■ ARTHOPLASTY

Prolonged incubation time does not increase sensitivity for the diagnosis of implant-related infection using samples prepared by sonication of the implants

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We have designed a prospective study to evaluate the usefulness of prolonged incubation of cultures from sonicated orthopaedic implants. During the study period 124 implants from 113 patients were processed (22 osteosynthetic implants, 46 hip prostheses, 54 knee prostheses, and two shoulder prostheses). Of these, 70 patients had clinical infection; 32 had received antibiotics at least seven days before removal of the implant. A total of 54 patients had sonicated samples that produced positive cultures (including four patients without infection). All of them were positive in the first seven days of incubation. No differences were found regarding previous antibiotic treatment when analysing colony counts or days of incubation in the case of a positive result. In our experience, extending incubation of the samples to 14 days does not add more positive results for sonicated orthopaedic implants (hip and knee prosthesis and osteosynthesis implants) compared with a conventional seven-day incubation period.

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Microbiological diagnosis of implant-related infection is essential for the selection of the ideal antibiotic therapy based on the individual susceptibility of any isolated micro-organisms.[1-3] In order to optimise this process, the best combination of available techniques must be used in order to minimise the number of infected joint replacements being regarded as 'culture-negative'. The classical diagnostic solution has been to culture several samples of peri-prosthetic tissue and/or synovial fluid.[1,3-5] Recently sonication of the implant (a technique that uses low-power ultrasound in order to remove all biofilm-embedded organisms from the implants) has been evaluated in the diagnosis of infection in these patients and in almost all cases, sonication has proved superior to conventional methods,[6-9] although there still remain some clinically infected cases with negative cultures. Combining this technique with molecular biology provides even better results.[10-12]

In 2008 Schäfer et al[13] described the usefulness of an extended incubation time of cultures of peri-prosthetic tissue samples. It was found that a conventional incubation time of seven days provided positive cultures in only 73.6% of cases in which no sonication was used. Between the seventh and 14th day slower growing isolated organisms, especially anaerobes like *Propionibacterium acnes* and *Peptostreptococcus spp*., together with other gram-positive rods, were identified. These findings were supported by Lutz et al[14] who reported that *P. acnes* grew in a mean period of 11.4 days (a mean of 8.37 days in early infections and 13.53 days in late ones). As gram-positive organisms described by Schäfer et al[13] are commonly found in prosthetic joint infections,[1,3] it might be logical to increase the incubation period of cultures of sonicated implant samples. However, this could create logistical problems in the laboratory due to the high number of plates that would need to be incubated. Moreover, the daily inspection of plates could be confounded through contaminations by naturally occurring environmental organisms, especially where counts of suspect organisms were low.

We have evaluated the combination of sonication and long incubation time in order to evaluate if an incubation time of two weeks decreased the number of negative cultures when compared with conventional seven-day incubation.

Patients and Methods

After approval by our local hospital ethics committee, this prospective study was performed between March 2011 and September 2012. Implant samples submitted to the Microbiology Department as part of diagnostic protocols for implant-related infection from our hospital were included in the study. Polymethylmethacrylate spacers were excluded

because of the lack of data regarding the significance of the culture results. During this period 124 implants were processed (22 osteosynthesis implants, 46 hip prostheses, 54 knee prostheses and two shoulder prostheses) from 113 patients (41 men and 93 women) with a mean age of 69.9 years (SD 14.8; 24 to 93). All samples were processed using a previously described protocol,[6,11] with the following modifications. Samples (except intramedullary nails, which were sonicated in plastic bags because of their size) were sonicated in air-tight sterile rigid plastic cylindrical containers (9.5 cm diameter), which were filled with phosphate buffer (bioMérieux, Marcy L'Etoile, France) until the samples were fully immersed. The sonication fluids were then centrifuged at 2500×g for 20 minutes, and sediment was resuspended in 5 ml of the same buffer.

Inoculated tryptic soy-5% sheep blood agar and chocolate agar plates (bioMérieux) were incubated at 37°C for 15 days in a 5% CO_2-enriched atmosphere. Schaedler-5% sheep blood agar plates (bioMérieux) were incubated for 48 hours under anaerobic conditions before the first examination, and then were incubated in the same atmosphere and examined daily for a total 15 days. McConkey agar plates were incubated at 37°C in normal atmosphere for 24 hours and Sabouraud-chloramphenicol agar tubes were incubated at 30°C in normal atmosphere for one month (both bioMérieux). One calibrated loop (10 µl) of the resuspended sonicate was inoculated onto each medium. The minimum amount of detectable organisms was 100 colony-forming units (CFU)/ml (a total of 500 CFU/sample, because sediment was suspended in 5 ml of buffer). Isolated organisms were identified using conventional techniques, and susceptibility testing was performed using standard procedures.[6]

Together with the prosthesis, between three and five peri-prosthetic samples per patient were also sent to the microbiology laboratory. These samples were ground under sterile conditions and inoculated in the same media as the sonicated implants and incubated over the same time frame. Peri-prosthetic tissues were also processed for mycobacterial culture according to commonly used protocols including automated liquid medium (Versatrek; bioMérieux), Lowenstein-Jensen and Coletsos slants (all media from bioMérieux). Solid media for mycobacteria were incubated for eight weeks at 37°C, while liquid media was kept for 42 days incubation at 37°C in an automated incubator.

Isolated organisms were identified using conventional techniques, and susceptibility testing was performed using standard procedures.[6] A strain isolated from the prosthesis was considered to be an identical biotype to a strain isolated from peri-prosthetic tissues based on chemical reactions and antimicrobial susceptibility testing . The date when a positive culture was confirmed with the different media was prospectively recorded. The first day of positivity was used in the different analyses.

Clinical notes of the patients were reviewed using a predefined protocol that included demographics, clinical examination, C-reactive protein (CRP) values, diagnostic imaging, treatment and outcome. Patients were diagnosed as having implant-related infection when at least one of the following commonly accepted criteria[15] were met: 1) a draining sinus; 2) presence of acute inflammation identified by histopathological examination; 3) presence of macroscopic purulence around the implant; 4) presence of two or more positive cultures from high-quality samples (synovial fluid, peri-prosthetic tissue, blood cultures); or 5) presence of acute or chronic pain in the absence of a mechanical problem AND at least one altered blood parameter (including erythrocyte sedimentation rate (ESR), CRP, or synovial cell count).

Statistical analysis. This was performed using the chi-squared and Fisher's exact test for qualitative variables, and Bartlett's test for inequality of population variances, analysis of variance (ANOVA) and Kruskal-Wallis tests for quantitative variables. For those samples with > 100 000 CFU/ml, a value of 100 000 CFU/ml was used for statistical calculations. All these tests were performed using the Epi-Info 3.5.3 software (CDC, Atlanta, Georgia). The McNemar non-parametric test was performed to compare the proportion of positive cultures between seven and 14 days of incubation, and a sample size estimation was performed, which revealed 52 samples were required in order to detect a difference of 20% between the two incubation times between both groups. A p-value of < 0.05 was considered statistically significant.

Table I. Relationship between tissue culture and sonicate culture for the 124 implants

	Tissue culture positive (n)	Tissue culture negative (n)
Infection present (78 samples)		
Sonicate culture positive	39 (*50*)	14 (*18*)
Sonicate culture negative	8 (*10*)	17 (*22*)
No infection present (46 samples)		
Sonicate culture positive	0 (*0*)	3 (*7*)
Sonicate culture negative	1 (*2*)	42 (*91*)

Results

Of the 113 patients from whom specimens were taken for culture, 70 were infected and 33 were non-infected. A total of 32 patients had received antibiotic therapy for a duration ≥ seven days before removal of the implant.

Of the 124 implants processed, infection was found to be present in 78 (62.9%) and no infection was found in 46 (37.1%). The comparison of data from cultures of sonicates and peri-prosthetic tissues are shown in Table I.

Of the 56 culture-positive implants (including both sonicate and tissue cultures; Table I), no differences were detected in terms of positive results when the seven- and 14-day cultures were compared ($p = 1.0$, non-parametric MacNemar bilateral test).

No differences were obtained in the mean colony counts between joint prostheses from patients with or without previous antibiotic treatment (56 210 CFU/ml (100 to > 100

Table II. Days of incubation needed and number of colony-forming units (CFUs) for sonicated samples

Implant	Positive samples (n)	Bacterial species*	Mean days (range)	Mean CFUs/ml (range)
Joint prostheses	45			56 403 (50 to > 100 000)
Hip (n = 31)	24	*Staphyloccocus aureus* (n = 9) *Staphylococcus epidermidis* (n = 5) *Pseudomonas aeruginosa* (n = 3) *Escherichia coli* (n = 2) *Enterococcus faecalis* (n = 2) *Propionibacterium acnes* (n = 1) *Enterobacter cloacae* (n = 1) *Proteus mirabilis* (n = 1) *Providencia stuartii* (n = 1) *Streptococcus agalactiae* (n = 1) *Staphylococcus simulans* (n = 1) *Comamonas testosteroni* (n = 1) *Bacillus spp.* (n = 1)	1.96 (1 to 5)	54 373.75 (50 to > 100 000)
Knee (n = 48)	20	*Staph. aureus* (n = 5) *Staph. epidermidis* (n = 6) *Ralstonia pickettii* (n = 4) *Sphingomonas paucimobilis* (n = 2) *E. faecalis* (n = 1) *S. agalactiae* (n = 2) *Corynebacterium striatum* (n = 2) *Staphylococcus lugdunensis* (n = 1) *E. coli* (n = 1) *E. cloacae* (n = 1) *Morganella morganii* (n = 1)	2.35 (1 to 6)	55 570 (100 to > 100 000)
Shoulder (n = 2)	1	*P. acnes* (n = 1)	4 (-)	500
Osteosynthesis implants	11			
Nails (n = 11)	5	*Staph. aureus* (n = 3) *P. aeruginosa* (n = 2) *Staph. epidermidis* (n = 1) *Corynebacterium spp.* (n = 1) Mixed anaerobic microbiota (n = 1)	1.33 (1 to 2)	90 000 (40 000 to > 100 000)
Plates/screws (n = 11)	6	*Staph. aureus* (n = 3) *Staph. epidermidis* (n = 1) *S. agalactiae* (n = 1) *E. cloacae* (n = 1) *Clostridium spp.* (n = 1)	2.35 (1 to 7)	52 371.92 (1000 to > 100 000)

* polymicrobial samples: hip prosthesis (n = 4), knee prosthesis (n = 6), nails (n = 2), plates and screws (n = 2)

000) *versus* 44 771.4 CFU/ml (50 to > 100 000); p = 0.48, ANOVA), but a relationship was found between detection of a positive culture and the absence of previous antibiotic treatment ($p < 0.001$, Fisher's exact test). These findings were the same for tissue culture results (p = 0.02, Fisher's exact test). All patients with positive results for osteosynthetic materials had received no previous antibiotic treatment.

The most frequently isolated species were *Staphylococcus aureus* (n = 20) and *Staphylococcus epidermidis* (n = 13). Table II shows the relationship between type of implant, isolated species and mean duration until detection in culture for each implant type. In 14 samples more than one type of organism was identified (Table II).

In eight samples there were positive results from the peri-prosthetic tissue and/or synovial fluid whilst the implant sonication fluid had no growth (Table I). These organisms included *Staph. aureus* (n = 1), *P. acnes* (n = 1), *Streptococcus agalactiae* (n = 1), *Escherichia coli* (n = 1), *Pseudomonas aeruginosa* (n = 1), *Corynebacterium spp.* (n = 1), and *Staph. epidermidis* (n = 2). In all but one case the organism required culturing for ? three days in order to grow, and in the remaining case (of *P. acnes*) required seven days of culture. All strains from the same species identified both in peri-prosthetic tissue and sonicate were identical. A strain was identified as unique according to the results of biochemical tests and also susceptibility tests. If all data

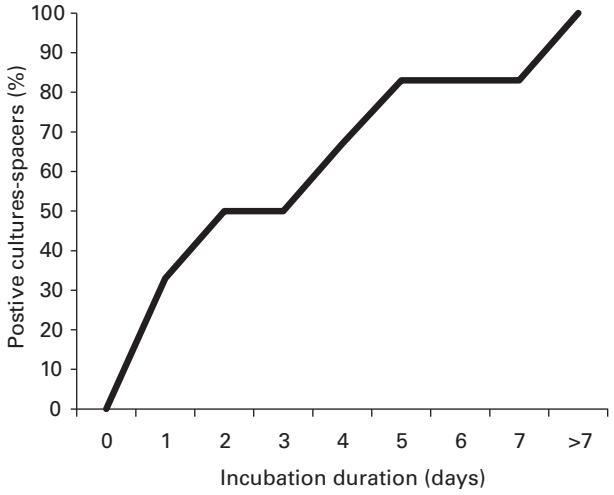

Fig. 1a

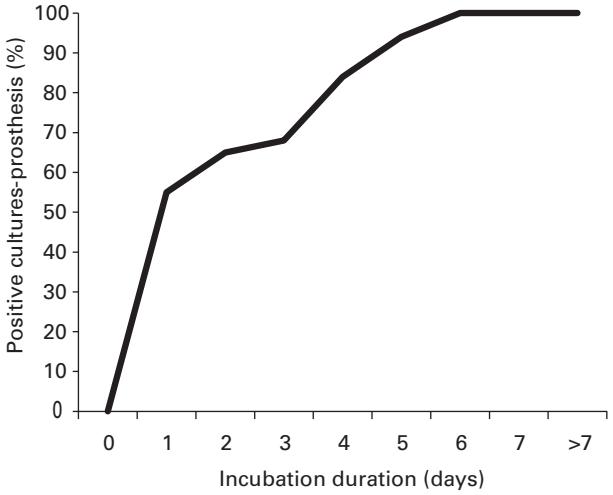

Fig. 1b

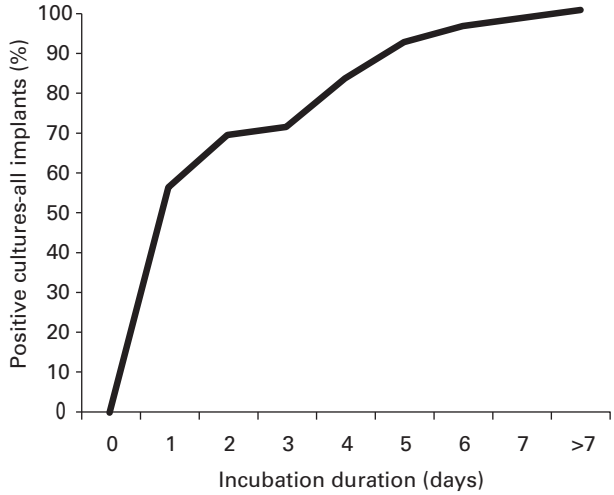

Fig. 1c

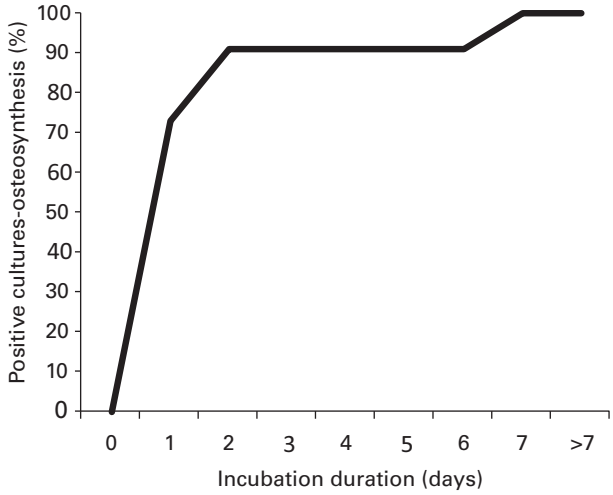

Fig. 1d

Graphs showing the proportion of positive samples by the duration of incubation for a) all samples, b) joint prosthesis samples, c) osteosyntheti implants, and d) samples from peri-prosthetic tissues.

between two strains from the same patients were the same, the strain was considered identical.

The mean number of days to obtain a positive culture was 2.4 (SD 2; 1 to 11) for joint prosthesis and 1.7 (SD 1.7; 1 to 7) for osteosynthetic materials. Figure 1 shows the increase in the proportion of positive samples related to the number of incubation days and demonstrates that no clear differences can be found amongst the different implants. An isolate of *Clostridium spp*. was detected at day seven, and no anaerobee was detected beyond seven days.

No mycobacteria or any other slowly growing organism was detected from implants during the study period but two of the peri-prosthetic tissue samples grew two of these organisms; *Mycobacterium tuberculosis* from one patient and *Mycobacterium fortuitum* from another.

Discussion

According to our results, all isolates detected by sonication appeared before the seventh day of incubation, including some slowly growing organisms such as *P. acnes*, *Corynebacterium spp*. or *Clostridium spp*. These findings were applicable to joint prostheses but also for other implants, such as osteosynthetic devices, where anaerobic bacteria are usually more frequently isolated.[16] Most reports regarding the use of sonication have used a seven-day incubation period with good results,[6,7,9-11,17,18] and only one report regarding prosthetic shoulder infections has used a more prolonged incubation period,[8] probably due to the fact that *P. acnes* is one of the most prevalent organisms in these cases.[17]

Only the study by Schäfer et al[13] compared seven and 14 day incubation times, with an increase of almost 30% in detected positive cultures. We have not observed such an increase in our study with all positive results from sonicated samples appearing within seven days. However, the low number of shoulder prostheses (where *P. acnes* is prevalent) in our series makes it difficult to make a general recommendation of shorter incubation times for these samples. A seven-day period could probably be recommended for hip and knee prostheses, and also for osteosynthetic implants, and a 14-day period needs to be evaluated in further studies for shoulder and elbow prostheses.

We have not detected a statistical difference between patients with or without previous antibiotic treatment, as we previously reported.[11] This could be due to the use of antibiotics that were ineffective against isolated bacteria. However, another report has found a difference,[9] so it is possible that the number of treated patients in our series is lower that that required to detect a difference.

Of interest are the cases with positive cultures in peri-prosthetic tissue and negative results in sonicated samples. It is reported that the use of long sonication times or high intensity of ultrasound can influence the results of this procedure.[19] However, our methodology has previously been tested and the reported results[6,11,16,20] are similar to those obtained with other protocols. The only change that we have made in this study was to use air-tight rigid plastic containers in order to minimise the risk of contamination,[21] so we believe the use of sonication in our study did not suppress the production of a positive culture. Another potential explanation is the presence of a higher amount of bacteria in the peri-prosthetic tissues, including intracellular bacteria in some samples. The presence of intracellular bacteria has been suggested as an important factor in the pathogenesis of prosthetic joint infections,[22] and the use of tissue grinding can enable these organisms to be detected. Finally, in some infections (*P. acnes*, *Corynebacterium spp*. and *Staph. epidermidis* isolates), although the organism grew in two samples, the significance remained doubtful, and it was probable that in two cases (*P. acnes* and *Corynebacterium spp*., both with scanty growth) the organisms were contaminants.

Our protocol can detect almost all bacteria implicated in orthopaedic implant-related infections, however, some organisms need special culture media (e.g., mycobacteria), and if these special media are not used, these organisms cannot be detected.[23] There are organisms that are difficult to detect even if this approach is used. In these cases, the use of molecular biology including 16S rDNA amplification and sequentiation can detect these organisms.[24] Unfortunately these techniques are limited to specially equipped laboratories at the present time.

In conclusion, the use of prolonged incubation times (> seven days) did not increase the detection of infection when culturing sonicated implants in our study. In our opinion, the best approach for the diagnosis of prosthetic joint infections currently is the combined use of sonication and peri-prosthetic tissue culture, using a seven-day incubation period for osteosynthetic devices and hip and knee prostheses. This reduces the number of patients with negative microbiological results while avoiding logistical problems in the microbiology laboratory. Further studies are needed to evaluate this approach for other implants, such as shoulder prostheses.

Supplementary material

A table providing further details of each patient diagnosed with infection and/or with positive cultures is available alongside the electronic version of this article on our website www.bjj.boneandjoint.org.uk

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