Bacteria-targeted fluorescence imaging for visualization of fracture-related infections in orthopaedic trauma surgery

Marina López-Álvarez*, Marjolein Heuker*, Klaas A. Sjollema, Gooitzen M. van Dam, Jan Maarten van Dijl †, Frank F.A. Ijpmans †, Marleen van Oosten †

* † Authors contributed equally

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Abstract

Fracture-related infection (FRI) is a serious complication in orthopaedic trauma surgery worldwide. Especially the distinction of infection from sterile inflammation and detection of low-grade infection are highly challenging. The aim of the present study was to explore the use of bacteria-targeted fluorescence imaging for enhanced detection of FRI on osteosynthesis devices as a step-up towards real-time image-guided trauma surgery. Extracted osteosynthesis devices from 12 patients, who needed revision surgery after fracture treatment, were treated with a near-infrared fluorescent tracer composed of the antibiotic vancomycin and the fluorophore IRDye800CW (i.e. vanco-800CW). Subsequently, the devices were imaged macro- and microscopically. The fluorescence signals were directly correlated to bacterial growth upon replica plating of the extracted devices on blood agar, and to the results of microbiological culturing. Importantly, compared to culturing, the bacteria-targeted fluorescence imaging of extracted osteosynthesis devices with vanco-800CW allows for prompt diagnosis reducing the time-to-result from days to less than 30 min. Moreover, bacteria-targeted imaging will provide surgeons with real-time visual information on the presence and extent of infection. Here we present the first clinical application of fluorescence imaging for detection of FRI. We conclude that imaging with vanco-800CW allows early, accurate and real-time visual diagnostic information of FRI in the clinical setting, even in case of low-grade infections.
Introduction

Fracture-related infection (FRI) is a devastating complication after bone fracture treatment, resulting in severe patient morbidity and loss of quality of life. FRIs can be differentiated in 1) acute infections with clear signs of infection caused by highly pathogenic bacteria, and 2) low-grade infections caused by less pathogenic bacteria. Low-grade infections often lack specific clinical symptoms, which complicates their diagnosis. Treatment of FRI usually consists of revision surgery, including debridement and removal of infected bone and osteosynthesis devices (e.g. plates, nails and screws), combined with long-term antibiotic therapy\(^1,2\). During surgery, samples are collected for culture to verify infections, and identify causative micro-organisms for specific antibiotic therapy\(^2-4\). A major challenge is the distinction between non-infected and infected areas during surgery, especially in case of low-grade infections. Surgeons rely on tactile and visual information to establish whether and to what extent wound areas are affected by the suspected infection. Further, a definitive pre-operative diagnosis of FRI is often not possible in case of low-grade infections due to the absence of clinical symptoms. In particular, the results from diagnostic modalities, such as white blood cell scintigraphy, fluorodeoxyglucose positron emission tomography (FDG-PET), or magnetic resonance imaging (MRI) are often inconclusive\(^3,5,6\). Therefore, a diagnostic imaging tool enabling early, accurate and real-time visualization of the infecting bacteria themselves, rather than inflammatory responses elicited by the infection, would provide a solution to this clinical problem.

Bacteria-targeted optical (i.e. fluorescence) imaging is an upcoming clinical imaging technique with great potential for the visualization of infections\(^7\). In particular, a conjugate of the antibiotic vancomycin and the near-infrared fluorophore IRDye800CW (i.e. vanco-800CW) was previously identified as an effective bacteria-targeted optical tracer in preclinical infection imaging studies\(^8,9\). Like vancomycin, vanco-800CW specifically targets the Gram-positive bacterial cell wall. Since FRIs are mostly caused by Gram-positive bacteria, such as *Staphylococcus aureus*, vanco-800CW seems particularly suited as a bacteria-targeted optical tracer for real-time diagnosis of FRI. Moreover, vanco-800CW efficiently binds Gram-positive bacterial biofilms encountered in FRI\(^10\).

The present study was aimed at investigating whether vanco-800CW allows discrimination between infected and non-infected osteosynthesis devices, and whether it can be applied to visualize bacterial biofilms in clinical practice. To this end, we investigated osteosynthesis devices from 12 patients, who needed revision surgery and extraction of plates and/or screws after fracture treatment.
Results

The investigated patient cohort consisted of 12 patients, who underwent revision surgery in the UMCG between January 2018 and August 2019 to extract osteosynthesis devices after bone fracture treatment. Patients were operated upon clinical suspicion of FRI (i.e. redness, swelling, persistent wound-leakage, non-union, plate breakage; n = 8; Fig. S1) or for removal of osteosynthesis devices for mechanical reasons (n = 4; culture-negative controls; Fig. S2). The extracted osteosynthesis devices were treated with vanco-800CW and imaged macro- and microscopically as schematically represented in Figure 1.

![Figure 1. Schematic overview of the fluorescence imaging workflow.](Image)

Evaluation of the imaging results obtained with the osteosynthesis devices from all 12 patients included in the present proof-of-principle study with vanco-800CW allowed a clear distinction of infected and non-infected devices, as was independently verified by microbiological culturing. Moreover, no true false-positives or false-negatives were identified (Fig. S1, S2). This is illustrated with two clinical cases of bacteria-targeted fluorescent imaging with vanco-800CW. Importantly, these cases demonstrate the first clinical application of bacteria-targeted fluorescent imaging.
**Bacteria-targeted imaging in an infected non-union of a tibial fracture**

A first clinical example of successful bacteria-targeted fluorescence imaging with vanco-800CW in a clinical setting was obtained for a patient with tibial non-union accompanied with acute FRI (Fig. 2). In this case, a 51-year-old man suffered a lower leg fracture for which he was initially treated with a cast. After 7 months, barely any fracture healing of the tibia had occurred and therefore operative debridement of the non-union site, followed by a cancellous bone graft and tibial plate fixation was performed. The post-operative course was complicated by FRI, characterized by wound dehiscence and eventually an exposed implant (Fig. 2A, B). Therefore, the infected plate and screws had to be removed. The plate was surrounded by debris and minimal cloudy seroma (Fig. 2C).

Figures 2C and 2D respectively show the tibial plate *in situ*, upon macroscopic analysis after extraction and 15 min incubation with vanco-800CW. The latter revealed a clear fluorescence signal with a patchy distribution, while control osteosynthesis devices of a confirmed infection-negative patient emitted no fluorescence signals (Figures 2E-G; Figure S1, patient 11; Figure S2, patient 10). Moreover, a bacterial biofilm stained with vanco-BODIPYTM FL was clearly visualized by two-photon microscopy, demonstrating the high binding specificity of vancomycin-based tracers (Figure 2H, I; for Video, see supplementary data, Movie S1). Sonication of extracted biomaterials and subsequent microbiological culturing identified *Corynebacterium tuberculostearicum*, *Staphylococcus aureus* and *Staphylococcus epidermidis* as causative agents of the infection.
Figure 2. Bacteria-targeted imaging with fluorescently labeled vancomycin of a fracture-related lower leg implant infection. Images relate to patient 11 unless indicated otherwise. A. Pre-operative clinical presentation with wound dehiscence and an exposed plate of the lower leg. B. X-ray of the delayed union of a crural fracture with tibial plate fixation. C. Intra-operative image showing the infected tibial plate prior extraction. D. Macroscopic fluorescence image (FL IVIS) of the extracted plate upon staining with vanco-800CW. E. White light (WL) image of both infected (POS) and confirmed culture-negative (NEG) extracted screws. Note that the culture-negative screws were obtained from a different patient (patient 10; Figure S2), but imaged simultaneously with infected screws of patient 11. F,G. Fluorescence images of the screws
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Presented in E upon staining with vanco-800CW performed with two different imaging devices, respectively an IVIS Lumina II (F. PerkinElmer Inc., USA; Excitation 710 nm; Emission filter ICG, Exposure time 10 s) and an intraoperative camera (G. Explorer Air coupled to a closed-field imaging box [Vault], SurgVision B.V. Groningen, NL; Exposure times 100-200 ms). H,I. Microscopic image of a negative control (H) and infected screw (I) upon staining with vancomycin-BODIPY™ FL showing the biofilm dimensions. Microscopy was performed with a two-photon confocal laser scanning microscope (Zeiss LSM 7MP). Images were analyzed with ImageJ, Living Image 4.7.3 and Imaris 9.5.0 software.

Detection of low-grade FRI with vanco-800CW

The high sensitivity and specificity of bacteria-targeted optical imaging with vanco-800CW is supported by a second case, where a patient with a fracture-related low-grade infection of the femur presented no clinical symptoms of infection prior surgery (Fig. 3A). The X-ray showed a non-union of a femoral fracture with a broken plate (Figure 3B). During surgery, no signs of bacterial infection were observed (Fig. 3C, D, F). Nonetheless, clear fluorescence signals were detectable upon 15 min incubation of the extracted plate and screws with vanco-800CW (Fig. 3E, G, I). Moreover, the fluorescence signals were directly correlated to bacterial growth upon replica plating of the extracted screws on blood agar (Fig. 3H-J). Microbiological culturing revealed that osteosynthesis devices of this patient carried *Cutibacterium acnes*. 
Figure 3. Bacteria-targeted fluorescence imaging and corresponding microbiological culturing of a fracture-related femur infection. Images relate to patient 5. A. Pre-operative clinical presentation with no signs of infection at the lateral thigh. B. X-ray of a non-union of a femoral fracture with a broken plate. C. Intra-operative image showing no signs of infection (e.g. no fluid, abscesses or diseased bone around the broken implant). D-G. Macroscopic white-light (WL) images of the back- and front sides of the extracted plate (D,F) with corresponding fluorescence images (E,G. IVIS Lumina II; Excitation 710 nm; Emission filter ICG, Exposure time 10 s). H,I. WL image (H) and corresponding fluorescence image (I) of suspected infected (POS) and new negative control (CNTRL) screws (IVIS Lumina II; Excitation 710 nm; Emission filter ICG, Exposure time 10 s). J. Correlation between fluorescence images (I) with bacterial colonies on a blood agar plate (J). Images were analyzed with ImageJ and Living Image 4.7.3 software.
Discussion

The present clinical investigation shows that detection of bacterial growth on osteosynthesis devices by optical imaging with vanco-800CW is highly specific, sensitive, fast and feasible in the clinical setting. On the contrary, the current diagnosis of FRI relies heavily on culture-based methods that may take several days. Hence, patients with suspected FRI are often treated empirically with revision surgery and broad-spectrum antibiotics until the causative organism(s) have been identified. Here we show that imaging of potentially infected osteosynthesis devices with vanco-800CW reduces the time-to-result from days to less than 30 min. This implies that bacteria-targeted fluorescence imaging may significantly enhance intra-operative clinical decision-making regarding empirical antibiotic treatment, extensive debridement, the choice between one- and two-stage revision surgery, or implant exchange\(^\textsuperscript{12}\). A possible limitation of the fluorescence imaging approach with vanco-800CW is that it cannot replace current diagnostic procedures, because it does not identify the causative agents of FRI and the associated antibiotic resistances. Hence, we regard optical imaging with vanco-800CW as a complementary tool to the current diagnostic methods for prompt visualization of an infection. Ideally, a definitive diagnosis of the causative agent is needed to direct antibiotic treatment, which may be achieved in the future by the use of multiple tracers next to vanco-800CW, i.e. targeting Gram-negative bacteria, or specific bacterial species.

Based on the present results, we envisage that intra-operative topical application of vanco-800CW, incubation and rinsing off excess and unbound tracer during surgery may provide surgeons with real-time visual information on the presence and extent of infection. Here it is noteworthy that imaging can be performed with doses of vanco-800CW that are \(\sim\)20-40-fold below the minimal inhibitory concentration (MIC) of vancomycin for staphylococci (https://eucast.org/clinical_breakpoints/\(^\textsuperscript{8,10}\)). Thus, the results of routine diagnostic procedures and culturing will not be affected by the imaging procedure. Another clear advantage of vanco-800CW is its fluorescence in the near-infrared range, which minimizes interference by tissue autofluorescence\(^\textsuperscript{6,10,13,14}\). In our study, an IVIS imaging system and an intra-operative camera coupled to a closed-field imaging box were used to detect the emitted fluorescence signal. The latter camera system is already in use for surgical applications in the operating theater\(^\textsuperscript{15,16}\). Yet, prior to any intra-operative application of vanco-800CW, it will be necessary to assess its safety, although vancomycin\(^\textsuperscript{17}\) and the IRDye800CW fluorophore have separately been approved for clinical implementation\(^\textsuperscript{18,19}\). A second limitation of vanco-800CW is that it will probably not detect FRI caused by Gram-negative bacteria, because vancomycin shows the highest specificity for Gram-positive bacterial cell walls. However, Gram-negative bacteria cause \(\sim\)30% of all cases\(^\textsuperscript{20}\), which implies that vanco-800CW will allow a correct diagnosis of FRI in most cases, a view that is supported by the present results.
Altogether, we conclude that bacteria-targeted optical imaging with vanco-800CW provides new perspectives in the fight against FRI. This technique allows early, accurate and real-time diagnosis of FRI in the clinical setting, even in case of low-grade infections. Moreover, our findings suggest that bacteria-targeted imaging may serve as a highly sensitive tool for rapid detection of implant infections, ranging from osteosynthesis devices to surgical meshes, stents, prosthetic valves and vascular grafts.

Materials and Methods

**Fluorescence imaging of extracted osteosynthesis devices**

Osteosynthesis devices extracted from 12 trauma patients were included in this study. During surgery, the removed plates were divided in two by using nippers. One half of the plate was used for fluorescence imaging, the other half was used for regular diagnostics (described in the following section). A number of extracted screws, all retrieved from the same surgical site, were used for regular microbial diagnostics, and some for fluorescence imaging. Upon extraction, the osteosynthesis devices of 12 patients were washed with phosphate-buffered saline (PBS), and incubated with vancomycin-IRDye800CW (vanco-800CW; 0.14 nmol mL\(^{-1}\); Li-COR Biosciences, Nebraska, USA) for 15 min at 37°C. Subsequently, the incubated devices were washed twice with PBS to remove unbound vanco-800CW and imaged in the near-infrared range with an IVIS Lumina II imaging system (Emission filter ICG, Excitation 710 nm, Exposure times 1-10 s; PerkinElmer Inc., USA) and an intra-operative Explorer Air camera coupled to a closed-field imaging box (Vault; Exposure times 100-200 ms; SurgVision B.V. Groningen, NL). Microscopic imaging was performed with a two-photon confocal laser scanning microscope (objective 5x/0.16, filters 500-550/575-610, wavelength 850 nm, Zeiss LSM 7MP) using vancomycin-BODIPY™ FL as a tracer (Thermo Fisher Scientific, USA). Of note, the use of a two-photon microscope was appropriate due to the shape and size of the materials tested (plates and screws) in order to have enough distance between the sample and objective. The fluorescence imaging workflow of extracted osteosynthesis devices is schematically represented in Figure 1.

**Sonication and cultivation of extracted osteosynthesis devices**

The presence of microorganisms on extracted osteosynthesis devices and collected tissues was independently investigated at the diagnostic microbiology laboratory of the University Medical Center Groningen (UMCG). Sonication of extracted osteosynthesis devices was performed as described by Trampuz et al.\(^{11}\), with subsequent culturing of the sonicate on aerobic blood agar, chocolate agar, anaerobic blood agar and in blood culture bottles (BD BACTEC™) as liquid culture. The decision whether osteosynthesis devices and/or tissues were infected was taken by the medical team, including a trauma surgeon, infectious disease specialist and a clinical microbiologist according to the standard protocol.
Data analysis

Macroscopic fluorescence images were analyzed using ImageJ (National Institutes of Health, Maryland, USA) and Living Image 4.7.3 (PerkinElmer Inc, USA) software. Microscopic images were analyzed using Imaris 9.5.0 software. Regions of interest (ROIs) were drawn around the stained osteosynthesis devices after which the fluorescence signal was quantified. The background signal was quantified by drawing ROIs in uncontaminated biomaterials. To determine target-to-background (T/B) ratios, ROIs were divided by the average background fluorescence.

Ethical approval

Only surplus osteosynthesis devices that were considered unnecessary for clinical diagnosis were used for the present bacteria-targeted imaging with vanco-800CW in accordance with the permission obtained from the Medical Ethical Review Board of the UMCG (permission number METc 2016/481). All experiments were performed with adherence to the guidelines of the Declaration of Helsinki and local regulations. Patient data were used pseudo-anonymously based on informed consent.

Patients gave informed consent, which included the use of pseudo-anonymized data and samples for the purpose of research and publication.

Transparency declaration

GMvD is consultant for OncoNano Medicine Inc. and CEO, founder and shareholder of the AxelaRx / TRACER BV group. The other authors have no conflicts of interest to disclose.

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Contributions

ML-A, MH, GMvD, JMVd, FFAIJ, and MVO conceived the study. JMVd, FFAIJ and MVO supplied materials. ML-A and MH performed the experiments. ML-A and KAS performed the microscopy. ML-A, MH, JMVd, FFAIJ and MVO wrote the manuscript. All authors reviewed and approved the manuscript.

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Supplementary materials
Figure S1. Bacteria-targeted fluorescence imaging of extracted osteosynthesis devices from patients with microbiologically confirmed fracture-related infections. Corresponding X-ray, pre-operative, intra-operative, white light (WL IVIS) and fluorescence (FL IVIS) images (IVIS Lumina II imaging system, PerkinElmer Inc., USA; Excitation 710 nm; Emission filter ICG, Exposure time 10 sec) of 8 patients and their extracted osteosynthesis devices with a microbiologically confirmed fracture-related infection (FRI) after surgery. The right column shows the raw images as can be viewed by the surgeon (Surgeon’s view). The presence of microorganisms on extracted osteosynthesis devices was investigated by culturing in the diagnostic microbiology laboratory of the University Medical Center Groningen, independently from the bacteria-targeted imaging with vanco-800CW. **Patient 1.** Diagnosis: FRI after intramedullary nailing of a cranial fracture. Clinical signs: local redness and swelling with elevated inflammation markers (C-reactive protein 347 mg/L). Surgery: removal of the nail, local debridement of the pretibial abscess and cavitary defect of the tibia. Culture: low growth density of *Streptococcus anginosus* (Gram-positive). Imaging: positive, however, weak signal due to a low bacterial density. **Patient 3.** Diagnosis: infected non-union of the distal tibia with plate breakage. Clinical signs: scar tissue on the medial side of the lower leg with some redness. Surgery: removal of the broken plate and screws, debridement and washout of the non-union site, temporary antibiotic cement spacer (Masquelet procedure) and application of an external fixator. Culture: *Enterobacter cloacae* (Gram-negative) and *Staphylococcus epidermidis* (Gram-positive) both in very low densities. Imaging: positive, however, weak signal due to low Gram-positive bacterial density. **Patient 4.** Diagnosis: FRI after multiple operations of a comminuted distal tibial fracture. Clinical signs: redness and fistula at the medial malleolus. Surgery: excision of the fistula, removal of osteosynthesis devices, local debridement and washout. Culture: *Acinetobacter radioresistens* (Gram-negative), *Citrobacter koseri* (Gram-negative), *Corynebacterium aurimucosum* (Gram-positive), *Corynebacterium jeikeium* (Gram-positive), *Dermabacter hominis* (Gram-positive), *Staphylococcus haemolyticus* (Gram-positive). Imaging: positive. **Patient 5.** Diagnosis: infected non-union of the left femur with a plate breakage. Clinical signs: no clinical signs of infection at the lateral thigh. Surgery: removal of the osteosynthesis devices, debridement of the non-union site and reosteosynthesis with an intramedullary nail. Culture: *Cutibacterium (Propionibacterium) acnes* (Gram-positive). Imaging: positive. Note that these data are also shown in Figure 3 of the main manuscript. **Patient 7.** Diagnosis: FRI after osteosynthesis of a Gustillo grade 3 complicated ankle fracture. Clinical signs: pain, swelling, redness, septic arthritis, elevated inflammation markers (CRP 280 mg/L). Surgery: removal of the osteosynthesis devices, washout of the ankle joint, reosteosynthesis of the medial malleolus. Culture: *Staphylococcus aureus* (Gram-positive). Imaging: positive. **Patient 8.** Diagnosis: FRI after osteosynthesis of a distal tibial fracture. Clinical signs: wound dehiscence with partially exposed plate. Surgery: removal of ventral plate and washout of the wound. Culture: *Enterococcus faecalis* (Gram-positive) and *Staphylococcus aureus* (Gram-positive). Imaging: positive. **Patient 11.** Diagnosis: infected non-union of a cranial fracture. Clinical signs: wound dehiscence and exposed implant. Surgery: removal of infected osteosynthesis devices and a washout of the non-union site. Culture: *Staphylococcus aureus* (Gram-positive). Imaging: positive. Note that these data are also presented in Figure 2 of the main manuscript. **Patient 12.** Diagnosis: infected non-union of the distal tibia. Clinical signs: local redness, swelling around the scar on the medial malleolus. Surgery: removal of plates and screws, debridement of the non-union, temporary antibiotic cement spacer (Masquelet procedure), soft tissue coverage with a latissimus dorsi flap. Culture: *Enterococcus faecalis* (Gram-positive), *Staphylococcus capitis* (Gram-positive) and *Staphylococcus epidermidis* (Gram-positive). Imaging: positive. Images were analyzed with ImageJ and Living Image 4.7.3 software.
Figure S2. Bacteria-targeted fluorescence imaging of four confirmed culture-negative osteosynthesis devices. Corresponding X-ray, white light (WL IVIS) and fluorescence (FL IVIS) images (IVIS Lumina II imaging system, PerkinElmer Inc., USA; Excitation 710 nm; Emission filter ICG, Exposure time 10 sec) of four patients without detectable implant infections. The absence of microorganisms on extracted osteosynthesis devices was concluded based on culturing in the diagnostic microbiology laboratory of our hospital, independently from the bacteria-targeted imaging with vanco-800CW. None of the patients showed clinical symptoms of inflammation and all imaging results of extracted osteosynthesis devices incubated with vanco-800CW were negative. **Patient 2.** Extracted lumbo-pelvic fixation device due to lower back pain. **Patient 6.** Removed endcap of a femur nail during exchange nailing for a non-union of a femoral fracture. **Patient 9.** Routinely removed K-wires of the right wrist after treatment of carpal injuries. **Patient 10.** Routinely removed lag screw after open reduction internal fixation of a Weber C ankle fracture. Images were analyzed with ImageJ and Living Image 4.7.3 software.

**Movie S1. Microscopic fluorescence imaging of an extracted screw from patient 5 with microbiologically confirmed fracture-related infection.** The supplemental movie visualizes a bacterial biofilm stained with vanco-BODIPY™ FL by two-photon microscopy, demonstrating the high binding specificity of vancomycin-based tracers (see also Figure 2H, I).

https://unishare.nl/index.php/s/AdDwswiF2cNzwPb
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