



Bacterial Analysis of Hamstring Autograft With Vancomycin Presoaking in Primary Anterior Cruciate Ligament Reconstruction Shows High Incidence of Colonization Without Early Clinical Infection

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Purpose: To determine the incidence of autograft bacterial colonization (BC) in anterior cruciate ligament reconstruction (ACLR) and to identify any bacteria type at the genus level. **Methods:** This prospective descriptive study included patients aged 18 years or older undergoing ACLR with hamstring autograft. Thirty-one patients (21 male and 10 female patients), with a mean age of 32.2 years, were included, with a mean follow-up period of 150 days (range, 90-208 days). Three samples were taken from every surgical procedure: a sample from the graft at the time it was obtained, a sample from the graft after tibial fixation, and a sample from the saline solution receptacle on the instrument table (control). All grafts underwent vancomycin presoaking. Bacterial composition was determined by next-generation sequencing of the 16S ribosomal RNA coding gene. Descriptive statistics were used to summarize data. The Fisher exact test was used to compare group samples (with $P < .05$ considered significant). **Results:** BC was detected in 11 of 31 patients (35.4%). BC was found in the first sample (sample from the graft at the time it was obtained) in 1 patient and in the third sample (control) in another. All 11 patients showed BC in the second sample (sample from the graft after tibial fixation), with statistical significance ($P = .003$). In these samples with BC, 47 types of gram-negative bacteria (GNB) were identified, averaging 6.8 bacteria per sample, with 3.97% relative abundance per bacterium. *Pseudomonas* was present in 5 of 11 samples, with 6.76% relative abundance. Additionally, 33 types of gram-positive bacteria (GPB) were found, averaging 6 bacteria per sample, with 9.62% relative abundance per bacterium; *Streptococcus* was present in 8 of 11 samples, with 26.23% relative abundance. All colonized samples exhibited both GPB and GNB. **Conclusions:** Bacterial contamination during ACLR is frequent, with the presence of both GNB and GPB. Although such colonization was common, no early clinical infections were noted in our patient cohort. **Level of Evidence:** Level III, prospective descriptive study.

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Anterior cruciate ligament reconstruction (ACLR) surgery is a common procedure to restore knee stability and function after ligament injury, with an incidence of 43.5 per 100,000 person-years in the United States.¹ Although the incidence of postoperative infection is low, ranging from 0.37 to 2.0%,^{2,3} it remains a severe complication that can lead to prolonged hospitalization; loss of articular cartilage; and an increased risk of arthrofibrosis, instability, and revision.³⁻⁶ Various sources of bacterial contamination exist in ACLR surgery, including the patient's skin and mucosal flora, as well as environmental sources such as the surgical team in the operating room. Although standard prophylactic measures such as preoperative skin preparation, antibiotic prophylaxis, and sterile surgical techniques are routinely

applied to reduce the risk of infection, bacterial colonization (BC) of grafts remains a concern.^{7,8}

In 2012, Vertullo et al.⁹ introduced a presoaking technique using vancomycin to soak the graft prior to ACLR. This method effectively reduces the incidence of clinically evident infections during ACLR.⁹ Subsequent investigations have shown that this approach diminishes the frequency of postoperative superficial and deep infections, yielding highly cost-effective outcomes.^{10,11} Currently, this approach to preventing infection is widely used in ACLR.

Recent studies have used molecular techniques, such as polymerase chain reaction (PCR), to detect bacterial DNA in samples taken from patients undergoing ACLR surgery, revealing a higher prevalence of BC than previously recognized.^{12,13} Moreover, some studies have reported an association between the presence of bacterial DNA and clinical outcomes such as graft failure and tunnel widening in patients without clinical infection, highlighting the potential impact of BC on long-term surgical success.^{14,15}

Given the potential impact of bacterial contamination on ACLR outcomes, it is important to understand the sources, prevalence, and clinical implications of BC in this patient population. However, traditional culture-based methods for bacterial detection, long considered the gold standard, have several drawbacks that limit their utility in comprehensively assessing surgical site colonization. They are not suitable for all bacteria, frequently missing fastidious, slow growing or viable but non-culturable organisms often associated with biofilm formation. In contrast, next-generation sequencing (NGS), PCR, and immunoassay are advanced molecular techniques that overcome these limitations. These methods provide rapid, highly sensitive, and specific detection of bacterial DNA, regardless of bacterial viability or growth requirements, offering detailed information regarding the presence and abundance of a broader spectrum of bacterial species. For instance, NGS can identify diverse polymicrobial communities that may be missed by conventional culture, and it can be crucial in detecting pathogens in culture-negative infections. Nevertheless, it is important to acknowledge that molecular methods such as NGS detect bacterial DNA, which may include nonviable organisms or environmental contaminants, potentially overestimating the presence of active colonization. Furthermore, although highly sensitive, NGS can be susceptible to contamination from reagents or laboratory environments, necessitating rigorous controls for accurate interpretation.

The purposes of this study were to determine the incidence of autograft BC in ACLR and to identify any bacteria type at the genus level. We hypothesized that BC of the graft would be frequent in primary reconstruction procedures.

Methods

Study Design and Patient Selection

We conducted a descriptive study in a single center between 2019 and 2023. After receiving institutional review board approval, we included patients aged 18 years or older who underwent ACLR with hamstring autograft, performed by 7 different knee surgeons. To ensure a focused analysis on BC during a standardized ACLR procedure and to control for potential variations in surgical time and contamination exposure, we established specific exclusion criteria for combined procedures. Patients receiving an allograft or autograft different from hamstring graft (e.g., bone–patellar tendon–bone) were excluded. Furthermore, any combined intra- or extra-articular procedures that were expected to significantly prolong surgical duration, alter the primary ACLR operative field, or introduce additional potential sources of contamination were excluded. This specifically included procedures such as extra-articular tenodesis, osteotomy, and complex multiligament repair. Conversely, common concomitant procedures often associated with anterior cruciate ligament (ACL) injury, such as meniscal repair or meniscectomy and treatment of minor chondral defects, were included. A total of 31 patients provided informed consent to participate in the study (Fig 1). The age range of participants was 19 to 49 years, with a mean age of 32.2 years. Among the 31 subjects, there were 21 male and 10 female patients. The mean surgery time was 93.32 minutes (range, 65–135 minutes), and the mean tourniquet time was 83.32 minutes (range, 54–125 minutes).

The patients were followed up by the operating surgeon and underwent monthly follow-up examinations until they were deemed fit to resume their occupational duties, with a mean duration of 150 days (range, 90–208 days). Medical consultations were continued until 180 days after intervention.

During the follow-up period, the primary clinical outcomes assessed were clinical infection, arthrofibrosis, and ACLR failure. Clinical infection was monitored through physical examination for signs such as persistent pain, erythema, swelling, warmth, purulent drainage, and fever. Arthrofibrosis was assessed by physical examination for limitations in knee range of motion. ACLR failure was defined as anterior knee instability on physical examination, confirmed by magnetic resonance imaging suggesting graft failure or requiring revision surgery.

Surgical Procedure

Before surgery, prophylactic antibiotics were administered to each patient. The standard dose was 1 g of cefazolin administered 30 minutes before the procedure. Prior to surgery, the patient's skin was prepared

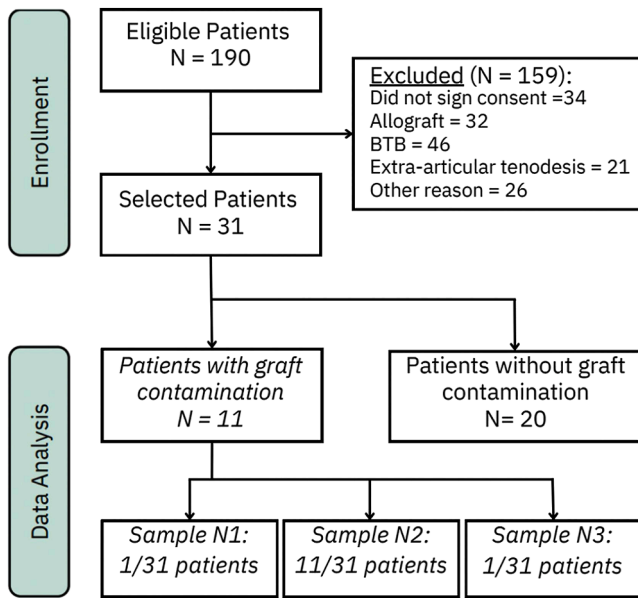


Fig 1. Flowchart of patient selection. All patients undergoing anterior cruciate ligament reconstruction with autograft were included. Of 190 patients, 31 were included in the study. Of these 31 patients, 11 had graft contamination in their samples. (BTB, bone-tendon-bone surgical technique; N1, sample obtained during harvest; N2, sample obtained after tibial fixation; N3, control sample.)

according to a standard protocol involving the sequential application of 2% chlorhexidine gluconate in both a soapy solution and an alcohol-based solution (Difem Pharma, Santiago, Chile).

A longitudinal incision (2–3 cm) was made over the pes anserinus insertion at the proximal medial tibia. The sartorius fascia was opened longitudinally, and the gracilis and semitendinosus tendons were identified and individually harvested using a tendon stripper. Care was taken to ensure full-length harvest of both tendons. The harvested hamstrings were taken to the back table. Muscle was removed with a blunt dissector and prepared by applying running sutures to both ends with nonabsorbable, high-strength, braided composite suture (FiberWire; Arthrex, Naples, FL). Once prepared, the graft was secured to an adjustable button (TightRope RT; Arthrex). Subsequently, the graft was soaked in a vancomycin solution at a concentration of 5 mg/mL and wrapped in sterile gauze saturated with vancomycin solution.

Sample Collection and Processing

During the surgical procedure, 3 samples were obtained from each patient at distinct time points: a segment of each tendon immediately after graft harvesting from the donor site (N1), a segment of each tendon subsequent to tibial fixation once the remnant was excised from the bone surface (N2), and a sample from a saline solution receptacle on the instrument

table that functioned as an environmental contamination control (N3). Samples were collected using 2-mL deoxyribonuclease- and ribonuclease-free cryovials and stored at -80°C .

Amplicon Sequencing and Bioinformatics Analysis

DNA extraction was performed in 100-mg tendon samples and 500 μL of saline solution using the Power Soil extraction kit (MoBio Laboratories, Carlsbad, CA) according to the manufacturer's instructions. Total isolated DNA was analyzed by agarose gel electrophoresis (1% wt/vol). Electrophoresis was performed at 80 V in $1\times$ TAE running buffer. The DNA concentration in each sample was measured by fluorometry (Quanti-Fluor, Promega, Madison, WI) with the DNAs Quant-iT PicoGreen Kit (P7589; Invitrogen, Waltham, MA) according to the manufacturer's instructions.

Amplification of the hypervariable regions of the V3–V4 16S ribosomal RNA (rRNA) gene was performed using the 357F and 806R primer pairs. PCRs were performed in Taq buffer, $1\times$ final concentration; 2-nmol/L magnesium chloride; 0.3-nmol/L deoxynucleotide triphosphates; 0.3 $\mu\text{mol/L}$ of each primer; 2.5 U of GoTaq Flexi DNA Polymerase (Promega); and 4 ng of template DNA. The amplification conditions were as follows: initial denaturation at 94°C for 3 minutes; 28 cycles at 94°C for 30 seconds, 57°C for 1 minute, and 72°C for 1.5 minutes; and final extension at 72°C for 10 minutes. Illumina primer constructs (San Diego, CA) were obtained from the Earth Microbiome Project. Combined amplicons were quantified by a standard quantitative PCR assay using the Illumina Library Quantification Kit (Kapa, Wilmington, MA) according to the manufacturer's instructions. The amplified library was analyzed on a Bioanalyzer 2100 system (Agilent Technologies, Palo Alto, CA) using a DNA 1000 chip (Agilent Technologies) according to the manufacturer's instructions. Samples were sequenced on the Illumina MiSeq platform, using a 600-cycle kit, generating paired-end sequences of 300 nucleotides long.

Amplicon sequences were processed using DADA2 (Divisive Amplicon Denoising Algorithm 2)¹⁶ to generate amplicon sequencing variants. Taxonomic assignment of the amplicon sequencing variants was performed against the Systematic identification of living organisms version 138 database.¹⁷ Data cleaning and visualization were performed using the phyloseq package in R.¹⁸

Detection of Bacterial DNA

Bacterial DNA was detected by PCR amplification of the 16S rRNA gene. DNA was extracted from each swab using a commercial DNA extraction kit (QIAamp DNA Mini Kit; Qiagen, Hilden, Germany) according to the manufacturer's instructions. PCR was performed

using universal 16S rRNA gene primers, and the amplified products were sequenced using Sanger sequencing (Macrogen, Seoul, Republic of Korea). The resulting sequences were compared with those from the GenBank database using BLASTn (Basic Local Alignment Search Tool for Nucleotides; NCBI, Bethesda, MD).

Data Analysis

Descriptive statistics were calculated using the R statistical package to summarize the data, focusing on the frequency and percentage of positive bacterial culture results, as well as the PCR results. The relative abundance of bacterial species was determined and expressed as a percentage. The Fisher exact test was implemented in R to detect statistically significant differences among sample groups, with a P value threshold of $<.05$ considered significant.

Results

Of the 31 patients studied, 11 (35.4%) showed evidence of BC. Most of these cases ($n = 9$) exhibited bacteria exclusively in the N2 sample ($P = .003$). One individual had bacterial presence in both the N1 and N2 samples, whereas one case showed BC in the N2 and N3 samples (Fig 1).

From the N2 samples with BC, 47 types of gram-negative bacteria (GNB) were identified, with a mean of 6.8 bacterial types per sample and a mean relative abundance of 3.97% per bacterium. Furthermore, 33 types of gram-positive bacteria (GPB) were observed, with a mean of 6 bacteria per sample and a mean relative abundance of 9.62% per bacterium. All colonized samples exhibited the presence of both GNB and GPB. Among the GNB, *Pseudomonas* was present in 5 of 11 samples, with a relative abundance of 17.38%. *Paracoccus* colonization was observed in 3 of 11 samples, with a relative abundance of 17.38%. *Photobacterium* showed the highest relative abundance (23.66%), being present in 2 of 11 samples. Among the GPB, *Streptococcus* was present in 8 of 11 samples, with a relative abundance of 26.23%. *Staphylococcus* was identified in 8 of 11 samples, with a relative abundance of 20.41%. *Lactobacillus* ranked third, being present in 6 of 11 samples, with a relative abundance of 12.89% (Fig 2).

Discussion

The most relevant finding in this study was the detection of bacterial material in ACL grafts in 35.4% of the cases, which confirms our hypothesis of frequent BC. It is important to note that this rate was observed despite the routine use of vancomycin presoaking for all grafts, and no patient experienced a clinical infection during the mean 150-day follow-up period. The detected bacteria with known pathogenic potential

included gram-negative organisms, such as *Stenotrophomonas*, *Acinetobacter*, and *Pseudomonas*, as well as gram-positive *Staphylococcus* and *Streptococcus*. Furthermore, colonization by dozens of other bacteria, whose pathogenic activity is less established, was identified.

Our detected colonization rate of 35.4% is consistent with some recent molecular-based studies that reported rates of bacterial DNA presence in ACL grafts ranging from 30% to 80%.^{12-14,19,20} This variation likely reflects differences in detection techniques and patient populations. Whereas vancomycin presoaking is well established for its effectiveness in reducing the incidence of clinical infections by targeting viable bacteria,⁹⁻¹¹ the use of NGS allows for the detection of bacterial DNA from both viable and nonviable (dead) organisms. NGS would still detect genetic material of bacteria killed by antibiotics or other prophylactic measures.

Recent publications suggest that bacterial contamination could lead to complications beyond acute infection—and could ultimately contribute to ACL graft failure. The use of allografts in ACLR has been associated with a higher risk of BC, as well as an increased risk of graft failure.^{19,20} The impact of bacterial contamination on graft biomechanics has been described by Sorensen et al.,²¹ who showed that *Staphylococcus epidermidis* contamination adversely affected allograft resistance. Furthermore, Flanigan et al.¹⁴ found a significant correlation between the presence of bacterial DNA and tunnel widening in failed ACLR, with 87% of revision cases presenting bacterial DNA in the graft. A recent study has analyzed intra-articular knee fluid and identified a significant association between ACL graft rupture and the presence of bacterial metabolites in patients without clinical infection.¹⁹ This growing body of evidence suggests that BC may contribute to graft failure through multiple mechanisms, including induction of an inflammatory response. However, the study's limited follow-up period precludes drawing conclusions on long-term outcomes such as arthrofibrosis, tunnel widening, and graft failure.

Given the potential risks associated with bacterial contamination in ACL surgery, it is important to develop and implement effective strategies to reduce the risk of graft colonization. One possible approach involves antibiotic presoaking of grafts, as explored by Vertullo et al.⁹ Subsequently, numerous researchers have examined the use of vancomycin presoaking, confirming its effectiveness in lowering infection rates after ACLR.²² Although the most frequent and abundant bacteria identified are gram positive (*Staphylococcus* and *Streptococcus*), the isolation of GNB such as *Stenotrophomonas*, *Acinetobacter*, and *Pseudomonas* and other bacteria not susceptible to vancomycin treatment raises concerns about the effectiveness of current

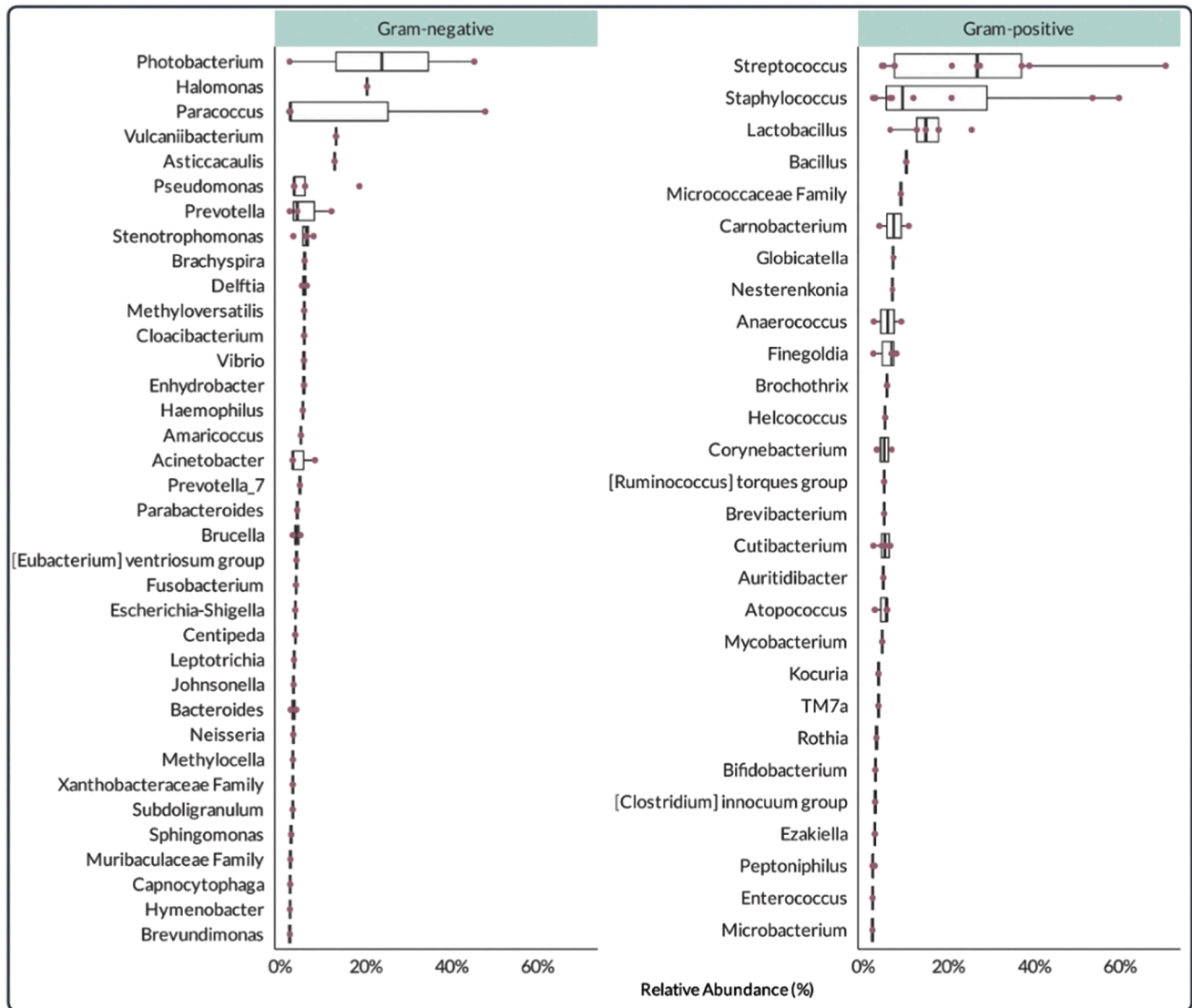


Fig 2. Relative abundance at genus level for samples obtained after tibial fixation. Boxplots show interquartile ranges (boxes), medians (horizontal lines), values within 1.5 x IQR (whiskers), and outliers (dots). The most abundant group in the gram-negative samples was *Photobacterium*. Overall, most of the organisms found to be prevalent in the samples (i.e., present in most of them) and in high abundance were gram positive, including *Streptococcus*, *Staphylococcus*, and *Lactobacillus*.

antibiotic prophylaxis protocols in eradicating the full spectrum of detected colonization. Our findings are consistent with those of previous studies that observed GNB, particularly *Pseudomonas aeruginosa* and *Acinetobacter*, in hamstring graft cultures.²⁰ The potential implementation of combined local antibiotic therapies, specifically those incorporating aminoglycosides, which have broad gram-negative coverage, merits exploration as a future strategy. However, such approaches would require rigorous clinical trials to ascertain their necessity and effectiveness in mitigating graft colonization risk and their impact on graft longevity, given the potential risks associated with expanded antibiotic use.

An alternative strategy involves the implementation of more aggressive systemic prophylactic antibiotic regimens, particularly in high-risk patients or cases with suspected BC. This topic has been investigated in the context of arthroplasty. For instance, Liu et al.²³ reported that dual antibiotic prophylaxis (vancomycin plus a second-generation cephalosporin) is associated with a significantly lower risk of infection in patients undergoing knee arthroplasty. However, other studies did not observe differences in the use of dual antibiotic prophylaxis in knee arthroplasty.²⁴ Even so, the use of systemic broad-spectrum antibiotics may also have adverse consequences, including the promotion of antibiotic-resistant bacterial strains

and disruption of the microbiome, which may negatively impact overall patient health.²⁵ Therefore, any modification to current prophylactic protocols must be supported by clinical evidence to avoid unintended consequences.

The findings of this study indicate that BC was primarily observed in the N2 samples (leftover graft after implantation and tibial fixation), with statistically significant differences from N1 (graft harvest) and N3 (control). This suggests that contamination likely occurs after graft manipulation, during later stages of the surgical procedure. To minimize contamination risks, surgical teams should consider reducing graft handling and exposure duration while also improving sterile field maintenance techniques, such as increasing the frequency of glove changes. Future research should also investigate specific bacterial load thresholds and their correlation with clinical symptoms or long-term complications and explore early indicators that might raise suspicion for subclinical infection, such as persistent unexplained inflammation, early signs of graft laxity, and atypical recovery patterns.

Limitations

Several limitations of this study should be considered. First, the limited sample size of 31 patients may have restricted the broader applicability of the findings. Second, the single-center design of the study may restrict the generalizability of its results to other health care centers with different surgical protocols, patient demographic characteristics, or environmental factors. Furthermore, our study exclusively included hamstring autografts, which may limit the generalizability of our findings to other graft types (e.g., bone–patellar tendon–bone graft or allograft). Third, the absence of a control group not receiving vancomycin presoaking precludes a direct assessment of vancomycin's independent effect on preventing infection. Additionally, whereas this research focused on BC during the surgical procedure, it did not explore clinical outcomes or complications associated with contamination beyond the mean 150-day follow-up period. Finally, the highly sensitive molecular detection methods applied may have identified nonviable bacteria or contaminants, potentially overestimating the true colonization rate. Although NGS offers superior sensitivity and a broader detection spectrum compared with conventional cultures, the absence of such a direct comparison means that we cannot directly correlate our findings with those of previous studies predominantly using cultures or provide a head-to-head assessment of detection rates between the two methodologies. However, it is worth noting that a control (N3 sample from the instrument table) was included, which produced positive bacterial detection in only 1 patient, providing some context to the level of environmental background contamination.

Conclusions

Bacterial contamination during ACLR is frequent, with the presence of both GNB and GPB. Although such colonization was common, no early clinical infections were noted in our patient cohort.

Disclosures

The authors declare the following financial interests/ personal relationships which may be considered as potential competing interests: M.S. reports that financial support was provided by Government of Chile Superintendency of Social Security (Grant ID P200-245). All other authors (R.B., R.A., J.U., M.J.T., G.E.) declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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