effective and can simulate particle dissemination in contaminated soft tissue after high- or low-pressure irrigation in a reproducible way. This model represents a promising approach for investigating the influence of different cleaning methods on bacterial transmission in open human wounds. The study showed a higher effectiveness of high-pressure irrigation compared to low-pressure flushing in particle elimination. Both methods were more effective in reducing the amount of bacteria than the positive control. *Nevertheless, a challenge for the validity of the study is the use of avital tissue and therefore a possible negative influence of high-pressure irrigation on tissue healing and further dispersion of particles cannot be evaluated.*

**Acknowledgements**

The department of trauma surgery received funding of Manfred-Roth-Stiftung for their research on soft tissue infections.

**Author contributions statement**

Christoph Brochhausen, Christian Pfeifer, Maximilian Kerschbaum and Volker Alt conceived experiments. Heiko Siegmund, Isabella Weiβ and Agnes Mayr carried out experiments. Franziska Froschermeier analysed data. All authors were involved in writing the paper and had final approval to the submitted and published versions.

**References**


Figure legends

Figure 1: A): Measuring fields with a size of 4cm² (2 cm x 2 cm) were prepared in fresh human muscle tissue samples from an amputated lower leg. B): Measuring fields with applied iron particle solution. C/D: Five biopsies were taken from each area.

Figure 2: Particles were visible in light microscopy. Microparticles were detectable in every staining type in light microscopy as regularly brown particles. Contrast between particles and background was best in Berlin Blue (C) stainings compared to H&E (A) and EvG (B) stainings.
Figure 3: Electron-microscopic analysis was performed and iron nano particles were detected by EELS (electron energy loss spectroscopy). The distribution was shown by ESI (electron spectroscopic imaging).

Figure 4: A): Running the script allowed detection of iron particles in Berlin Blue stained slices due to their different size and coloration compared to the background. B): Small microparticles were marked by circles with a diameter of 100 µm.

Figure 5: This figure shows an original whole slide image sample (Figure 5A). In Figure 5B, the tissue-specific regions (superficial particle layer, subcutis, fascia, muscle, subfascial connecting tissue) are marked in different colours. The particles can be displayed in the overview after applying the automated particle detection script (Figure 5C). We excluded those tissue sections from analysis that had detached from the tissue composite at the edge of the biopsies.

Figure 6: Whole slide image overviews as visual presentation of punch biopsies after (A) high-pressure and (B) low-pressure irrigation as well as of the (C) positive control samples.

Figure 7: Comparison of high-pressure (HP) and low-pressure (LP) group with the positive control samples (PC) of particle density after automated particle count. The
positive control samples showed significantly higher particle density compared to both HP and LP. The high-pressure group also showed significantly less particles than the low-pressure group.

Supporting information

Script: A script for automated quantitative evaluation of the scans was employed with the help of the bioimage analysis software QuPath. The resulting script for particle detection is shown in this figure. It considers not only the particle size, but also their different coloration compared to the background.
Response to Reviewers

Dear reviewers and editor,

We would like to thank you very much for your valuable comments and suggestions. Changes are highlighted as bold red letters in the manuscript. If any further questions or any dubiety appears, please do not hesitate to contact us.

Reviewer #1:

General Errors/comments not listed in the sections above: The authors have re-introduced the term "germ" in their revised text. This was a word requested to be removed from the manuscript as it is not a formal scientific term.

Response:
Thank you for this comment. We changed this term.

Reviewer #2:

I have one final comment regarding the conclusions. As I stated in the previous revision, please nuance the conclusion. I suggest the authors therefore include the following sentence at the end of the abstract and conclusions of the manuscript:

"Nevertheless, a challenge for the validity of the study is the use of avital tissue and therefore a possible negative influence of high-pressure irrigation on tissue healing and further dispersion of particles cannot be evaluated."

Response:
Thank you for this comment. We added this conclusion as suggested.

Thank you very much for the constructive comments and the opportunity to revise our paper. We hope that the changes can convince you to publish this paper.

Kind Regards,

Maximilian Kerschbaum
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