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A commentary by Dirk Jan F. Moojen, MD, PhD, is linked to the online version of this article at jbjs.org.

Providing an Evidence Base for Tissue Sampling and Culture Interpretation in Suspected Fracture-Related Infection

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Background: The recent consensus definition for the diagnosis of fracture-related infection (FRI) includes the identification of indistinguishable microorganisms in at least 2 surgical deep-tissue specimens as a confirmatory criterion. However, this cut-off, and the total number of specimens from a patient with suspected FRI that should be sent for microbiological testing, have not been validated. We endeavored to estimate the accuracy of different numbers of specimens and diagnostic cut-offs for microbiological testing of deep-tissue specimens in patients undergoing surgical treatment for possible FRI.

Methods: A total of 513 surgical procedures in 385 patients with suspected FRI were included. A minimum of 2 surgical deep-tissue specimens were submitted for microbiological testing; 5 or more specimens were analyzed in 345 procedures (67%). FRI was defined by the presence of any confirmatory criteria other than microbiology. Resampling was utilized to model the sensitivity and specificity of diagnostic cut-offs for the number of surgical specimens yielding indistinguishable microorganisms and for the total number of specimens. The likelihood of detecting all clinically relevant microorganisms was also assessed.

Results: A diagnostic cut-off of at least 2 of 5 specimens with indistinguishable microorganisms identified by culture was 68% sensitive (95% confidence interval [CI], 62% to 74%) and 87% specific (95% CI, 81% to 94%) for the diagnosis of FRI. Two out of 3 specimens were 60% sensitive (95% CI, 55% to 66%) and 92% specific (95% CI, 88% to 96%). Submitting only 3 deep-tissue specimens risked missing clinically relevant microorganisms in at least 1 in 10 cases.

Conclusions: The present study was the first to validate microbiological criteria for the diagnosis of FRI, supporting the current confirmatory diagnostic criteria for FRI. Analysis of at least 5 deep-tissue specimens in patients with possible FRI is recommended.

Level of Evidence: Diagnostic Level III. See Instructions for Authors for a complete description of levels of evidence.

Fracture-related infection (FRI) is a complication that occurs in up to one-third of complex open limb fractures[1,2]. Infection following fracture fixation is associated with marked impairment in quality of life, in excess of that conferred by fracture nonunion or amputation[3]. Measures of incidence have been limited by the lack, until recently, of an agreed-upon definition of FRI[1,4].

The clinical spectrum of FRI is varied. Some cases present only with fracture nonunion or painful fracture-fixation devices, without obvious local or systemic signs of infection. In other cases, sinuses, exposed metalwork and bone, and purulence confirm the presence of infection. The anatomical location of FRI, associated with differences in the soft-tissue envelope, perfusion, and approach for internal fixation, as well

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as bacterial quiescence, biofilm formation, and intracellular persistence, contribute to protean clinical signs[1,5].

Preoperative diagnosis of FRI is limited by the poor predictive value of superficial swabs, poor radiographic discrimination between normal fracture-healing and infection, and limited sensitivity of preoperative biopsy. Infection has a patchy microscopic and macroscopic distribution[5-9]. Even in patients with a low risk of FRI, positive microbiology can be found in >40% of femoral and tibial nonunions[10].

Recent consensus guidance recommends microbiological criteria for the diagnosis of FRI based on 5 separately harvested intraoperative tissue specimens. Positive microbiology is defined as identifying phenotypically indistinguishable microorganisms from at least 2 specimens. Other clinical confirmatory criteria for the diagnosis of FRI include a fistula, sinus or wound breakdown, purulent drainage or the presence of pus at the time of surgical debridement, and the presence of microorganisms or a significant neutrophil infiltrate on histopathological examination of intraoperative deep-tissue specimens[4,11].

However, in contrast with periprosthetic joint infection, this microbiological sampling strategy has yet to be validated in practice among patients with suspected FRI, to our knowledge.

We aimed to identify the optimal microbiological criteria for FRI through the retrospective analysis of 3 patient cohorts with suspected FRI, using the other confirmatory diagnostic criteria recommended by the international consensus guidance[4,11,12].

Materials and Methods

Patient Population

We retrospectively analyzed anonymized clinical and microbiological data from cases identified through 3 prospective cohort studies. FRI was not ascertained prior to inclusion in these cohorts, which were prospectively recruited from surgical lists and for evaluation of sonication fluid culture; therefore, patients who did not have infection were included in the present analysis[13,14]. Patients received surgical treatment for fracture nonunion, suspected fracture implant-associated infection, and unexplained pain at a U.K. tertiary referral center for orthopaedic infection. Cases for which <2 samples were submitted were excluded because it would not be possible to assess the presence of identical pathogens. Duplicate procedures were removed. Approval for this analysis was granted by the institutional review board (Clinical Infection; Datix number 5936).

Diagnostic Test Procedure

Standard protocols for surgical deep-tissue specimen collection and laboratory processing were applied for microbiological investigation[13-15]. Briefly, deep-tissue specimens were taken intraoperatively as soon as possible after skin incision, with individual sterile instruments; the specimens were transported in individual dry sterile containers and processed within 4 hours. Specimens were homogenized separately in 5 mL of saline solution with glass beads and then inoculated into blood culture bottles (BACTEC Plus Aerobic/F bottle and BACTEC Lytic/10 Anaerobic/F bottle; Becton Dickinson) for incubation up to 10 days. Any identified microorganisms underwent antimicrobial susceptibility testing. Quantitative sonication fluid culture was considered identical to a surgical-tissue specimen for the purposes of this analysis, following the results of previous modeling[13]. Tissue for histopathology was cut into 5-μm paraffin-embedded sections, stained with hematoxylin and eosin and Gram stain, and examined to find areas of inflammation containing neutrophils. At least 10 high-power fields (×400) were examined in each section of tissue.

Diagnostic Definition

We retrospectively applied a standard clinical definition of FRI, including any of the following: a sinus, fistula, or discharging wound at the site of suspected infection at clinical examination; intraoperative visible purulence or purulent discharge; or positive histology indicated by at least 5 polymorphonuclear cells per high-power field or visible microorganisms on special staining[4,11,12]. Microbiological criteria for the diagnosis of definite fracture-related infection, based on identification of microorganisms from culture of operative specimens, were omitted in order to avoid incorporation bias[13].

Statistical Analysis

We determined the number of specimens from which an indistinguishable isolate (i.e., a microorganism of the same species with comparable antimicrobial susceptibility) was required to confirm infection. Additional analysis of the number of deep-tissue specimens yielding any microorganism and the number of deep-tissue specimens required to identify all "significant" microorganisms, defined as those present in >1 deep-tissue specimen, was performed.

We used each surgical procedure as the primary unit of analysis. However, a sensitivity analysis with only the index

TABLE I Proportion of Cases Meeting the Definition of FRI* According to the Number of Surgical Specimens for Microbiological Analysis†

	No. of Specimens					
	2	3	4	5	6	>6
Total cases	23	49	96	298	37	10
No. (%) of cases meeting definition of FRI	11 (48%)	26 (53%)	55 (57%)	228 (77%)	30 (81%)	6 (60%)

*Excluding the microbiological criterion. †Cases meeting the diagnostic definition of FRI had a greater number of surgical specimens submitted for microbiological testing, compared with those without a diagnosis of infection ($p < 0.0001$).

procedure for each patient was also performed and is reported in the Appendix.

The difference in the numbers of surgical specimens submitted for microbiological analysis for patients with and without reference standard criteria for FRI was analyzed with use of a chi-square test (Table I).

Exploring the Effect of the Number of Specimens Obtained for Culture

To model the effect of the number of specimens obtained for culture, because the distribution of sample number was not independent of the likelihood of FRI diagnosis, we utilized a 2-stage bootstrap resampling computer algorithm.

Bootstrap resampling is a technique that allows unbiased estimation of sensitivity, specificity, and 95% confidence intervals (CIs) in data for which the distribution of true and false positive and negative results is not known. By taking repeated samples with replacement that are the same size as the original data, repeated empirical estimates for sensitivity and specificity are derived. The distribution of these estimates is the same as if they came from samples taken directly from the original population[16].

First, we resampled all procedures for which at least *n* specimens were submitted for microbiological analysis with replacement, generating 100 bootstrap samples. In those 100 bootstrap samples, for each procedure, *n* specimens were selected at random without replacement in order to model

TABLE II Baseline Patient and Surgical Characteristics*

	Met Definition of FRI	No Definite Criteria for FRI
No. of procedures	356	157
Patient characteristics†		
Male	272 (76%)	93 (59%)
Female	84 (24%)	64 (41%)
Mean age (range) *(yr)*	49.7 (17 to 86)	47.9 (16 to 87)
Median age *(yr)*	49	47
Anatomical site of infection		
Lower limb	284 (79%)	119 (75%)
Upper limb	63 (18%)	38 (24%)
Axial or other	9 (3%)	0
Metal implants at the time of surgery		
Yes	214 (60%)	105 (67%)
No	135 (38%)	43 (27%)
Unknown	7 (2%)	9 (6%)
Specimens received for microbiological testing		
Median no. of specimens (range)	5 (2 to 7)	5 (2 to 9)
Mean no. of specimens	4.7	4.3
Patients with ≥5 specimens	264 (74%)	81 (52%)
≥2 positive microbiological samples	245 (68%)	21 (13%)
1 positive microbiological sample	30 (8%)	30 (19%)
No positive microbiological samples	81 (23%)	106 (68%)
Histology		
Suggestive of infection	302 (85%)	0
Not suggestive of infection	8 (2%)	107 (68%)
Nondiagnostic or equivocal	23 (7%)	32 (20%)
Missing	23 (7%)	18 (11%)
Clinical features of FRI		
Sinus or fistula	243 (68%)	0
Purulence or purulent drainage at operation	141 (40%)	0
No known sinus, fistula, or purulence†	70 (20%)	157 (100%)

*Values are given as the count with the percentage in parentheses, unless otherwise indicated. †Note that some patients had >1 surgical procedure; data are presented using surgical procedure as the unit of analysis, so some patients have data included more than once. ‡Yet meeting the definite criteria for FRI, including histopathology but excluding microbiology.

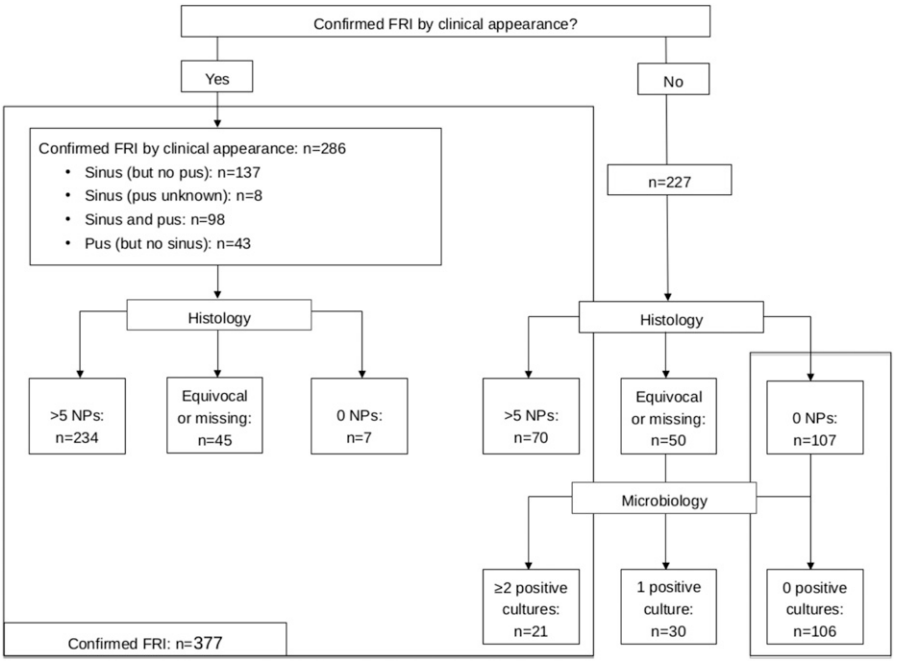

Fig. 1

Flowchart demonstrating the criteria for the diagnosis of FRI in this study. Procedures for which microbiological results were positive according to definite FRI diagnostic criteria have not been defined as meeting the definition of FRI because microbiological testing was excluded from the diagnostic gold standard for this study in order to avoid incorporation bias; procedures in this category were defined as negative for FRI. NPs = neutrophils per high-power field.

the effect of performing microbiological analysis on just those *n* specimens. Sensitivity and specificity were modeled for each bootstrap sample on the basis of the isolation of indistinguishable organisms from 2 or more specimens from each resampled procedure, and 95% CIs for sensitivity and specificity were estimated with use of 1.96 times the standard error of the observed bootstrap estimates. Statistical analysis and modeling were performed with use of R (version 3.6.2; R Foundation for Statistical Computing)[17,18].

Defining the Optimal Diagnostic Cut-Off

With use of the bootstrap modeling method described above, we determined the number of specimens from which an indistinguishable isolate (i.e., a microorganism of the same species with comparable antimicrobial susceptibility) was required to confirm infection (i.e., the diagnostic cut-off).

Assessing the Impact of Reducing the Number of Specimens Submitted for Analysis on the Ability to Identify All Clinically Relevant Microorganisms

For each procedure included in the study, all indistinguishable microorganisms identified in at least 2 independent specimens were noted. Modeling used the bootstrap resampling approach described. The proportion of resampled surgical procedures for which all relevant microorganisms were identified was modeled, out of 100 bootstrap samples, to estimate the sensitivity of *n* surgical specimens to detect all clinically relevant microorganisms, irrespective of whether the diagnostic definition was met. This modeled the effect of reducing the sample number.

Results

A total of 513 surgical procedures in 385 patients with suspected FRI were included in the analysis. Five or more deep-tissue specimens were obtained for microbiological analysis in 345 (67%) of the cases.

Baseline patient characteristics and surgical details are described in Table II, and criteria for the diagnosis of FRI are shown in Figure 1. On average, fewer specimens were obtained from patients without definite clinical criteria for the diagnosis of FRI (Table I) ($p < 0.0001$).

Figures 2 and 3 show the sensitivity and specificity of the number of specimens yielding indistinguishable microorganisms on microbiological culture out of the total number of specimens submitted for analysis, as modeled with use of bootstrap resampling. Optimal sensitivity for the diagnosis of FRI was obtained when 2 or more of 6 surgical deep-tissue specimens (69%; 95% CI, 54% to 84%). A diagnostic cut-off of at least 2 of 5 specimens with indistinguishable microorganisms identified by culture was 68% sensitive (95% confidence interval [CI], 62% to 74%) and 87% specific (95% CI, 81% to 94%) for the diagnosis of FRI. Two out of 3 specimens were 60% sensitive (95% CI, 55% to 66%) and 92% specific (95% CI, 88% to 96%).

The specificity for FRI of any number of specimens submitted and with a diagnostic cut-off of 2 or more specimens yielding indistinguishable microorganisms was >85%. As microorganisms from the skin frequently contribute to both FRI and sample contamination, the presence of an organism in a single sample is difficult to interpret. Microorganisms

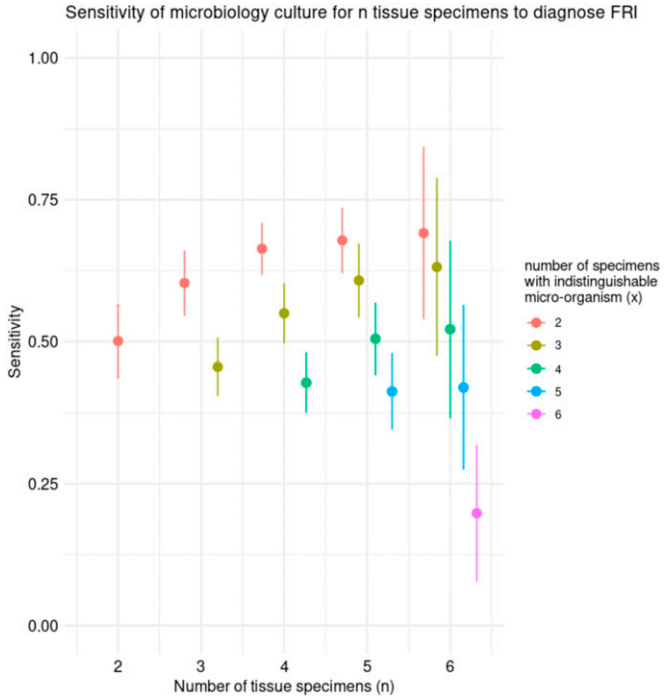

Fig. 2

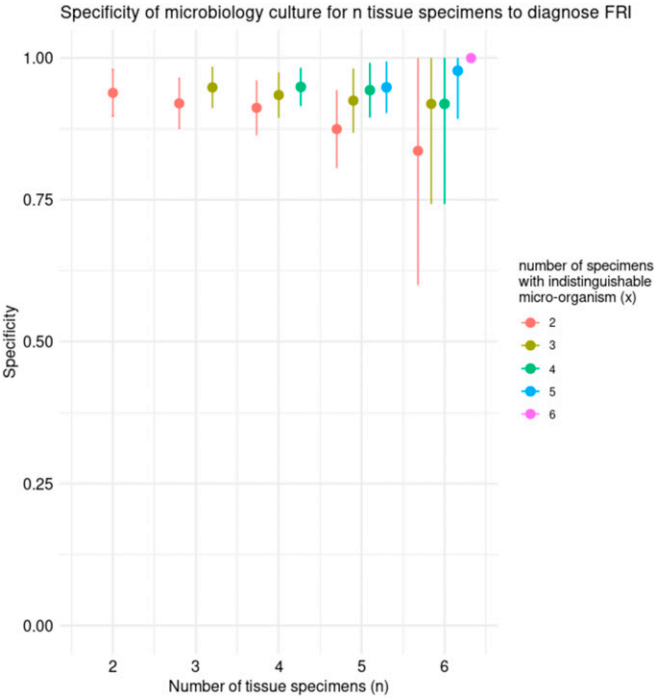

Fig. 3

Fig. 2 Sensitivity for the modeled number of surgical deep-tissue specimens yielding ≥1 indistinguishable microorganism from microbiological culture for the diagnosis of FRI. Error bars show 95% CIs estimated through bootstrap resampling. Sensitivity was estimated with use of resampling from 513 surgical procedures. A total of 513 procedures had ≥2 tissue specimens, 490 had ≥3, 441 had ≥4, 345 had ≥5, and 47 had ≥6 submitted for microbiological testing. **Fig. 3** Sensitivity for the modeled number of surgical deep-tissue specimens yielding ≥1 indistinguishable microorganism from microbiological culture for the diagnosis of FRI. Error bars show 95% CIs estimated through bootstrap resampling. CIs were not estimated for 6 samples.

identified in only 1 specimen were observed in 8% of patients with FRI and 19% of patients without FRI, according to the diagnostic definition.

When analyzing the index procedure for each patient only (see Appendix), at least 2 of 5 specimens with indistinguishable microorganisms identified by culture were 66% sensitive (95% CI, 60% to 73%) and 93% specific (95% CI, 87% to 99%) for the diagnosis of FRI. Two out of 3 specimens were 61% sensitive (95% CI, 55% to 67%) and 95% specific (95% CI, 90% to 100%).

Table III shows the modeled sensitivity for identifying all significant microorganisms, for cases where at least 1 significant microorganism was identified. This analysis showed that if only 3 deep-tissue specimens were obtained for microbiological analysis, more than 10% of clinically relevant microorganisms were likely to be missed.

TABLE III Estimated Sensitivity According to the Number of Surgical Deep-Tissue Specimens*

No. of Samples	Estimated Sensitivity to Identify All "Significant" Microorganisms (95% CI)
2	68% (62% to 72%)
3	84% (79% to 90%)
4	92% (89% to 96%)
5	97% (95% to 100%)
6	93% (84% to 100%)

*To ensure that all "significant" microorganisms are identified in the simulated sample set. CI = confidence interval.

Discussion

To our knowledge, the present study is the first to evaluate the existing recommendations for microbiological diagnosis of FRI among patients who required diagnosis or exclusion of FRI, using the recent consensus definition of definite clinical criteria for FRI diagnosis as a reference.

Significantly fewer specimens were submitted for microbiological analysis for patients without a diagnosis of FRI compared with those with a diagnosis of FRI (Table I). This disparity supported the resampling modeling strategy because direct comparison between specimens in the FRI and non-FRI groups would otherwise not have been valid.

The recommendation to submit at least 5 surgical deep-tissue specimens for microbiological testing is less likely to miss clinically relevant microorganisms than is submitting fewer specimens (Table III). Missing pathogens is an important consideration for not reducing the number of operative specimens when investigating FRI. Microbiological testing in FRI is needed not only to confirm infection but to direct antimicrobial treatment. More than 20% of cases of FRI are mixed infections[2,14,15] (i.e., those involving >1 pathogen identified as

"significant" on microbiological culture and present in 2 or more specimens each). Although the most common pathogen found in FRI is *Staphylococcus aureus,* many FRIs involve gram-negative organisms or mixed gram-positive and gram-negative species; thus, antimicrobial therapy for FRI without identifying a pathogen risks being both too narrow and too broad[14]. In a large multicenter prospective study, Bémer et al. described adequate sensitivity of 3 surgical specimens for the diagnosis of PJI (94% when compared with the gold standard of 5 or more specimens) but did not discuss the limitations of diagnosing polymicrobial infection[19]. This aspect of microbiological diagnosis should not be discounted in surgical sampling recommendations.

Additionally, this modeling approach supports the use of a minimum of 2 surgical specimens yielding an indistinguishable microorganism as a definite criterion for the diagnosis of FRI, to optimize sensitivity and specificity (Figs. 2 and 3).

For the 134 procedures in which patients had no sinus or fistula (i.e., no definite preoperative clinical signs of infection), FRI was diagnosed by at least 1 other confirmatory criterion (i.e., microbiology, histopathology, or intraoperative purulence). Thus, in 26% of cases, FRI was present but there were no unequivocal preoperative clinical signs of infection. This is similar to a previous study of fracture nonunion, in which Hackl et al. reported a rate of infection of 40% in cases with no sinus or fistula[10]. In the present study, surgeons took fewer specimens in cases without obvious signs of infection (Table I), but this practice should be discouraged because it risks missing pathogens. Hackl et al. noted that secondary surgical procedures and delayed wound healing were more common among patients who were not initially suspected to have FRI but who subsequently had a positive microbiological culture in 2 specimens, as compared with those with negative culture results[10]. This finding emphasizes the need to harvest several specimens (i.e., ≥5) from all cases of potential FRI, including those without a sinus or fistula.

The present study reflects a typical mix of procedures for suspected FRI, in which sterile fracture nonunion or persistent pain following fracture union often cannot reliably be distinguished from FRI preoperatively. The majority of patients in this situation have confirmed FRI, as in our study sample (71%).

The diagnostic reference standard was chosen based on the international consensus confirmatory criteria for FRI. The test under investigation (i.e., microbiological culture) was not included in the confirmatory criteria for FRI for the purposes of this study in order to avoid incorporation bias, which would artificially inflate sensitivity and specificity.

At our institution, tissue specimens are collected separately, avoiding cross-contamination with other specimens and the skin, and processed independently in the microbiological laboratory. A median of 5 deep-tissue specimens were submitted for both procedures with confirmed FRI and those for which FRI was excluded on the basis of definite criteria, so this analysis reflects the recommended surgical sampling procedures.

The limitations of this study include the use of retrospective data collection and the use of procedures as the unit of analysis. Data clustering was found to have a minor effect on the estimates of sensitivity and specificity (see Appendix). Including repeat procedures allowed the representation of a greater spectrum of diagnostic uncertainty; the different numbers of specimens, surgical approaches, and pathogens from repeat procedures will have reduced apparent within-patient clustering. The specificity of microbiological culture was elevated when only index surgical procedures were analyzed.

Modeling with use of bootstrapping, although it may amplify error in the data, was essential to avoid underestimating sensitivity and specificity in smaller numbers of specimens, as resampling made use of a greater pool of procedures to model fewer specimens[14].

The sensitivity of microbiological culture was low, despite meticulous specimen handling and the recommendation against preoperative antimicrobial therapy for at least 2 weeks[12,20]. In previous studies, up to one-third of chronic osteoarticular infections treated surgically were culture-negative[5,14,15]. In the present study, 31% of procedures in which the patient had confirmatory criteria for FRI other than microbiology yielded ≤1 microbiological culture-positive deep-tissue specimen. This finding supports the use of a complementary diagnostic test in culture-negative cases. Histology with >5 neutrophils per high-power field has been shown to be a useful test in clinical practice, with very high specificity and positive predictive value or FRI[11].

We included quantitative sonication culture (which represented 2% of specimens) for cases in which prosthetic material or bone was submitted for analysis, but treated the results of this diagnostic test as an additional surgical-tissue specimen, which may underestimate the true sensitivity, as previously published studies have reported better sensitivity for sonication[21].

Conclusions

The present study supports including microbiological testing in the confirmatory criteria for the diagnosis of FRI, with use a minimum of 2 surgical deep-tissue specimens collected and processed independently and yielding indistinguishable organisms. We recommend that ≥5 separate specimens are submitted for microbiological testing in all cases in which FRI is possible. Such testing, compared with that of fewer specimens, improves sensitivity for diagnosing FRI, reduces the risk of missing important pathogens, and is necessary to choose appropriate antimicrobial therapy. If a microorganism of low virulence is not seen in multiple deep-tissue specimens, it may be assumed to be a contaminant, and not targeted. Reducing the total specimen number submitted for analysis from 5 to 3 specimens is likely to miss at least 10% of "significant" microorganisms. Microbiology should be supplemented by histology to facilitate diagnosis in culture-negative cases.

We recommend microbiological testing of ≥5 independent surgical tissue specimens, taken with separate instruments before antimicrobial therapy, even when the patient does not have unequivocal preoperative clinical signs of FRI. Otherwise,

if clinical findings are used to determine which cases proceed to full microbiological diagnostic testing for FRI, up to one-third of possible cases may receive suboptimal diagnostic testing, which could lead to an infection being missed or to inadequate postoperative antimicrobial therapy.

Appendix

eA Supporting material provided by the authors is posted with the online version of this article as a data supplement at jbjs.org (http://links.lww.com/JBJS/G424). ■

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Update

This article was updated on July 1, 2021, because of a previous error. On page 983, a section entitled "Appendix" with a link to the data supplement was previously not included and has now been added.

An erratum has been published: J Bone Joint Surg Am. 2021 August 4;103(15):e62.