Beyond the Colony-Forming-Unit: Rapid Bacterial Evaluation in Osteomyelitis

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Abstract: Examination of bacteria/host cell interactions is important for understanding the aetiology of many infectious diseases. The colony-forming-unit (CFU) has been the standard for quantifying bacterial burden for the past century, however, this suffers from low sensitivity and is dependent on bacterial culturability *in vitro*. Our data demonstrate the discrepancy between the CFU and bacterial genome copy number in an osteomyelitis-relevant co-culture system and we confirm diagnosis and quantify bacterial load in clinical bone specimens. This study provides insight into improving the quantification of bacterial burden in such cases.

Co-culturing mammalian host cells with bacteria *in vitro* is an important tool for examining the host-pathogen interaction in infectious disease modelling [1]. One of the essential readouts is the intracellular bacterial burden carried by the infected host cells. The most commonly used method for enumerating such bacterial load is counting the colony-forming-unit (CFU) number by agar plate culturing following the lysis of host cells and release of bacteria. However, the reliability of such measurements is impacted by various factors, including, but not limited to, host cell/tissue type, bacterial strain, bacterial load, and culturing conditions for CFU enumeration [2, 3]. Osteomyelitis (OM), an infectious disease with pathogen-mediated infection in bone tissues, features a considerable proportion of infected but culture-negative cases, ranging between 20-40% [4-6]. This clear deficiency in CFU evaluation create difficulties for the accurate diagnosis and therefore treatment of OM.

Staphylococci, including coagulase-negative species, such as *S. epidermidis,* and *S. aureus*, comprise the major pathogens in adult OM [7]. In this study, we employed a previously established osteocyte-like cell model, differentiated SaOS2 (SaOS2-OY) and the single most common causative pathogen in OM, *S. aureus*, to simulate the *in vitro* infection of osteocytes, the most abundant cell type in bone tissue [8, 9]. Two previously characterised *S. aureus* strains, a high virulence strain, WCH-SK2 (SK2), shown previously to establish an intracellular infection in human osteocytes [10] and a low virulence strain, WCH-SK3 (SK3) [11], were chosen for experimentation. In addition to CFU enumeration to quantify bacterial number, a polymerase chain reaction (PCR) approach measuring genomic DNA copies represented by a single copy gene within the *S. aureus* genome, was also performed in parallel. In the past, real time quantitative PCR (qPCR) assays have been widely used for the quantification of bacteria. However, the working principle of standard qPCR is dependent on the establishment of a

standard curve using a known number of bacterial gene copies and the fitting of test samples into a linear regression to estimate the bacterial number for each assay [12, 13]. Here, we utilised digital droplet PCR (ddPCR) for this measurement. By its nature, the latter technique offers the advantage of counting the absolute target sequence copy number within the sample and removes the need for normalisation [14]. For all PCR-based assays, the quality of DNA containing the target sequence for amplification and quantification is critical for the sensitivity and reproducibility of readout. Therefore, methods for DNA preparation from both human and bacterial cells were examined. Here, we introduced the usage of DirectPCR™ Lysis Reagent (Direct buffer), a lysis buffer that maximises the release of genomic DNA from samples and is compatible with the downstream PCR analysis from raw cell culture lysate without any purification step. With the application of Direct buffer for DNA preparations in comparison to a commonly used DNA extraction spin column kit (DNA kit), the absolute bacterial genome copy number quantified by ddPCR was measured to be 5-fold higher in SaOS2-OY samples and 100-fold higher in samples from two strains of *S. aureus*, SK2 and SK3; better overall reproducibility among biological replicates was also achieved using Direct buffer over the standard approach (**Fig. 1A-C**). Serial 10-fold dilutions of bacterial log-phase suspension cultures were used to generate DNA lysates for testing the extent of DNA release. Plotting genome copy number against CFU from the same cultures showed near perfect linear regression relationships (**Fig. 1D-E**), confirming the complete and highly reproducible release of bacterial DNA. With this validating readout, we sought to determine if the elimination of purification steps by using the Direct buffer might minimise the sample loss associated with the utilisation of conventional column-based extraction methods [15]. Further, the release of genomic material by this reagent appeared to be complete and unbiased in our experiments, which led to higher consistency of inter- and intra- assays.

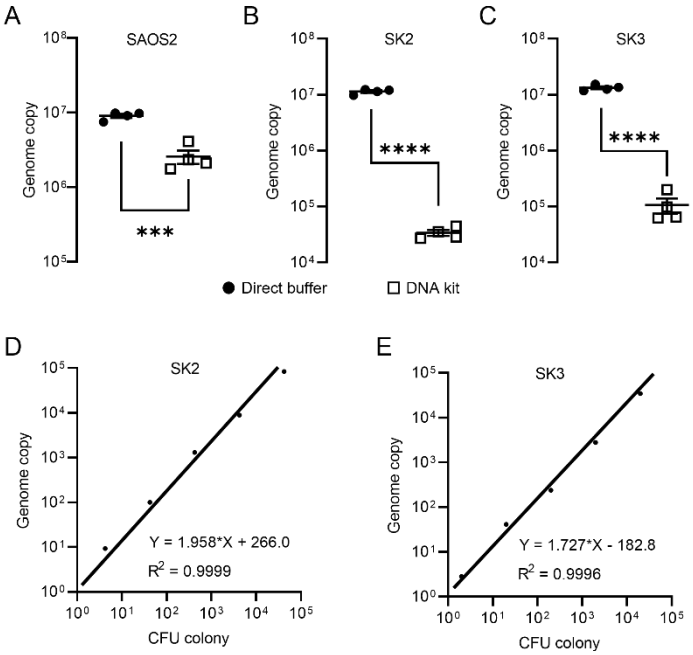

Fig. 1: ddPCR genome counting of SaOS2-OY (A), SK2 (B) and SK3 (C), comparing the Direct buffer approach (●) and a standard DNA kit (□) (4 biological replicates with mean and standard errors were shown on graphs, ***p < 0.001 and ****p < 0.0001); demonstration of complete bacterial chromatin release from SK2 (D) and SK3 (E): correlation with CFU plating. Each data point represented the comparison of one CFU recovery and one total DNA measurement; three independent experiments were carried out for each strain and similar results were achieved; representative results of one experiment are presented.

Using the combined approach of direct lysis DNA preparation and ddPCR quantification, we then examined the *S. aureus* persistence in the intracellular environment in infected SaOS2-OY cells. Following infection, the SaOS2-OY and *S. aureus* co-cultures were lysed to break down host cell structures for the release of intracellular *S. aureus* for CFU development on agar plates. Consistent with previous findings, significant reductions ranging between 10^3-fold and 10^6-fold in cultured CFU on agar plates over the 5-day co-culture time period were observed in both SK2 and SK3 infected SaOS2-OY groups (**Fig 2. A-F**). This trend was also found in low multiplicities of infection (MOI) (~10^7 total CFU/well, **Fig 2. C & E**) and high MOI (~10^8 total CFU/well, **Fig 2. D & F**) for initial bacteria-inoculated groups with either strain. However, when total DNA preparations from the whole culture lysates were taken for ddPCR quantification of bacterial genome copy number, differences in the dynamic changes of bacterial load were found. In the high virulence strain SK2-infected group, the genome copy numbers by 5 days post-infection were maintained at the levels of 10^7 and 10^8 cells/well for the low and high MOI groups, respectively (**Fig 2. C & D**), similar to the original input levels.

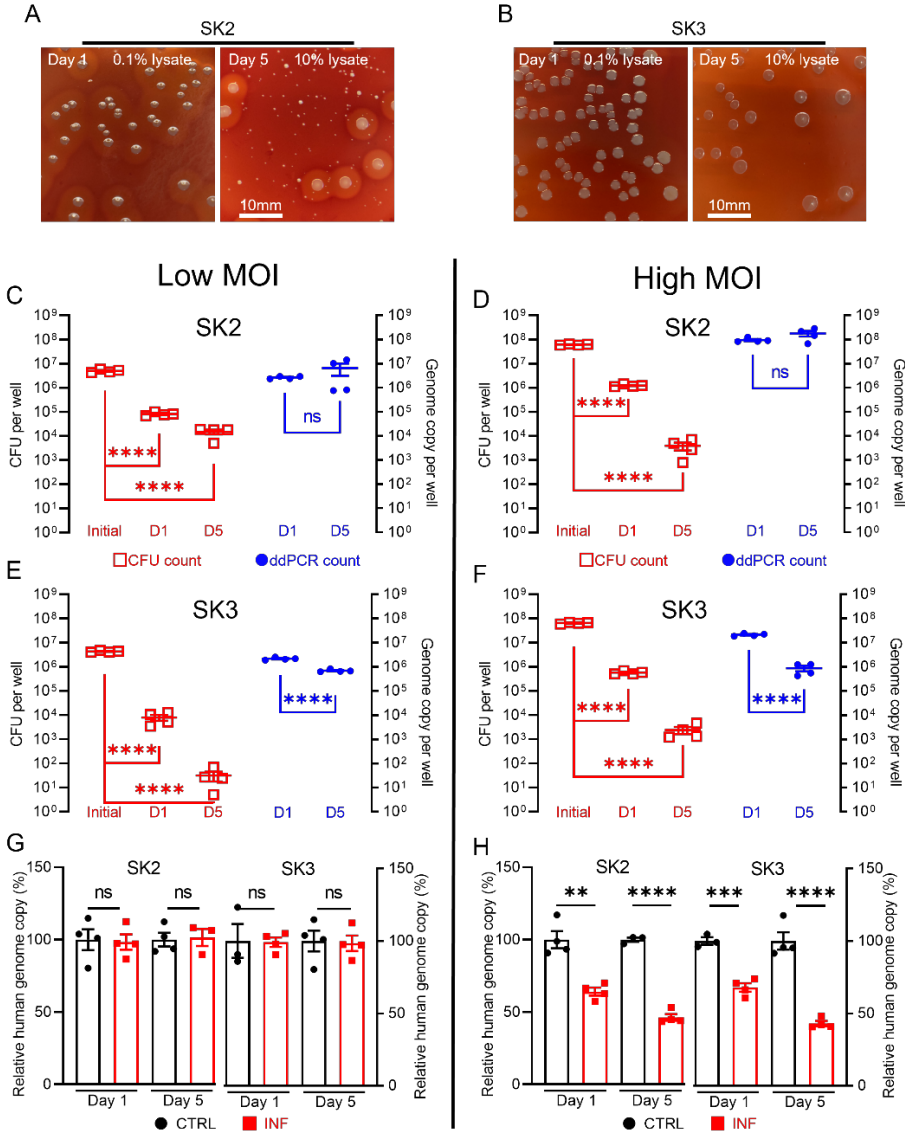

Fig. 2: CFU recovery of SK2 (A) and SK3 (B) from host SaOS2-OY cells; quantification of SK2 and SK3 using CFU count (□, left Y) and ddPCR count (●, right Y) from low (C & E) and high (D & F) MOI groups; relative human genome copy (%) with CTRL (●) *VS* INF (■) quantified by ddPCR from low (G) and high (H) MOI groups. (4 biological replicates with mean and standard errors are shown; ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; ns = not significant)

However, the low virulence strain SK3-infected group demonstrated a decrease in genome copy counts. Interestingly, regardless of initial input amount, by 5 days post-infection, the remaining SK3 genome counts dropped to approximately 10^6 cells/well for both groups (**Fig 2. E & F**). The quantification of human genome copy number was also performed in all groups to estimate the remaining host cells in culture and this also served as an indicator of host cell viability. With the low

85 MOI co-cultures, the human genome copy counts in the presence of either strain of *S. aureus* were
86 not statistically different from uninfected controls, across 1- and 5-days post-infection. When the
87 initial bacterial inoculum was increased 10-fold to 10^8 cells per well, reductions in the human genome
88 copies of ~30% and 50% were measured in 1- and 5- day post-infection groups, respectively; such a
89 trend was found to be similar in both SK2- and SK3-infected groups. Overall, our observations
90 demonstrated profound differences in the readout of the presence of intracellular bacteria when
91 using CFU and ddPCR quantification methods. Such inconsistency between these readouts in our
92 model was as high as 10^6-fold, with interaction of factors including initial bacterial load and strain
93 variation. In contrast, comparison of the same quantification methods performed on bacteria
94 suspension cultures yielded differences under 2-fold, indicated by the respective slope factor (less
95 than 2) in the linear regression curves for both SK2 and SK3 strains (see **Fig 1. D & E**). Together, our
96 results indicated the dramatic change in bacterial culturability when comparing growth in ideal
97 microbial suspension culture conditions and the growth limiting intracellular environment. Therefore,
98 we propose that the interaction between the host cell response to infection and pathogen adaptation
99 to the intracellular environment will compromise the reliability of the standard CFU counting method
100 alone for the evaluation of bacterial persistence in a bacteria/host cell co-culture experiment.
101 However, phenotypic variations were also observed in the recovered colonies from agar plating, with
102 variations in haemolytic activity and small colony variant (SCV) formation, particularly in the SK2 group
103 (**Fig. 2A**). SCV variants of *S. aureus* feature reduced metabolic activity and growth but are an indication
104 of adaptation and the establishment of a chronic infection. This is consistent with the findings of
105 reductions in CFU recovery but maintained levels of detectable genome copies, at least in the SK2
106 infected group.

107

108 To examine the applicability of this workflow to a clinical setting, we next extended the tests to clinical
109 human bone specimens. Three clinically culture-negative prosthetic joint infection (PJI) cases were
110 chosen for analysis. All three cases were confirmed infected by clinical observation for typical PJI
111 symptoms by clinicians, using the current gold-standard Musculoskeletal Infection Society (MSIS)
112 criteria [16]; neither the culturing of the synovial membrane biopsies nor synovial fluid around the
113 prosthesis returned any positive cultures from a clinical laboratory. The bone tissue sections from the
114 3 patients were examined by Masson's trichrome staining in comparison to tissue section from an
115 osteoarthritis (OA) patient as non-infection control, to examine the bone matrix degradation,
116 according to our previous study [17]. With this method, the red colour associates to intact bone matrix
117 collagen and blue colour to degraded collagen. Within all the histological observations, matrix
118 degradation was found in sections of all three PJI patients (**Fig. 3 B-D**), whereas the staining of the
119 control OA patient bone indicated intact collagen (**Fig. 3 A**), consistent with infection in the 3 PJI
120 patient specimens [17]. In addition to the gold-standard diagnostic approach and histological staining,
121 we performed CFU analysis using homogenised bone tissue, and negative culture results were also
122 recorded. In this study, the samples containing total genomic DNA were prepared from histological
123 sections in conjunction with the use of the DirectPCR™ Lysis reagent. The prepared DNA samples were
124 put through a PCR amplification using primer set targeting a highly conserved bacterial genomic
125 region, elongation factor Tu (*tuf*) [18], for the enrichment of bacterial signal within host/pathogen
126 genomic mixture. The generated amplicons were then sequenced using an Oxford Nanopore
127 Technology (ONT) Minion sequencer for the identification of bacterial species. All three patient bone
128 DNA samples were confirmed to be bacterial genomic positive, with combinations identified of the
129 coagulase-negative staphylococcal species *S. haemolyticus*, *S. hominis* and *S. epidermidis* (**Fig. 3 E-G**).
130 The respective bacterial load for each of the samples was then quantified by ddPCR. Each bone sample
131 examined contained between 2×10^4 and 1×10^6 bacterial genomic copies per million human genomic

132 copies (**Fig. 3 E-G**). To confirm that the detected bacteria species were not introduced during the
133 handling procedures, bone specimens from five primary total hip replacement (non-infected) patients
134 were processed at the same time along with the PJI specimens; PCR reactions with negative *tuf*
135 amplification for these bone samples indicated that operational contamination was unlikely
136 (**Supplementary Fig. 1**).

137

138 In this study, by the analysis of genomic material, we have addressed the discrepancy between the
139 ineffectiveness of the classical bacterial CFU development approach in determining a confirmed
140 infection by both an *in vitro* experimental model and by examining human clinical cases. With the
141 introduction of a direct DNA release approach, less handling is required while delivering increased
142 accuracy. The addition of ddPCR to this approach provided the advantage of achieving absolute
143 quantification without the need for a PCR standard curve, which is both cost and time effective. For
144 the purposes of unknown pathogen diagnosis in clinical cases, the exact bacterial species readout is
145 required from sequencing the generated amplicons. Here, the utilisation of Oxford Nanopore
146 technology dramatically lowered the equipment and skill demands for performing the analysis, since
147 the MinION sequencer is a portable device and the sequencing results are analysed by the on-board
148 software with little bioinformatics requirement. Together, this workflow is potentially applicable as a
149 point-of-care diagnostic method plus the prospect of a rapid turnover: within hours comparing to the
150 current procedure in the magnitude of days.

151

152 To conclude, for the purpose of quantifying the intracellular bacterial load in a bacteria/host co-
153 culture setting, we have developed a workflow with the advantages of: a) rapid and labour-saving with
154 the elimination all the DNA extraction steps by virtue of using the direct lysis approach; b)
155 minimisation of potential sample loss encountered with conventional DNA isolation methods,
156 enabling better accuracy and reproducibility; c) absolute number quantification by ddPCR for
157 achieving superior consistency of intra-assay experiments; d) further sequencing analysis by ONT
158 MinION sequencer has the potential to become a point-of-care diagnostic approach when performing
159 unknown pathogen identification in a clinical setting. Nevertheless, we suggest that the CFU plating
160 method should not be ignored for the evaluation of bacterial phenotypic adaptation in such
161 experimentation.

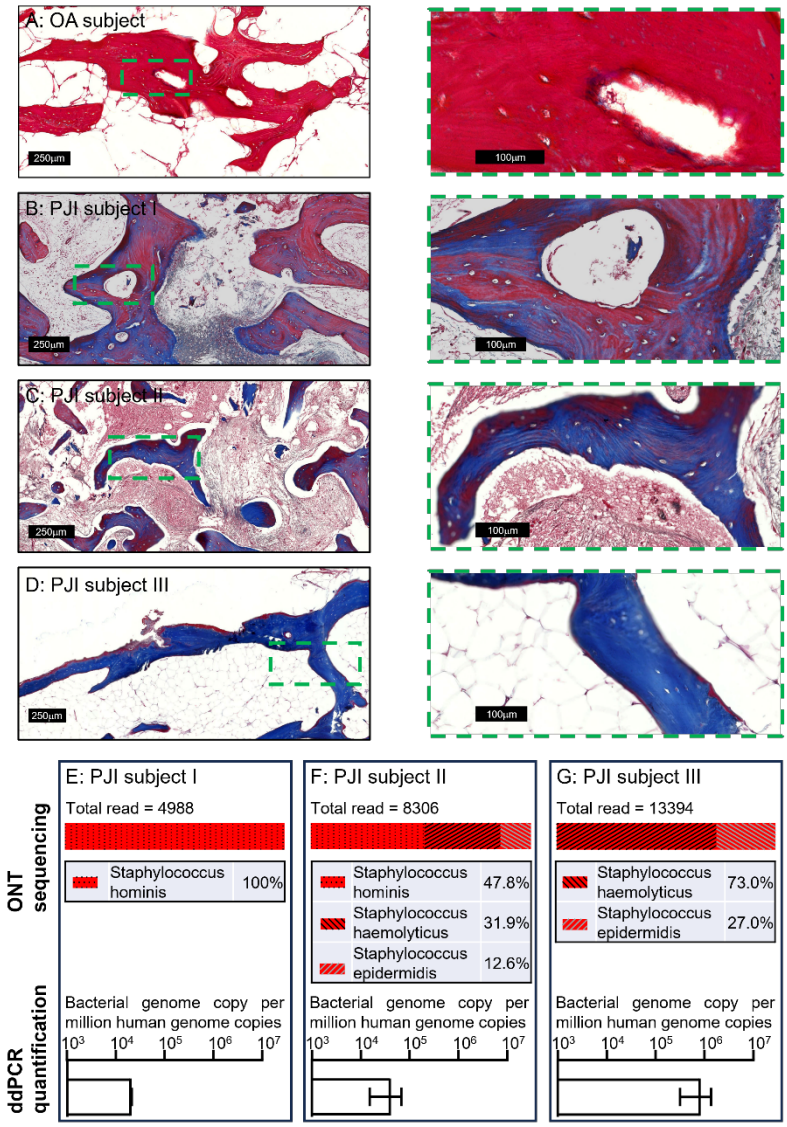

Fig. 3: Masson's trichrome staining on bone tissue sections of osteoarthritis (OA) subject (A) and culture negative PJI subject I-III (B-D). Pathogen profiling using total DNA of bone specimens from the above 3 PJI patients (E-G) by the methods of ONT sequencing for the readout of exact persisting species and ddPCR to quantify bacterial load as a ratio of bacterial : human genomic copies (error bars shown are the combination of the standard error of the mean from two individual DNA preparations and the device generated error from each of the ddPCR runs, with over 15,000 droplets read per sample).

Materials and methods

SaOS2 cell culture and differentiation

The human osteosarcoma cell line SaOS2 was employed for performing the bacteria/host co-culture experiments, as previously described [9]. Cells were seeded at a density of 2 x 10^4 cells per well in 48-well multi-well plates and cultured at 37°C/5% CO_2, in growth media consisting of αMEM (Thermo-Fisher, VIC, Australia) supplemented with 10% v/v foetal calf serum (FCS) (Thermo-Fisher), 2mM L-Glutamine (Thermo-Fisher), 1U/ml penicillin/streptomycin (Thermo-Fisher). At 90% confluence, SaOS2 cultures were switched to differentiation media comprising αMEM supplemented with 5% v/v FCS, 100mM ascorbate 2-phosphate (Sigma-Aldrich, St Louis, USA), 1.8 mM potassium di-hydrogen phosphate (Sigma-Aldrich), 1mM HEPES (Thermo-Fisher), 2mM L-Glutamine and 1U/ml

182 penicillin/streptomycin. Cells were then maintained under differentiation conditions for 28 days to
183 achieve osteocyte-like phenotype (SaOS2-OY) [8].

184

185 *S. aureus* preparation

186 Two pre-characterised *S. aureus* strains, a methicillin-resistant *S. aureus* (MRSA), WCH-SK2 (SK2) [11]
187 and a methicillin-sensitive *S. aureus* (MSSA), WCH-SK3 (SK3), were grown in terrific broth (Thermo-
188 Fisher) on a 37°C /200 rpm rocking platform to achieve log-phase suspension cultures individually.
189 Bacteria were pelleted by 3000g for 5 min centrifugation and then resuspended in sterile phosphate-
190 buffered saline (PBS) to estimate cell number by the optical density (OD) at 600nm light absorption.
191 The two bacteria suspensions were then taken to undergo 10-fold serial dilutions and the diluents
192 were individually plated on blood agar plates containing 10% v/v defibrinated sheep blood (Thermo-
193 Fisher) to determine the colony-forming-unit (CFU) number from original bacterial suspension
194 preparations. The calculated CFU numbers from agar plates were compared to the ddPCR quantified
195 genome copy numbers.

196

197 Host cell infection by co-culturing of SaOS2-OY and *S. aureus*

198 For host cell infection, bacteria suspension cultures were reconstructed in sterile PBS as described
199 above to achieve the density for low and high multiplicities of infection (MOI). The differentiation
200 media for SaOS2-OY was removed from cells and the cells were washed with PBS twice. The resulting
201 bacterial inoculums were added to SaOS2-OY cells to allow invasion for 1 hour at 37°C/5% CO_2. Post
202 infection, SaOS2-OY cells were washed twice with PBS and incubated at 37°C/5% CO_2 with 20 μg/ml
203 lysostaphin (Sigma-Aldrich) in antibiotic-free media for 24h to eliminate extracellular bacteria before
204 supplied with normal differentiation media. Supernatants were cultured on an agar plate to verify that
205 extracellular bacteria were absent at 24 hours and 120 hours post-infection.

206

207 Measurements of intracellular bacterial number by CFU counting

208 The intracellular bacteria numbers were estimated at 24-hour and 120-hour post-infection time points
209 by spreading cell lysates on blood agar plates. For optimal CFU reading, cell lysates were serially
210 diluted and individually plated on blood agar plates for 37°C /5% CO_2 incubation. CFU numbers were
211 recorded after 48 hours of colony development.

212

213 DNA extraction from host cells and bacteria

214 For the quantification of the genome, the total DNA was isolated using either DNeasy Blood & Tissue
215 Kits (Qiagen Inc., VIC, Australia), or DirectPCR™ Lysis Reagent (Direct buffer) (Viagen Biotech Inc., CA,
216 USA), as per the manufacturer's instructions. For this new approach using the Direct buffer, 500 μl
217 buffer together with 200 μg/ml proteinase K (Thermo-Fisher) was used for the lysis of one SaOS2-OY
218 sample in one well from 48-well tissue culture plate or one pelleted bacterial sample prepared from
219 the serial dilutions from suspension culture. Individual cell lysates were transferred to 1.5 ml tubes
220 and digested at 55°C for 35 min and then heat inactivated at 85°C for 15 min to terminate enzyme
221 digestion. The processed lysates were ready for PCR analyses.

222

223 Digital droplet polymerase chain reaction (ddPCR)

224 The ddPCR assay was performed using QX200 Digital Droplet PCR System (Bio-Rad Laboratories, USA)
225 (Bio-Rad Laboratories, CA, USA) as per manufacturer's instruction. Primer sets targeting a human
226 genome specific sequence within type X collogen (*COL10A1*) gene and a *S. aureus* genome specific
227 sequence within sigma factor B (*sigB)* gene, were used for the amplification of PCR products from DNA
228 samples from SaOS-OY and *S. aureus*, respectively. The sequences of primer sets are listed as below.

229 *COL10A1*: forward 5'-ccaccaggtcaagcagtcat-3', reverse 5'- gttggcactaacaagaggggt-3'

230 *sigB*: forward 5'-ggggcaacaagatgaccatt-3', reverse 5'-tgccgttctctgaagtcgtg-3'

231

232 Analysis of human bone tissues

233 All human studies received institutional research ethics approval (Royal Adelaide Hospital Human
234 Research Ethics Committee Approval No. 14466). Bone biopsies were collected from patients
235 undergoing either primary total hip replacement or revision surgery for PJI, with informed written
236 patient consent. Each bone specimen was separated into two parts. One of these was homogenised
237 in a bead-beating tissue homogeniser (Bead Ruptor Elite, Omni International, Kennesaw, GA, USA),
238 with 2 cycles of beating at 3m/s for 30s to disassociate the bone structure for the release of potential
239 viable bacteria. Bone homogenates were then sent to a clinical pathology laboratory (SA Pathology,
240 Adelaide, Australia) for CFU analysis. Clinical soft tissue and fluid samples were sent to SA Pathology
241 directly from surgery for pathogen analysis. The second piece of bone biopsy was processed and de-
242 mineralised using OSTEOSOFT® (Sigma-Aldrich) solution for standard paraffin embedding procedure.
243 Post-embedded, the bone tissues were sectioned under DNase/RNase-free conditions. One 5μm
244 section from each bone specimen was used for Masson's Trichrome staining, as described in our
245 previous study [17] and six sections were pooled together in DirectPCR™ Lysis Reagent for the isolation
246 of total genomic DNA, as described above. Prepared DNA samples were then subjected to 1)
247 conventional PCR amplification for an ONT sequencing readout (MinION sequencer, Oxford Nanopore
248 Technology, Oxford, UK), according to the manufacturer's instructions; 2) ddPCR for the absolute
249 quantification for both human and bacterial genome copy number. The primer sets targeting the
250 human *COL10A1* genomic sequence, as listed above, and broad bacterial *tuf* sequence (forward 5'-
251 ttctcaatcactggtcgtgg-3', reverse 5'-ggagtatgacgtccaccttc-3') were used for measuring human and
252 bacterial genome copies, respectively. The output sequencing data were analysed by Epi2ME software
253 within the WIMP workflow (Oxford Nanopore Technologies) for bacterial taxonomical analysis.

254

255 Statistics

256 The results were graphed as means ± standard errors of the means (SEMs). The significance between
257 two treatment groups was evaluated using two-tailed T-test by GraphPad Prism 9.5.1 (GraphPad
258 Software, MA, USA), where applicable.

259

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Author contributions

D.Y., G.J.A. and L.B.S. conceived the study. D.Y. adapted key protocols and developed the workflow; Q.S. performed most of the experimental work; K.H. contributed to molecular analysis and D.M. contributed to histological analysis; N.J.G and A.R.Z validated the methods developed in this work; Q.S. and D.Y. drafted the manuscript; G.J.A and D.Y provided scientific insight and supervised the study; L.B.S. consented patients and performed biopsy retrieval; all authors contributed to manuscript preparation and data presentation and agreed on the submitted version of the manuscript.

Competing interest

The authors declare no competing interests.

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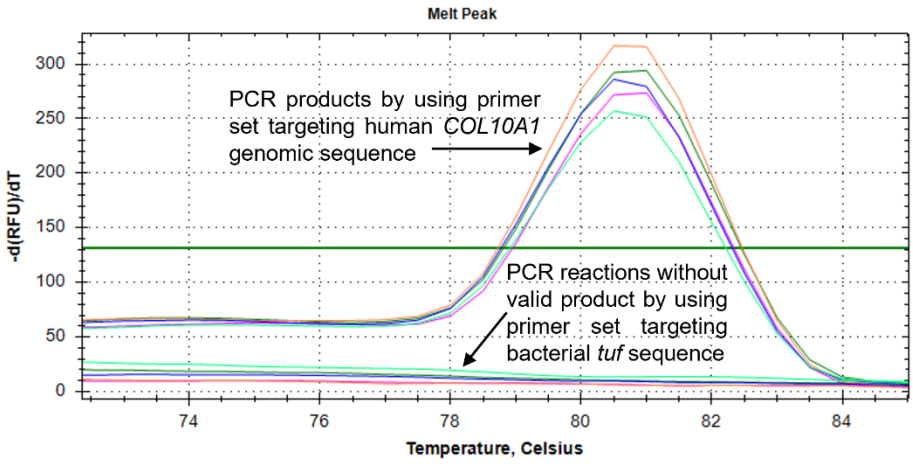

Supplementary figure 1: PCR analysis of bone samples from primary total hip replacement cases. DNA isolated from 5 patient bone samples were analysed by PCR for the presence of human *COL10A1* and bacterial *tuf*. The negative presence of bacterial *tuf* PCR product was confirmed by melt analysis post-quantitative PCR reactions using the DNA samples of five primary total hip replacement (non-infected) patients (coded with five different colours).