**Bacterial deoxyribonucleic acid (DNA) is often present in failed revision anterior cruciate ligament (ACL) reconstructions**

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**Introduction**: Failure following primary anterior cruciate ligament reconstruction (ACLR) is multifactorial. Colonization of tendon grafts with low virulence bacteria could cause graft tissue attenuation without overt clinical symptoms and predispose patients to ACL graft failure. Polymerase chain reaction (PCR) is a sensitive method for detecting bacterial DNA in very low concentrations. We hypothesize that bacterial DNA will be detectable via PCR in torn graft tissue at time of revision ACLR at higher rates than in primary ACLR graft tissue.

**Methods**: A total of 31 consecutive revision ACLR cases and 5 primary ACLR controls from one center were included. No patients had clinical signs of infection on presentation. A torn graft tissue sample was obtained with sterile instruments (not used earlier in the procedure) in revision cases and from excess hamstring graft in primary cases. A PCR analysis was performed with a universal bacterial primer and microscopy was utilized to confirm presence of a biofilm.

**Results**: Bacterial DNA was detectable in torn graft tissue in most revision ACLR cases 27/31 (87.0%) and less commonly 1/5 (20%) in primary ACL autograft controls (p=0.002, Chi-square test); biofilm presence was confirmed with microscopy. Median bacterial DNA concentration in torn grafts at time of revision ACLR was low overall at 17.5 ng/sample (range 0-101). A trend toward higher bacterial DNA concentrations was observed with prior autograft (median 19 ng/sample range 0-101) vs. allograft (median 13 ng/sample range 0-21; p=0.13, Wilcoxon rank sum).

**Conclusions**: Bacteria are often present in torn graft tissue at time of revision ACLR and at higher numbers than seen from similar graft tissue samples from primary ACLR’s. These findings suggest likely bacterial colonization of many failed ACLR grafts, though the causal relationship between graft colonization and failure remains unclear.

**Key words:** bacterial biofilm, revision ACL reconstruction, failed ACLR

**Short title:** Bacterial DNA in failed ACLR

**Introduction**:

Clinically significant postoperative infection related to anterior cruciate ligament reconstruction (ACLR) is a rare occurrence, with two recent studies showing rates between 0.49% and 1.7%.1, 19 Staphylococcus species are by far the most common causative organisms.19 Septic arthritis following ACLR commonly presents with a fever and modest local signs of infection as well significantly elevated inflammatory markers, particularly synovial white blood cell count. 19 Judd et al. report that clinically evidence intraarticular infections after ACLR presented with elevated serum ESR (mean 67) CRP (mean 14) and markedly elevated synovial WBC counts (mean 52,000).9

Failure following primary ACLR remains a significant problem. A 2003 meta-analysis reported rates of primary ACLR failure of 1.9% for patellar tendon grafts and 4.9% for hamstring grafts.5 Despite extensive study of graft failure, the mechanism of graft failure remains poorly understood. Traumatic loading, infection, and surgical error have all been suggested to play a role.10 Recent work by Hiller et al suggests there may be bacterial colonization of these ruptured grafts in the absence of clinical symptoms.7 Colonization of the ACL reconstruction graft or non-biodegradable materials used for fixation such as suture with low virulence bacteria could cause graft tissue attenuation without overt clinical symptoms and predispose patients to ACL graft failure.

While clinically apparent infection post-ACLR is rare, subclinical bacterial colonization of orthopaedic graft material have shown colonization rates as high as 23%,12 In other procedural fields, biofilms are noted to be frequently present in certain culture-negative soft tissue lesions; Bjarnsholt et al. demonstrate biofilms on 7/8 of culture negative long-lasting nodules after soft tissue filler injections. 2 Colonization and biofilm formation with low virulence organisms such as *P. acnes* is now a well-recognized entity in the shoulder literature. Millet et al. published a case series of patients with *P. acnes* post-operative shoulder infections with presented no clinical signs of infection other than pain.16 Hou et al. performed a case-control study of patients presenting for revision arthroplasty with positive versus negative *P. acnes* cultures, and the culture positive group was more likely to have glenoid sided loosening and a soft tissue membrane between the humeral component and endosteum. 8 In 2015, Hiller et al. demonstrated presence of bacteria in failed ACL grafts and found significantly different species specific markers when comparing failed ACLR grafts to control ACLs removed during arthroplasty. 7

Polymerase chain reaction (PCR) is a highly sensitive method for detecting bacterial DNA present in very low concentrations and detecting species that cannot be reliably cultured in a clinical laboratory. When investigating low virulence bacteria or bacteria that reside within a biofilm, this is a useful screening method for detecting bacterial DNA in settings that would otherwise likely be culture-negative. We hypothesize that bacterial DNA will be detectable via PCR in torn graft tissue at time of revision ACL reconstruction at higher rates than in primary ACL reconstruction graft tissue.

**Methods**:

*Recruitment:*

A total of 31 consecutive revision ACL reconstructions and 5 primary ACL reconstruction controls (all hamstring autograft) from one center were included (Table 1) Institutional review board approval was obtained prior to patient enrollment. Among revision cases, 23 (64%) had an autograft, 5 (14%) had an allograft, and 3 (9.3%) had an unknown graft placed during the prior ACL reconstruction (Table 1). Among revision cases, the time from the previous reconstruction to graft failure was a median of 5.4 years (range, 105 days-20.6 years). All patients were treated by three sports-medicine fellowship trained surgeons within a single academic medical center practice. No patients had clinical signs of infection as demonstrated by clinical exam. Additionally, no included patients had elevated serum inflammatory markers. Synovial aspirate was not routinely performed on first time revisions, but patients presenting for a second or third revision ACL reconstruction or those who had ever had a history of post-operative knee infection did undergo pre-operative aspiration and were all found to have normal white blood cell counts.

*Sample procurement and clinical testing:*

 For revision cases, tissue biopsies of the from the tendon graft were obtained from the femoral tunnel, intraarticular segment, and tibial tunnel. A set of instruments previously unused during the procedure were utilized for sample procurement. A portion of the sample from each of the three sites (tibial tunnel, intraarticular, and femoral tunnel) were sent to the clinical microbiology lab for routine aerobic, anaerobic, and fungal cultures as well as gram stain. The remaining portion was kept for later PCR analysis. For primary ACL reconstruction controls, a portion of the excess tendon graft was removed and transferred to a specimen container with instruments previously unused during the procedure. Clinical cultures were not obtained on primary ACL reconstructions.

*PCR analysis and microscopy:*

A PCR analysis was performed with a universal bacterial primer (16S rRNA gene) on all tissue samples using a previously described protocol.21 The forward primer 27F 5′-AGAGTTTGATCMTGGCTCAG-3′ as described by Lane et al.14 and 907R reverse primer 5′-CCGTCAATTCMTTTRAGTTT-3′ as described by Muyzer et al.17 were used. The PCR was performed in 25 μL reactions containing 50 nmol each of two primers and 23 μL of PCR supermix (Invitrogen). The reactions received 1 μL of DNA preparation as template. The PCR was performed with 30 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min followed by a hold sequence at 4°C. Aliquots taken from reactions at the final cycle were electrophoresed on 1.2% agarose gel at 20 volts/cm for 30 min and stained with 1 μg/mL ethidium bromide, added to the agarose.

All tendon graft specimens will be subjected to DNA purification using MoBio Laboratories Inc. PowerSoil**®** DNA Isolation Kit. To facilitate DNA extraction, the samples were pulverized per the kit recommendations. We were unable to obtain a dry weight of the graft tissue to standardize the amount of tissue utilized. Additionally, the physical characteristics of the torn graft tissue itself added substantial variability to the degree to which the sample could be pulverized by the PCR kit-recommended method. Therefore, reporting of the amount of bacterial DNA detected via PCR is limited to semi-quantitative reporting of nanograms per sample (rather than nanograms bacterial DNA per unit weight of tissue graft).

 To confirm the presence of bacteria in a biofilm state, a subset of revision ACLR tissue specimens were subjected to dual fluorescent staining with SYTO59 for nucleic acids and WGA-alexa488 for extracellular polysaccharide. Imaging was obtained for both soft tissue as well as recovered inert material (suture and fixation devices).

*Statistical analysis:*

Data was analyzed using a standard statistical software program (STATA 12.1, StataCorp, College Station, TX). Differences between demographic groups with respect to bacterial colonization rates were determined by student's t-test or Fisher’s exact test. Non-parametric testing (Wilcoxon rank-sum) was used as appropriate for variables with non-normal distribution. The associations between bacterial colonization and number of revisions, and time to failure of the previous reconstruction were assessed via ANOVA or Pearson correlation.

**Results**: *Presence of bacterial DNA*

Bacterial DNA was detectable in torn graft tissue in most revision ACL cases 27/31 (87.0%) and less commonly 1/5 (20%) in primary ACL autograft controls (p=0.002, Fisher’s exact test) (Table 2). One patient had positive aerobic bacterial cultures (coagulase negative staphylococcal species) and was treated with IV antibiotics per the recommendations of an infectious disease specialist. This patient did not have antecedent clinical symptoms of infection.

Microscopy confirmed the presence of bacterial biofilms on failed ACLR grafts. Biofilms were identified on both soft tissue portions of the specimens (Figure 1) as well as suture material (Figure 2).

*Association between bacterial DNA load, graft type, and time to failure*

Median bacterial DNA concentration in torn grafts at time of revision ACL was low at 18 ng/sample (range 0-101) (Table 2) with a trend toward higher concentrations found among revision patients with prior autograft (median 19 ng/sample range 0-101) vs. allograft (median 13 ng/sample range 0-21) used at time of the previous ACL reconstruction (p=0.13, Wilcoxon rank sum). There was no association between bacterial DNA concentration and time to failure (p=0.75, R-square=0.00) or number of prior ACL reconstructions (p=0.63, R-square=0.01).

**Discussion**:

The results of the current study confirm the hypothesis that bacterial colonization is often present on torn ACLR graft tissue and less commonly present on primary ACLR tendon autograft. The lack of antecedent clinical symptoms, delayed time to failure (median 5.4 years) and low clinical culture positivity rate indicate that these bacteria are primarily present in a biofilm state. The degree to which biofilm formation contributes to graft attenuation or loss of fixation following ACL reconstruction is unclear.

Non-adherent, planktonic (free-floating) bacteria tissue are the primary culprit in acute infections due to production of a large amount of virulence factors and resulting clinically significant infectious symptoms (Figure 3).22 Patients with deep infections after ACLR present with symptoms early after surgery and are usually culture positive19, consistent with infection due to planktonic bacteria.22 However, there is evidence that bacteria can switch phenotype early-on from a planktonic state to a biofilm state (Figure 3).22 If this occurs, bacteria in a biofilm state have far less interaction with surrounding host tissue and may remain undetected or years.3 The presence of bacterial biofilms within the surgical site is highly underestimated in orthopaedics due to reliance on clinical cultures as a primary diagnostic tool.4 Several studies of culture-negative surgeries for fracture nonunion or revision arthroplasty have shown high rates of bacterial DNA as well as direct visualization of bacteria with microscopy.6, 18, 20

The results of the current study as well as Hiller et al.7 indicate bacteria are frequently present in failed ACLR graft tissue in a biofilm state (Figure 3). It does not provide evidence for a causative link between bacterial colonization and graft failure after ACL reconstruction, though it does provide sufficient data to support further experiments on the topic. By establishing the presence of bacteria, there is justification for further work regarding characterization of any potential biofilms (where do they occur?) and DNA sequencing to determine whether the bacterial ‘community’ affects clinical behavior. Hiller et al. report that the bacterial species present in torn ACLR grafts are distinct from those found in ACL tissue at time of total knee arthroplasty,7 though the effect of these distinct communities on the surrounding tissue has yet to be determined. Of specific interest to ACL reconstruction is the effect of bacterial biofilms on adjacent bone within transosseous tunnels as well as the graft tissue itself (Figure 3). Tunnel widening commonly occurs after ACLR and has been associated with use of hamstring autograft9, 13 The mechanism of osteolysis and resulting tunnel widening around the tendon graft is not well understood, and no prior studies have investigated whether bacterial colonization is associated with tunnel widening. Alternatively, bacteria around the biofilm may have a direct effect on the tendon graft itself and result in decreased mechanical properties. Further research is needed to determine the degree to which biofilms on ACL grafts affect either tissue type.

There is some evidence of increased incidence of post-operative infection after ACL reconstruction performed with autograft vs. allograft. Katz et al report an incidence of clinically significant post-operative infection after ACL reconstruction of 1.2% after use of autograft and 0.6% after use of allograft.11 Maletis et al. further distinguished between types of autograft and found that hamstring autograft has an incidence of infection of 0.61% versus 0.07% for bone patella tendon bone (BPTB) autograft or 0.27% for allograft.15 In our study, we did observe a trend toward higher bacterial concentrations among cases in which autograft was utilized during the prior ACL reconstruction. Though not directly proven, the reduction of infection rates by Vertullo et al. after pre-soaking hamstring autograft in vancomycin solution23 suggests that higher infection rates with hamstring autograft may in part be due to higher bacterial loads on the graft at time of fixation.

There were several limitations to the current study. Due to variations in torn graft tissue structural properties and the inability to obtain an accurate dry weight of the sample, substantial variability was introduced in the initial tissue steps required for bacterial DNA extraction. This did not affect our ability to detect whether bacterial DNA was present though it did limit our ability to assess concentration of bacterial DNA (it was reported semi-quantitatively as nanograms per sample in this study rather than nanograms per unit volume). Our choice of control (primary ACL reconstruction cases) effectively controlled for environmental contamination in the operating room as a source of bacterial DNA. However, it does not control for the possibility of ubiquitous bacterial colonization of sites of previous soft tissue transosseous grafts about the knee. We cannot determine with the current study design whether bacterial DNA is equally as prevalent on intact ACL reconstruction grafts.

In conclusion, bacteria is often present in torn graft tissue at time of revision ACL reconstruction and at much higher rates than seen from similar graft tissue samples from primary ACL reconstructions. These findings suggest likely bacterial colonization of many failed ACL grafts though the causal relationship between graft colonization and failure remains unclear.

**Table 1.** Clinical data

|  |  |  |
| --- | --- | --- |
|  | **Revision cases (n=31)** | **Control cases (primary ACL) (n=5)** |
| **Age** | Mean 28.2 SD 11.9 | Mean 22.6 SD 4.6 |
| **Gender** | 18 (58%) Male13 (42%) Female | 4 (80%) Male1 (20%) Female |
| **Number of prior ACL reconstructions** | Failed primary ACLR: 24 (77%)Failed 1st revision ACLR: 6 (19%)Failed 2nd revision ACLR: 1 (3%) | N/A |
| **Graft used during previous ACLR** | Autograft: 23 (74%)Allograft: 5 (16%)Unknown: 3 (10%) | N/A  |
| **Time to failure** | Median 5.4 yearsRange 105 days-20.6 years | N/A |

**Table 2.**  PCR and clinical culture results

|  |  |  |  |
| --- | --- | --- | --- |
| **Patient number** | **Bacterial DNA concentration (ng/sample)** | **Detectable bacterial DNA**  | **Positive culture growth** |
| **Revision ACL patients** | Median 18 Range 0-101 | 27 (87%) Yes4 (13%) No | 1/31 (3%) |
| **Primary ACL controls** | Median 0, Range 0-14 | 1 (20%) Yes4 (80%) No | N/A (no cultures on controls) |



**Figure 1.** Fluorescent staining of a torn graft at time of revision ACLR. The red stain (SYTO59) represents nucleic acids and the green stain (WGA-alexa488) is for extracellular matrix. Scale bars are provided. **Left:** A portion of tendon graft (labeled host cells) and adjacent clusters of bacteria (white arrows). **Right:** A cluster of bacteria within a biofilm. The green polysaccharide around the red bacterial nucleic acid is a hallmark feature of a bacterial biofilm.



**Figure 2.** Fluorescent staining of suture with adherent bacterial biofilm recovered from the femoral tunnel at time of revision ACLR (scale bar provided). The red stain (SYTO59) represents nucleic acids and the green stain (WGA-alexa488) is for extracellular polysaccharide. The green polysaccharide around the red bacterial nucleic acid is a hallmark feature of a bacterial biofilm.



**Figure 3.** Conceptual diagram of the role of environmental bacterial contamination in ACL reconstructions. Deep infection following ACLR is rare (around 1%) and is caused by planktonic bacteria with early, clinically significant symptoms. An unknown proportion of ACLR grafts are colonized with environmental bacteria that switch early on to a biofilm phenotype and do not cause clinical symptoms. These bacterial biofilms have a subdued interaction with surrounding tissue and may: 1) increase risk of graft failure (through local osteolysis or decreased graft mechanical properties), or 2) have no appreciable effect on graft failure risk and be a clinically incidental finding.

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