Optimal culture incubation time in orthopedic device-associated infections – a retrospective analysis of prolonged 14-day incubation

Nora Schwotzer\textsuperscript{a,\#,*}, Peter Wahl\textsuperscript{b}, Dominique Fracheboud\textsuperscript{c}, Emanuel Gautier\textsuperscript{b}, Christian Chuard\textsuperscript{a,d,\#}

Department of Internal Medicine\textsuperscript{a}, Department of Orthopedic Surgery and Traumatology\textsuperscript{b}, Microbiology laboratory\textsuperscript{c}, Division of Infectious Diseases\textsuperscript{d}, Hôpital cantonal de Fribourg, Fribourg, Switzerland.

Running head: Optimal incubation in orthopedic tissue samples.

\#Address correspondence to: nora.schwotzer@chuv.ch or christian.chuard@h-fr.ch

*Present address: Department of Internal Medicine, Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland.
ABSTRACT

Accurate diagnosis of orthopedic device-associated infections can be challenging. Culture of tissue biopsies is often considered as the gold standard, however there is currently no consensus on the ideal incubation time for specimens. The aim of our study was to assess the yield of a 14 day incubation protocol for tissue biopsies from revision surgery (joint replacement and internal fixation devices) in a general orthopedic and trauma surgery setting. Medical records were reviewed retrospectively in order to identify cases of infection according to predefined diagnostic criteria. From August 2009 to March 2012, 499 tissue biopsies were sampled from 117 cases. In 70 cases (59.8%), at least one sample showed microbiological growth. Among them, 58 (82.9%) were considered as infections (82.9%), and 12 cases (17.1%) were classified as contaminations. The median time to positivity in the cases of infection was 1 day (range 1-10) as compared to 6 days (range 1-11) in cases of contamination ($p < 0.001$). Fifty-six (96.6%) of the infection cases were diagnosed within 7 days of incubation. In conclusion, the results of our study show that incubation of tissue biopsies beyond 7 days is not productive in the general orthopedic and trauma surgery setting. Prolonged 14-day incubation might be of interest however in particular situations, where the prevalence of slow-growing microorganisms and anaerobes is higher.
Surgical implants play a major role in orthopedic trauma surgery as well as in the management of degenerative and inflammatory joint diseases. However, the rising number of indwelling devices is related to an increase in related complications. Along with device loosening or malfunctioning and foreign material reactions, infection remains one of the most serious problems encountered with surgical implants. Although orthopedic device-associated infections (ODAI) are uncommon, occurring in only 1-2% of hip and knee replacements and up to 6% of patients after internal fixation of closed fractures, their management is difficult (1). It can require multiple revision surgeries and prolonged antibiotic treatment, may result in permanent disabilities and is associated with high costs (2, 3).

Despite the promising results reported with newer techniques such as sonication cultures and molecular testing, the diagnosis of ODAI remains a medical challenge as routinely used methods lack sensitivity and specificity (4-7). Synovial fluid culture, tissue biopsy culture, and histopathological examination show high sensitivities and are frequently considered as the gold standard. A reliable microbiological diagnosis is crucial for determining appropriate treatment (8).

There is currently no consensus on the appropriate incubation time for ODAI tissue biopsies. In most studies, the duration of
incubation is not specified, but a 5-day period has often been reported (9-11). Recently, some authors have proposed prolonging the incubation period to 7 or 14 days in order to reveal low-virulent microorganisms such as Propionibacterium acnes, Peptostreptococcus spp, and Corynebacterium spp (12-15). Low-virulence, foreign material-adherent bacteria are typically in a dormant, starved state with a slow replicating rate (16). This particular behavior may require a longer culture incubation time (16-19). However, prolonging the incubation time is costly, labor intensive and could increase the likelihood of detecting organisms that are not clinically relevant. Thus, the aim of our study was to determine if an incubation time of 14 days for tissue biopsies is useful in the diagnosis of ODAI.

MATERIALS AND METHODS

Study design

Microbiological samples of tissue biopsies taken from orthopedic device revision surgery (joint replacement and internal fixation devices) between August 2009 and March 2012, which had been incubated for 14 days, were analyzed. At our institution, 14-day incubation is standard for implant-associated samples and is performed on request for other bone and joint infections. In this study, case identification was prospective and continuous, while the study was retrospective. When there were several interventions
on the same joint, only the first revision surgery was taken into account. The time until microbial growth was recorded. In cases of polymicrobial growth, infection was diagnosed if at least one microorganism fulfilled the diagnostic criteria (see below). The day of growth of the slowest growing microorganism was used to avoid overlooking late growing bacteria.

The study was performed in a hospital acting as a primary care and referral center for a population of about 280,000 inhabitants. Elective orthopedic and trauma surgery each account for about half of the activity of the Department of Orthopedic Surgery and Traumatology at this hospital. Medical records were reviewed in order to determine if infection was present. Infection was diagnosed according to predefined diagnostic criteria (see below). Cases were reviewed by an infectious disease specialist and an orthopedic surgeon. Patient files were scanned for indications of clinical infectious signs (fever, erythema, edema, local hyperthermia, wound discharge, and/or the presence of a sinus tract). A temperature above 38.5°C was considered as fever and fracture non-union was taken as a potential sign of infection. Preoperative antimicrobial treatment was defined as the administration of any type of antibiotic for more than 24 hours during the 14 days preceding surgery. The histopathological findings were divided into 3 categories depending on the average number of polymorphonuclear cells (PMN) per high-power field (HPF, 400x) on microscopic analysis, as a mean value of at least
10 fields examined: <1 PMN/HPF, 1-5 PMN/HPF and >5 PMN/HPF.

Definition of infection

Infection was diagnosed if one of the following criteria was fulfilled:

1. Microbiological criterion: Positive culture with ≥3 positive samples showing identical microorganisms (20);

2. Histopathological criterion: Positive culture with any number of positive samples and histopathological examination showing >5PMN/HPF not explained by an acute fracture (21-23);

3. Clinical criterion: Positive culture with any number of positive samples and clinical signs of infection: erythema, edema, local hyperthermia, wound discharge, presence of a sinus tract, or fracture non-union (8, 24).

Patients who had not been treated post-operatively with antibiotics and showed no signs of infection after 12 months of follow-up were not considered to be infected, independently of the diagnostic criteria. Cases with positive cultures that did not fulfill the criteria of infection were classified as contamination.

Culture methods

Tissue sampling was performed in the operating room according to usual surgical methods. The standard procedure was to take 3 to 6 samples with priority given to tissue biopsies, if not limited by
anatomical restrictions as in the finger, hand and foot (20, 25-27). In order, tissues were sampled from the inflammatory membrane around the implant, joint capsule, and any macroscopically suspect tissue (28, 29). Each biopsy was stored in transportation medium to ensure the survival of all bacteria, including anaerobe microorganisms (BBC\textsuperscript{TM} Port-A-Cul\textsuperscript{TM}, Becton, Dickinson, and Company, Sparks MD, USA).

Homogenization of the tissue biopsies was carried out using a disposable closed tissue homogenizer system (gentleMACS\textsuperscript{TM} Dissociator, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) with the addition of normal saline as necessary to obtain a heavy suspension. All manipulations were performed under sterile conditions and under laminar airflow. One hundred microliters of this suspension was inoculated on each of the following agar plates: i) blood agar: Columbia-D agar base (bioMérieux, Marcy l'Etoile, France) with 5% sheep blood, ii) chocolate agar: Columbia-D agar base (bioMérieux, Marcy l'Etoile, France) with 5% sheep blood (heated to lyse blood cells) and supplemented with growth factors Vitox SR0090 (Oxoid-Thermo Fisher Scientific, Basingstoke, United Kingdom), iii) pre-reduced Brucella agar with 5% sheep blood, hemin and vitamin K1 (bioMérieux, Marcy l'Etoile, France). After inoculation, the plates were sealed with a Parafilm laboratory film (Bemis Company, Inc., Oshkosh, WI, USA) to avoid desiccation. The first 2 media were incubated at 35 °C in a 5% CO\textsubscript{2} atmosphere for cultivation of aerobic and facultative organisms.
The third plate was incubated at 35°C in an anaerobic atmosphere for the cultivation of anaerobic and facultative organisms. The remainder of the suspension was inoculated into a thioglycolate broth medium CM0173 (Oxoid-Thermo Fisher Scientific, Basingstoke, United Kingdom) and incubated at 35°C for enrichment of aerobic, anaerobic and facultative microorganisms. Quality control showed the presence of adequate anaerobe conditions in the lower part of the broth. Each medium was inspected for signs of growth every day over a period of 14 days.

Statistical methods

Continuous variables are presented as medians and ranges and categorical variables as rates. The statistical significance was assessed using the chi-square test or Fisher’s exact test for categorical variables and the Mann-Whitney U test (Kruskal-Wallis test) for continuous variables. All tests were performed using SPSS®, version 21 (SPSS Inc., Chicago, IL, USA). \( p < 0.05 \) was considered as statistically significant. For graphical representation, Microsoft Excel® 2008 (Microsoft Corporation, Redmond, Washington, USA) was used.

RESULTS

Study population

During the study period, 499 tissue biopsies were collected from
117 cases of revision surgery, corresponding to a median number
of 4.0 samples per case (range 1–12). In 70 cases (59.8%), a
minimum of one sample was positive for microbiological growth,
leaving 47 cases (40.2%) sterile during the incubation period.

The study population consisted of 50 women (42.7%) and 67
men (57.3%) with a median age of 68.0 years (range 14-94). The time
between index and revision surgery was <1 month in 31 cases
(26.5%), 1-12 months in 32 cases (27.4%), and >12 months in 54
cases (46.2%). Orthopedic devices included 62 cases (53.0%) of
joint prosthesis and 55 cases (47.0%) of internal fixation devices.
Localization of the devices varied from the hip in 51 cases (43.6%),
knee in 29 cases (24.8%), lower extremity in 22 cases (18.8%),
upper limb in 9 cases (7.7%), to the spine in 6 cases (5.1%).
Articular devices were labeled according to the joint region
involved. Histopathologic analysis was available for 85 cases
(72.6%). Among the 70 cases with positive culture, 58 cases
(82.9%) were classified as infections and 12 cases (17.1%) as
contaminations. The proportion of infections among the 117 cases
undergoing revision surgery was 49.6%; 41.9% for joint
replacement and 58.2% for internal fixation devices.

Microbiology

The majority of the isolated microorganisms were Gram-positive
bacteria, mainly Staphylococcus aureus in 22 cases (31.4%) and
coagulase negative staphylococci in 18 cases (25.7%).
Streptococcus spp accounted for 2 cases (2.9%), Enterococcus spp for 2 cases (2.9%), P. acnes for 3 cases (4.3%), Gram-negative bacteria for 6 cases (8.6%) and polymicrobial culture results for 17 cases (24.3%). The full spectrum of bacteria according to case classification is illustrated in table 1. Both types of orthopedic devices showed a similar spectrum of microorganisms, except for S. aureus, which were significantly more frequent in prosthesis than in internal fixation devices (15 cases compared to 7 cases; p=0.04).

Diagnostic criteria

Of the 58 cases of infection, the majority was diagnosed by at least 2 diagnostic criteria, leaving only 9 cases with a single criterion (microbiological criterion in 3 and clinical criterion in 6 cases).

Discrepancy between the study’s definition of infection and the treating medical team’s diagnosis occurred in one case that was treated as an infection based on one out of 8 samples showing P. acnes on day 7. This case was classified as contamination according to our criteria.

Time to culture positivity

Median time to culture positivity for the 70 cases with positive tissue biopsies was one day (range 1-11). A total of 47 cases (67.1%) became positive within the first 2 days of incubation, 57 cases (81.4%) within 5 days, and 65 cases (92.9%) within 7 days.
The median time to positivity in cases classified as infection was one day (range 1-10) as compared to 6 days (range 1-11) for cases considered as contamination (p <0.001). A total of 52 cases (89.7%) of infections were diagnosed within 5 days of incubation, and 56 cases (96.6%) within 7 days (figure 1). No infection was diagnosed beyond 10 days. Twenty-five percent of contaminants grew after 7 days, representing the majority (60%) of late-growing microorganisms. Because the absolute number of cases with infection or contamination is also relevant, graphical representation of the results is shown by histogram in figure 2.

Only 2 cases of infection were detected after 7 days of incubation, at day 8 and 10, respectively (table 2). The first case was a late post-operative infection, which showed a high amount of *Corynebacterium* sp (4 out of 5 positive tissue biopsies) growing within 4 days. This was associated with an *Escherichia coli* infection (one out of 5 positive tissue biopsies) growing at day 8, and both bacteria were considered as pathogens. The second case involved an early post-operative infection and demands special consideration. In this case, sampling occurred under antibiotic treatment, which had been initiated after a superficial wound swab had showed *S. aureus* (not recorded in our data base) and in the presence of clinical signs of acute infection (erythema, edema, wound discharge). A coagulase negative staphylococcus found to be growing in the tissue biopsy after 10 days was recorded as the etiological agent according to our study.
definition (histopathological and clinical criteria). However, considering the clinical picture, it is more probable that S. aureus was responsible for the infection. As a consequence, the late growth of the coagulase negative staphylococcus is probably not relevant.

Our sample showed no significant difference in median time to culture positivity (p = 0.84) with regard to the type of orthopedic device (joint replacement or internal fixation device).

DISCUSSION

Few studies have focused on the optimal incubation time for orthopedic surgery specimens. Most commonly, incubation of 5 to 7 days is used for ODAI (10, 11, 30-32). However, in recent studies (12, 13, 15), prolonging the incubation time for up to 14 days has been proposed. Schaefer et al. (13) have addressed the infectious component in aseptic loosening and have considered mostly elective surgery. Zappe et al. (15) and Butler-Wu et al. (12) have specifically explored an extended culture protocol for P. acnes. In studies on alternative diagnostic procedures, such as 16S rRNA PCR or sonication, 14-day incubation has sometimes been reported, although without assessment of its benefits (33, 34). The aim of our study was to explore the relevance of 14-day tissue biopsy culture incubation for the diagnosis of overall ODAI in the setting of general orthopedic and trauma surgery, where both acute and chronic infections are encountered.
We found that an incubation period of 7 days was sufficient to identify 56 out of 58 cases (96.6%) of infection. The major difference between our data and those of Schaefer et al. (13) is the proportion of low-virulent microorganisms such as Propionibacterium spp, coryneform bacteria and coagulase negative staphylococci which account for 80% of infections in their study population. These microorganisms are known to have a slow growth rate. In our study, only 54% of the cases showed low-virulence microorganisms. In particular, P. acnes accounted for 4.3% of isolated bacteria, which corresponds with reports from other general orthopedic and trauma surgery departments (8, 35, 36).

Diagnosis of ODAI is a well-known challenge. In our study, we have tried to provide clear and reproducible diagnostic criteria that are applicable to a retrospective analysis, which has well-established limitations. For the microbiological criterion, our threshold of at least 3 positive samples with identical microorganisms could be viewed as stringent compared to other studies in which 2 culture positive specimens are considered sufficient to diagnose infection (10, 37, 38). However, none of our contamination cases had more than one positive sample, meaning that our results would not have been any different even if we had adopted a lower threshold. The same is true for the histopathological criterion, as no contaminant showed an intermediate result of 1–5 PMN/HPF. Although the clinical
diagnostic criterion is somewhat subjective, all the cases classified as infection on this basis were quite evident; 10% of infections would have been missed without this strategy.

Overall, we identified a high proportion of infections (22, 39, 40). This can be explained by the inclusion of trauma cases, where early revision surgery was indicated on the basis of a high preoperative suspicion of infection, whereas the systematic sampling of loose prosthesis used in other studies obviously has a lower yield (39, 40).

Most reports focus on hip and knee prosthesis (1, 13, 24, 37). We believe that including both prosthesis and internal fixation devices in our study makes sense because similar pathogens have been described in both settings (41-43) and biofilm-formation is common to all types of foreign body infections (44-46). We found that slow-growing microorganisms such as coagulase negative staphylococci and *P. acnes* were equally represented in both groups.

In conclusion, the results of our study show that extension of culture incubation time beyond 7 days has a low yield in a general orthopedic and trauma setting where virulent bacteria predominate and post-traumatic infections are frequent. However, based on the current literature, prolonging incubation to 14 days or using molecular techniques might be useful in particular situations where the prevalence of slow-growing bacteria and anaerobes is higher.
REFERENCES


**Table 1:** Spectrum of microorganisms in tissue biopsies.

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Infection n (%)</th>
<th>Contamination n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>22 (37.9)</td>
<td>-</td>
</tr>
<tr>
<td>Coagulase negative staphylococci</td>
<td>10 (17.2)</td>
<td>8 (66.7)</td>
</tr>
<tr>
<td><em>Streptococcus</em> spp</td>
<td>2 (3.4)</td>
<td>-</td>
</tr>
<tr>
<td><em>Enterococcus</em> spp</td>
<td>2 (3.4)</td>
<td>-</td>
</tr>
<tr>
<td><em>Propionibacterium acnes</em></td>
<td>1 (1.7)</td>
<td>2 (16.7)</td>
</tr>
<tr>
<td>Gram-negative bacilli</td>
<td>6 (10.3)</td>
<td>-</td>
</tr>
<tr>
<td>Polymicrobial culture</td>
<td>15 (25.9)</td>
<td>2 (16.7)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>58 (100)</td>
<td>12 (100)</td>
</tr>
</tbody>
</table>

Table 2: Characteristics of 2 cases of infection with time to tissue culture positivity beyond 7 days of incubation.

<table>
<thead>
<tr>
<th>Microorganism(s)</th>
<th>Culture positive samples</th>
<th>Histology (PMN/HPF)*</th>
<th>Type of implant</th>
<th>Clinical signs</th>
<th>Day of growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Corynebacterium sp E. coli</td>
<td>4 / 5</td>
<td>0</td>
<td>Hip prosthesis</td>
<td>Ongoing pain</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>1 / 5</td>
<td></td>
<td></td>
<td></td>
<td>8</td>
</tr>
<tr>
<td>2 Coagulase negative staphylococcus</td>
<td>1 / 3</td>
<td>&gt; 5</td>
<td>Knee prosthesis</td>
<td>Erythema, wound discharge</td>
<td>10</td>
</tr>
</tbody>
</table>

*Number of polymorphonuclear cells (PMN) per high power field (HPF, 400x); more than 5 PMN/HPF is highly suggestive of infection.
Figure 1: Time to tissue culture positivity comparing cases of infection and contamination.
Figure 2: Absolute number of cases with positive tissue cultures at each day of incubation comparing cases of infection and contamination.