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ARTICLE

Prolonging culture to 15 days improves bacterial detection in bone and joint infections

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Abstract Since the optimal incubation period of cultures for diagnosis of bone and joint infections is still a matter of debate, the present study aimed to evaluate the effects of different incubation periods (5 and 15 days) on microbial isolation. Samples from 387 patients with bone and joint infections (including prosthetic ones) were analyzed from March 2012 to February 2014. In 197 patients (51 %) growth was obtained within 48 hrs, while in 124 (32 %) and 66 (17 %) patients cultures yielded positive results within and after 5 days of incubation, respectively. Of 449 microorganisms isolated, 247 grew within 48 hrs, 131 within the first 5 days of incubation while 71 were isolated after 5 days. *Staphylococcus aureus* was the most frequently isolated pathogen within 48 hrs, while *Propionibacteria* were prevalently isolated after 5 days of incubation. Interestingly, about 25 % of microorganisms isolated after 5 days of incubation were coagulase-negative staphylococci. Extending incubation period of broth cultures improves isolation rates of pathogens involved in bone and joint infections thus improving management of these infections.

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Introduction

Bone-joint infections are associated with difficult clinical and surgical management, severe complications, long hospital stay and high costs to society [1].

The cornerstone of the diagnosis of bone or joint infections is the identification of a microorganism from a bone, a contiguous anatomical structure or from a blood culture. Diagnosis of osteo-articular infections, particularly of the prosthetic ones (PJIs), is still a challenge for clinicians and microbiologists, although several improvements in their detection have been introduced in the recent past. Overall, cultures from bones, soft tissue biopsies, prosthetic components, subperiosteal exudates or joint fluids provide a microbiological diagnosis in only 30–80 % of cases [2]. Molecular methods may shorten the time needed for an etiological diagnosis [3], nevertheless a limit is that specific assays are not available for all microorganisms, especially in late infections. Nonetheless, culture methods are indispensable because they allow the determination of antibiotic susceptibility [4].

A variety of culture incubation periods have been reported in the literature. Typically, the majority of bacterial cultures of tissues and fluids obtained from sterile sites are incubated for 5 days or less [5, 6]. Antimicrobial therapy before sampling, slow and fastidious growth of microorganisms and changes in metabolism due to growth in biofilm are among the reasons for a high rate of false negative results of culture [3]. Therefore, a number of studies have demonstrated the importance of extending culture incubation beyond one week [7–9].

The aim of the present study was to evaluate if prolonging samples' incubation from 5 to 15 days may contribute to increased rate of microbial isolation.

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Methods

Sample collection and analysis

Samples (prostheses, tissue specimens, synovial fluids) were taken as part of standard care and sent to the Laboratory of Clinical Chemistry and Microbiology of IRCCS Galeazzi Orthopaedic Institute of Milan, Italy, from March 2012 to February 2014. The study was approved by the Ethics Committee of the Institute and was performed in accordance with the Helsinki Declaration. Written consent was obtained by all patients before enrolment into the study.

Prostheses were considered infected if the same pathogen was isolated by cultures from at least two separate samples obtained from the affected prosthetic joint [10, 11].

Tissue samples and prosthetic implants were aseptically collected in the operating theatre and put in plastic sterile containers with no transport media. Synovial fluid (at least 1 mL) was obtained through intraoperative aspiration and collected in sterile tubes. For each patient, 3–7 samples were collected.

Prostheses were processed by sonication until the introduction of treatment with dithiothreitol (DTT) in routine microbiological diagnosis in February 2013 [12]. Sonication was performed as described by others [13]. The sonicated fluid was accurately resuspended, collected in sterile tubes and centrifuged at 3000 rpm for 10 minutes at 4 °C. DTT treatment was carried out as previously described [13]. Synovial fluid was centrifuged at 3500 rpm for 10 minutes at 4 °C. Tissue specimens were washed with phosphate buffered saline (PBS), stirred with an orbital shaker for 10 minutes and then centrifuged at 3000 rpm for 10 minutes at 4 °C. The pellet obtained after centrifugation of all samples (sonicated or DTT-treated implants, tissues and synovial fluids) was resuspended in a volume of about 1.5 mL.

One hundred microliters from each sample were plated onto Chocolate Agar (CA), McConkey Agar (MC), Mannitol Salt Agar (MSA), Sabouraud Agar (SAB), Brain Heart Infusion Broth (BHI) (BioMerieux, Marcy L'Etoile, France) and Thioglycollate Broth (TH) (Oxoid, Milan, Italy). CA was incubated at 37 °C for 24 hours in 10 % CO_2 enriched atmosphere, MC at 37 °C for 24 hours, MSA and SAB at 37 °C for 48 hours, while BHI and TH were incubated for 15 days at 37 °C and daily controlled for microbial growth. Aliquots from broths showing visible turbidity were plated on Blood Agar (BA) and, in the case of TH, also on Schaedler Blood Agar (SBA) (BioMerieux). BA was incubated at 37 °C for 24 hours in 10 % CO_2 enriched atmosphere, while SBA was incubated in anaerobiosis at 37 °C for 48–72 hours. On the 15th day each broth was vortexed for 30 seconds and 100 μL were plated on BA and SBA plates. Identification of the isolates was carried out by Vitek2 Compact (BioMerieux).

Comparison of 5 days incubation between chocolate agar plates and broths

To compare prolonged incubation of agar plates with that of broths, in a parallel arm of the study, samples from 250 patients were plated on CA and inoculated in BHI and TH broths. CA plates were incubated for 5 days at 37 °C in 10 % enriched atmosphere while broths were maintained for 15 days at 37 °C in aerobiosis. Plates and broths were daily checked for microbial growth. Broths were plated on BA plates in case of turbidity and after 5 and 15 days of incubation.

Results

Prolonged incubation of broths

Samples collected from 1,039 patients (mean age±SD: 63.9± 15.6 years, range 24–82 years; 628 females and 411 males; 810 with suspected PJI and 229 with suspected osteomyelitis) were analyzed. In 652 patients (62.8 %) no microbial growth was observed, while samples from the remaining 387 (37.2 %; 291 with suspected PJI and 96 with chronic/acute osteomyelitis) yielded positive cultures. These latter were retrospectively analyzed: in 197 patients (51 %) growth was obtained both on agar plates and in broth subcultures, while 124 (32 %) and 66 (17 %) patients had positive broths within and after 5 days of incubation, respectively, without differences between 5 and 15 days of incubation. Positive cultures obtained from agar plates, 5 days of broth incubation and more than 5 days of broth incubation were 21.6, 68.4 and 9.5 % for prostheses, 23.5, 67.4 and 9.1 % for biopsies and 20.8, 67.4 and 11.3 % for synovial fluids, respectively.

On the whole, 449 microorganisms were isolated: 247 (55 %) grew on agar plates, 131 (29.2 %) were obtained from broths in the first 5 days of incubation while 71 (15.8 %) were isolated when incubation lasted more than 5 days. Sixty-two patients (16 %) presented a polymicrobial infection.

All the microorganisms isolated are listed in Table 1: the species most frequently isolated was *S. aureus* (25.7 %), which in most cases grew on agar plates (82.8 % vs 13.8 % within 5 days and 3.4 % after 5 days). *Propionibacterium acnes* (32.4 %) was the microorganism mostly identified from broths after 5 days of incubation; it was isolated in 23 (92 %) patients after 5 days and only in two (8 %) within 5 days of incubation. Also coagulase-negative staphylococci (CoNS) other than *S. epidermidis* were often isolated after 5 days (18.3 %), while *S. epidermidis* was more frequently isolated within 5 days of incubation (39.7 %).

Other bacteria that were found almost exclusively after 5 days of enrichment were *Lactococcus spp.*, *Granulicatella*

Table 1 Microorganisms isolated from patients with bone-joint infections

Microorganism	Total (n)	Agar plates		Within 5 days		After 5 days	
		n	%	n	%	n	%
S. aureus	116	96	82.8	16	13.8	4	3.4
S. epidermidis	77	20	26	52	67.5	5	6.5
Other CoNS	60	17	28.3	30	50	13	21.6
Streptococci	26	18	69.2	6	23.1	2	7.7
Enterococci	26	18	69.2	7	26.9	1	3.9
Pseudomonas spp	25	20	80	4	16	1	4
E. coli	13	13	100	–	–	–	–
Other Enterobacteria	26	21	80.8	5	19.2	–	–
Acinetobacter spp	11	10	90.9	–	–	1	9.1
Corynebacterium spp	9	1	–	3	–	5	–
Bacillus spp	6	1	–	2	–	3	–
Sphingomonas paucimobilis	4	–	–	1	–	3	–
Candida spp	5	5	–	–	–	–	–
Ralstonia pickettii	2	–	–	1	–	1	–
Micrococcus spp	2	–	–	–	–	2	–
S. maltophilia	2	2	–	–	–	–	–
Granulicatella spp	2	–	–	–	–	2	–
Aerococcus viridans	2	1	–	1	–	–	–
Lactococcus spp	1	–	–	–	–	1	–
Arcanobacterium spp	1	–	–	–	–	1	–
Moraxella spp	1	–	–	1	–	–	–
Pasteurella spp	1	1	–	–	–	–	–
P. acnes	25	–	–	2	8	23	92
Actinomyces	4	1	–	–	–	3	–
Total	449	247	55	131	29.2	71	15.8

spp., *Sphingomonas paucimobilis*, *Actinomyces spp.* and corynebacteria.

Comparison between 5 days incubation of solid media and enrichment broths

Results obtained from agar plates and broths after 5 days incubation are reported in Table 2. Concordance between agar plates and broths was obtained in 186/250 patients (74.4 %): in 29 (11.6 %) cases microbial growth was observed for both plates and broths, while for the remaining 157 patients, samples did not yield microbial growth. Discordant results were observed for samples from 64 patients. In 14 of them, growth on solid media was not followed by that in broths; bacteria isolated were CoNS and propionibacteria, and these microorganisms were considered contaminants in most cases, because they grew only from one of multiple samples. Moreover, these patients did not show any other clinical sign suggestive of infection. Instead, in the remaining 50 patients only broths became positive. For these patients, we considered number and type (prosthesis, tissue or synovial fluid) of specimen, pre-operative C-reactive protein and erythrocytes sedimentation rate values and clinical signs to determine if they were suggestive for infection or for merely contamination, according to common definition of prosthetic infection [11], whereby 41 of them (82 %) met these criteria.

Table 2 Comparison between results obtained after 5 days incubation of solid media and broths

Solid media	Broths	Number (*n*)	Percent (%)
Negative	Negative	157	62.8
Positive	Negative	14	5.6
Negative	Positive	50	20
Positive	Positive	29	11.6

Discussion

Apart from the notable technical improvement of recent years with development of methods able to dislodge microorganisms from biofilms, the optimal period of culturing samples

for diagnosis of bone and joint infections is still a matter of debate.

Generally, standard culture techniques contemplate a short period of broth incubation (5 days) or prolonged incubation of agar plates, which may not meet the requirements for reliable identification of slowly growing organisms [9]. On the other hand, a few authors suggested that an incubation period of 2 weeks is a promising approach toward optimization of infection diagnostics [8, 14].

Therefore, the aim of this study was to investigate the utility of extended culture incubation in an orthopaedic institute and to evaluate the differences between bacterial species isolated within and after 5 days of media incubation.

Our results showed that without the prolongation of incubation times, for a significant number of patients (66, i.e. 17 % of positive patients, 6.4 % of all patients) cultures would have yielded negative results. Therefore, extending incubation period really improves isolation rates of pathogens involved in bone and joint infections.

Microorganisms isolated on agar plates, within and after 5 days of incubation, were not the same. The most noticeable difference between agar plates and enrichment broths after 5 days was that bacterial species commonly associated with orthopaedic infections (*S. aureus*, *Pseudomonas aeruginosa*, *Escherichia coli* and other enterobacteria, enterococci) were mainly isolated from agar plates, while prolonging incubation allowed the isolation of unusual bacteria. The real clinical significance of lactococci, micrococci, *Ralstonia spp.* or *Sphingomonas* spp. may be questionable since, in some cases, their retrieval could be caused by sample contamination, which might occur during sample collection or subsequent treatment before plating. The role of *Arcanobacterium* and *Bacillus spp* has not been clearly established and is limited to a few reports [15–19], while *Corynebacterium, Actinomyces* and *Granulicatella* species are occasional causes of PJIs and *Acinetobacter* is an emerging nosocomial pathogen, which has also been isolated from osteomyelitis and PJIs [20, 21].

In our study, the microorganism most frequently isolated after 5 days of incubation was *Propionibacterium spp*, a commensal bacterium of the deep layers of skin, respiratory, digestive and eye mucosa with low level of virulence, but able to cause a range of postoperative and device-related infections [22]. *P. acnes* was isolated with high frequency in our setting, compared with other investigations. The 15-day incubation period in our study provides a reasonable explanation for this finding, because propionibacteria were detected almost exclusively during the second week of culture.

Interestingly, about 25 % of microorganisms isolated after 5 days of incubation were coagulase-negative staphylococci (CoNS). This is a very interesting result, since in literature there are scarce data on the importance of prolonged cultures for the isolation of CoNS; only a very old study reports that the most common "broth only isolates" (microorganisms which grew only in the inoculated thioglycollate broth and not on the directly inoculated solid media) were CoNS [13]. The role of CoNS in healthcare-associated infections has been acknowledged in recent years, accounting for 30–40 % of PJIs [23–25].

A recent study states that the use of prolonged incubation times for solid media failed to increase the detection of implant-related infections [5]. Therefore for 250 patients we extended incubation of AC plates. Our findings only partially confirm results of Esteban et al.; in fact, AC plates of 17.2 % of patients became positive after the first day of incubation. Only in a few cases the growth on solid media was not followed by a growth in broths and microorganisms grown only on AC plates were often contaminants.

Anyway, in the remaining cases, in which growth on AC was confirmed by broths, prolonged incubation times for solid media allowed the detection of a certain percentage (11.6 %) of positivity.

However, since in most cases growth on AC plates after the first day of incubation was confirmed by that in broths, longer incubation times of broths seems to be preferable to a prolongation of solid media incubation.

In conclusion, extending incubation period of broths improves isolation rates of pathogens involved in bone and joint infections thus allowing determination of susceptibility profiles in order to ameliorate management of these infections. Moreover, prolonged incubation of broths greatly increases the detection of infections in comparison to solid media.

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Marín M, Esteban J, Meseguer MA, Sánchez-Somolinos M (2010) Microbiological diagnosis of bone-joint infections. Enferm Infecc Microbiol Clin 28:534–540
2. Mitha A, Boulyana M, Hue V, Pruvost I, Martinot A, European French-speaking expert group, Dubos F (2012) Consensus in diagnostic definitions for bone or joint infections in children by a Delphi method with European French-speaking experts. Acta Paediatr 101: 350–356
3. Rak M, Barlič-Maganja D, Kavčič M, Trebše R, Cor A (2013) Comparison of molecular and culture method in diagnosis of prosthetic joint infection. FEMS Microbiol Lett 343:42–48
4. Larsen LH, Lange J, Xu Y, Schønheyder HC (1995) Optimizing culture methods for diagnosis of prosthetic joint infections: a summary of modifications and improvements reported since 1995. J Med Microbiol 61:309–316
5. Esteban J, Alvarez-Alvarez B, Blanco A, Fernández-Roblas R, Gadea I, Garcia-Cañete J, Sandoval E, Valldazo M (2013) Prolonged incubation time does not increase sensitivity for the diagnosis of implant-related infection using samples prepared by sonication of the implants. Bone Joint J 95(Suppl 7):1001–1006

6. Thomson RB (2007) Specimen collection, transport, and processing: bacteriology. In: Murray PR, Baron EJ, Jorgensen JH, Landry ML, Pfaller MA (eds) Manual of clinical microbiology, 9th edn. ASM Press, Washington, pp 291–333
7. Butler-Wu SM, Burns EM, Pottinger PS, Magaret AS, Rakeman JL, Matsen FA III, Cookson BT (2011) Optimization of periprosthetic culture for diagnosis of *Propionibacterium acnes* prosthetic joint infection. J Clin Microbiol 49(Suppl 7):2490–2495
8. Schäfer P, Fink B, Sandow D, Margull A, Berger I, Frommelt L (2008) Prolonged bacterial culture to identify late periprosthetic joint infection: a promising strategy. Clin Infect Dis 47:1403–1409
9. Sendi P, Frei R, Maurer TB, Trampuz A, Zimmerli W, Graber P (2010) *Escherichia coli* variants in periprosthetic joint infection: diagnostic challenges with sessile bacteria and sonication. J Clin Microbiol 48:1720–1725
10. Parvizi J, Zmistowski B, Berbari EF, Bauer TW, Springer BD, Della Valle CJ, Garvin KL, Mont MA, Wongworawat MD, Zalavras CG (2011) New definition for periprosthetic joint infection: from the Workgroup of the Musculoskeletal Infection Society. Clin Orthop Relat Res 469:2992–2994
11. Musculoskeletal Infection Society (2013) International consensus on periprosthetic joint infection. http://www.msis-na.org/international-consensus/. Accessed 17 Nov 2014
12. Drago L, Signori V, De Vecchi E, Vassena C, Palazzi E, Cappelletti L, Romanò D, Romanò CL (2013) Use of dithiothreitol to improve the diagnosis of prosthetic joint infections. J Orthop Res 31:1694–1699
13. Kobayashi H, Oethinger M, Tuohy MJ, Procop GW, Bauer TW (2009) Improved detection of biofilm-formative bacteria by vortexing and sonication: a pilot study. Clin Orthop Relat Res 467:1360–1364
14. Levy PY, Fenollar F, Stein A, Borrione F, Cohen E, Lebail B, Raoult D (2008) *Propionibacterium acnes* postoperative shoulder arthritis: an emerging clinical entity. Clin Infect Dis 46:1884–1886
15. Cazanave C, Greenwood-Quaintance KE, Hanssen AD, Patel R (2012) Corynebacterium prosthetic joint infection. J Clin Microbiol 50:1518–1523
16. Hedke J, Skripitz R, Ellenrieder M, Frickmann H, Köller T, Podbielski A, Mittelmeier W (2012) Low-grade infection after a total knee arthroplasty caused by *Actinomyces naeslundii*. J Med Microbiol 61:1162–1164
17. Mougari F, Jacquier H, Berçot B, Hannouche D, Nizard R, Cambau E, Zadegan F (2012) Prosthetic knee arthritis due to *Granulicatella adiacens* after dental treatment. J Med Microbiol 62:1624–1627
18. Loïez C, Tavani F, Wallet F, Flahaut B, Senneville E, Girard J, Courcol RJ (2009) An unusual case of prosthetic joint infection due to *Arcanobacterium bernardiae*. J Med Microbiol 58:842–843
19. Reboli AC, Bryan CS, Farrar WE (1989) Bacteremia and infection of a hip prosthesis caused by *Bacillus alvei*. J Clin Microbiol 27:1395–1396
20. Hischebeth GT, Wimmer MD, Molitor E, Seifert H, Gravius S, Bekeredjian-Ding I (2015) Multidrug resistant *Acinetobacter baumanii* reaches a new frontier: prosthetic hip joint infection. Infection 43:95–97
21. Zurawski DV, Thompson MG, McQueary CN, Matalka MN, Sahl JW, Craft DW, Rasko DA (2012) Genome sequences of four divergent multidrug-resistant *Acinetobacter baumannii* strains isolated from patients with sepsis or osteomyelitis. J Bacteriol 194:1619–1620
22. Zeller V, Ghorbani A, Strady C, Leonard P, Mamoudy P, Desplaces N (2007) *Propionibacterium acnes*: an agent of prosthetic joint infection and colonization. J Infect 55:119–124
23. Morris AJ, Wilson SJ, Marx CE, Wilson ML, Mirrett S, Reller LB (1995) Clinical impact of bacteria and fungi recovered only from broth cultures. J Clin Microbiol 33(Suppl 1):161–165
24. Zimmerli W, Trampuz A, Ochsner PE (2004) Prosthetic joint infections. N Engl J Med 351:1645–1654
25. Drago L, De Vecchi E, Cappelletti L, Mattina R, Vassena C, Romanò CL (2014) Role and antimicrobial resistance of staphylococci involved in prosthetic joint infections. Int J Artif Organs 37:414–421