



# An update about molecular biology techniques to detect orthopaedic implant-related infections

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- Despite different criteria to diagnose a prosthetic joint infection (PJI), aetiological diagnosis of the causing microorganism remains essential to guide treatment.
- Molecular-biology-based PJI diagnosis is progressing (faster, higher specificity) in different techniques, from the experimental laboratory into clinical use.
- Multiplex polymerase chain reaction techniques (custom-made or commercial) provide satisfactory results in clinical series of cases, with specificity close to 100% and sensitivity over 70–80%.
- Next-generation metagenomics may increase sensitivity while maintaining high specificity.
- Molecular biology techniques may represent, in the next five years, a significant transformation of the currently available microbiological diagnosis in PJI.

**Keywords:** microbiological cultures; molecular diagnosis of PJI; prosthetic joint infection (PJI)

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## Introduction

Orthopaedic prosthetic joint infection (PJI) is a specific type of infection related to joint replacement and associated with biofilm formation on the surface of the inert implant. With an overall incidence between 1% and 5% depending on the joint, PJI is a complex entity that differs from bone and joint infections because the colonized implant becomes a persistent reservoir of microorganisms, increasing the difficulty to successfully diagnose and treat the infection.<sup>1</sup>

Microbiological analysis is currently the most reliable tool to orient PJI treatment. The etiological diagnosis, based on microbiology, leads to the specific antibiotic treatment, which is the most important adjuvant treatment to surgery in PJI since the early days of implant arthroplasty in orthopaedics. More than 40 years ago, problems

in the diagnosis of PJI were already recognized.<sup>2,3</sup> Particularly, adequate sampling and processing was praised as a method to obtain the most effective diagnosis,<sup>4</sup> while Gram staining, microbiological cultures and histopathology were the most recognized diagnostic techniques.<sup>5</sup> Bacterial colonization of orthopaedic implants was also identified as a major challenge in the prevention, diagnosis and treatment of PJI. The ‘race for the surface’ and the recognized pathophysiological role of the biofilm were fundamental steps towards today’s conception of PJI.<sup>6</sup>

The prominent role of microorganism identification to confirm infection also prevails in PJI, and different diagnosis guidelines have been discussed and proposed<sup>7–10</sup> by different organizations (Infectious Diseases Society of America – IDSA; Musculoskeletal Infection Society – MSIS; International Consensus Meeting of Philadelphia – ICM; European Bone and Joint Infection Society – EBJIS). Widely accepted major criteria of PJI include sinus tract communicating with the prosthesis or the identification of the same microorganisms isolated from two or more cultures, although some differences are found in the minor criteria, under continuous revision and improvement.<sup>9</sup>

Even if repeated twice to confirm, conventional culture techniques have limitations in terms of identifying microorganisms in prosthetic infection. False negatives (negative cultures) range from 5% to 42% of PJI, as seen in a recent systematic review,<sup>11</sup> due to sampling, prior antibiotics, insufficient culture time, chronic infections and probably other unknown causes,<sup>11–13</sup> jeopardizing the diagnosis and treatment of PJI. But cultures and diagnostic tests also produce false positives, detecting contaminant and secondary microorganisms or indirect infection signs that may not guide the most adequate treatment. Sampling may compromise the primary causing microorganism and alter the priorities to adequately treat each case. Without an appropriate aetiological diagnosis identifying the causing microorganism in PJI, the adequate treatment may not be established, including timely surgery and a precisely oriented antibiotic chemotherapy. Sensitivity and specificity have substantially improved, particularly

**Table 1. Techniques applied in prosthetic joint infection novel diagnostic strategies**

Cultures <sup>21–26</sup>
– Culturing after sonication/rinsing
– Culturing in blood-culture enriched media
Imaging techniques <sup>27–29</sup>
– Fluorescent in situ hybridization (FISH)
– Confocal laser scanning microscopy (CLSM)
– Scanning electron microscopy (SEM)
Biomolecular techniques <sup>30–36</sup>
– PCR-based methods, including multiplex PCR and DNA microarrays
– Electrospray ionization (ESI-TOF) and matrix-assisted laser desorption ionization (MALDI-TOF) time of flight mass spectrometry
– Fourier transformed near infrared (FT-NIR) spectroscopy
– Next-generation sequencing (NGS) based on shotgun metagenomics

Note. PCR, polymerase chain reaction.

with sonicate fluid after centrifugation<sup>14</sup> and samples in blood culture bottles.<sup>15</sup> However, the inherent delays of these diagnostic techniques still suppose a limitation to accurately guide decisions on antibiotics and surgery for an appropriate management of these patients.

As the diagnosis of the microorganism causing PJI is frequently delayed and incomplete, with variable false negatives that limit the treatment orientation and its efficacy, this review will focus on molecular diagnosis methods claimed to potentially improve this aetiological diagnosis.

## From culture-based methods to new strategies

Almost all microorganisms can be the cause of PJI. Despite Gram-positive bacteria (especially *Staphylococci*) being the most frequently isolated organisms, an increase of infections caused by Gram-negative has been described in recent years.<sup>16,17</sup> Another special concern today is the growing development of antimicrobial resistance among these organisms, definitively impacting on the selection of antibiotics or treatment strategies, but also compromising the outcome.<sup>18,19</sup> Furthermore, polymicrobial infection, often underdiagnosed because of technical limitations and competing growth in cultures, poses a supplementary problem in understanding the course of some recalcitrant infections. The presence of biofilm as an essential pathogenic factor is also an important issue for the selection of microbiological diagnostic methods.

To diagnose biofilm-related infections, novel techniques have been proposed to improve bacteria detection. These include modified culturing techniques (from samples obtained through sonication or other methods), visualization of biofilm through different microscopy techniques (basically for experimental studies), and finally, molecular diagnosis (Table 1). Some of these sophisticated techniques mostly rely on experimental data, and

thus are not, or may not be, appropriate for standard clinical application. Others are just not well-known or have not been broadly introduced into hospitals because of logistics or organizational issues. New techniques have direct associated costs that may limit their expansion, although in the medium term, increased diagnostic accuracy will probably prove cost-effective if it helps to avoid direct and indirect extra costs due to overtreatment when the specific infection is not ascertained, or delayed treatment when infection is not detected. As effectiveness is further improved and knowledge about the clinical meaning increases, these novel techniques will spread and cost-benefit analysis will probably confirm their interest.<sup>20</sup> A better understanding of those that can be clinically applied may offer the clinician more grounds to decide what is to be expected from new diagnostic techniques and eventually decide on their application.

## Biomolecular techniques

The use of molecular biology techniques, perhaps the most important advance for decades in microbiological diagnosis, is quickly spreading for PJI diagnosis, as in other areas of clinical microbiology and particularly in virology. Ideally, all infections could be detected with the highest sensitivity and specificity of these techniques, but the reality is not so clearly positive. Current polymerase chain reaction (PCR)-based techniques detect traces of microorganism nucleic acids. When applied in sonicate fluid,<sup>33,34</sup> these techniques have shown notable effectiveness in diagnosing the infective microorganisms from biofilm, with higher sensitivity than PCR from periprosthetic tissue,<sup>37</sup> but mostly under experimental conditions. Two different approaches (custom-made or commercial) can be described.

### Custom-made PCR

Custom-made methodologies, based mainly in 16S rDNA amplification and sequencing, have high sensitivity and specificity,<sup>38,39</sup> and even high reproducibility is described in some reports.<sup>40,41</sup> These later studies are extremely important, because a well-known claimed limitation of this technology is the lack of reproducibility. In the study by Plouzeau et al<sup>40,41</sup> a control was submitted to different laboratories that have previously published the multicentre study.<sup>40,41</sup> The results of reproducibility showed that, in well-trained experienced laboratories, this approach could be very useful and reproducible. Moreover, because this method could identify almost all existing bacteria, it has a very high potential for diagnosis. However, a high-quality molecular biology laboratory with experimented technicians is needed, and most medium and small-sized hospitals may not encompass these facilities. Besides, in case of polymicrobial infections, a custom-made technique

**Table 2.** Comparison of studies on multiplex PCR kits commercially available, both specific for bone and joint infection or adapted blood-culture kits

Reference	Kits in use (bone and joint infection specific†, or adapted ††)	Type of samples for PCR	Patients total and PJI	Sensitivity	Specificity	PPV	NPV
Esteban et al <sup>33</sup>	Adapted <sup>4</sup>	Sonicate fluid	126 pt (47 PJI)	71.6	81.9	74.3	79.7
Achermann et al <sup>42</sup>	Adapted <sup>3</sup>	Sonicate fluid	47 pt (37 PJI)	78.4	100.0	100.0	55.5
Portillo et al <sup>43</sup>	Adapted <sup>3</sup>	Sonicate fluid	86 pt (24 PJI)	96.0	100.0	100.0	98.4
Metso et al <sup>44</sup>	Specific <sup>2</sup>	Synovial fluid, tissue	81 pt (38 PJI)	81.6	100.0*	100.0	74.1
Vasoo et al <sup>45</sup>	Adapted <sup>5</sup>	Sonicate fluid	216 pt (98 PJI)	53.0 <sup>58**</sup>	99.0	—	—
Borde et al <sup>46</sup>	Specific <sup>1</sup>	Tissue	28 pt (7 PJI)	42.8	95.2	75.0	80.0
Hischebeth et al <sup>47</sup>	Specific <sup>1</sup>	Sonicate, synovial fluid	31 pt (18 PJI)	66.7	100.0	100.0	68.4
Renz et al <sup>48</sup>	Specific <sup>1</sup>	Synovial fluid, tissue	111 pt (78 PJI)	53.3	94.0	95.0	47.0
Prieto-Borja et al <sup>49</sup>	Specific <sup>1</sup>	Sonicate fluid	68 pt (29 PJI)	60.5	98.0	95.8	76.6
Mandalain et al <sup>50</sup>	Specific <sup>1</sup>	Tissue, synovial fluid	239 pt	49.1	99.4	99.3	51.5
Morgestern et al <sup>51</sup>	Specific <sup>1</sup>	Synovial fluid	142 pt (77 PJI)	65.8	92.1	91.2	65.2
Renz et al <sup>52</sup>	Specific <sup>1</sup>	Tissue, sonicate, synovial fluid	51 pt (38 PJI)	77.0	92.0	96.0	60.0
Sigmund et al <sup>53</sup>	Specific <sup>1</sup>	Tissue, sonicate, synovial fluid	90 pt (38 PJI)	71.1	96.2	93.1	82.0
Suren et al <sup>54</sup>	Specific <sup>1</sup>	Synovial fluid	26 pt (15 PJI)	78.6	100.0	91.7	84.6

Note. PCR, polymerase chain reaction; PJI, prosthetic joint infection; PPV, positive predictive value; NPV, negative predictive value.

†Bone and joint specific kits used in these studies: Unyvero i60 ITI (Curetis AG, Germany),<sup>1</sup> Mobidiag (Mobidiag, Finland).<sup>2</sup>

††Adapted (general kits initially conceived for blood-borne microorganisms), used in these studies for PJI: SeptiFast™ (Roche, Switzerland),<sup>3</sup> GenoType™ (Hain, Germany),<sup>4</sup> or Filmarray™ (Biofire, USA).<sup>5</sup>

\*81 pt (only 38 confirmed PJI, only 20 confirmed controls, six false positive PCR in non-confirmed PJI, no false positives in controls).

\*\*53% overall sensitivity, improved to 58% when considering only microorganisms included in the panel (non-specific test).

could have problems identifying different pathogens. And finally, the arrival of commercial multiplex PCR has limited the use of custom-made techniques, although commercial kits initially conceived for blood-borne microorganisms have also been adapted for PJI and published from many laboratories (Table 2).

### Commercial multiplex PCR

Commercial techniques may be more robust, and do not require special infrastructures. Table 2 offers a comparison of studies with commercially available multiplex PCR kits. Early studies were based on customized kits already designed for the identification of microorganisms isolated from blood culture bottles, such as SeptiFast™ (Roche, Switzerland), GenoType™ (Hain, Germany), Xpert™ (Cepheid, USA), or Filmarray™ (Biofire, USA).<sup>33,42,43,45,55</sup> These include most of the microorganisms causing PJI. Despite relatively good results (high specificity in all cases), they are not used in most laboratories beyond the experimental studies. The reasons for this may include the cost, logistics for more cumbersome procedures, worries about the clinical significance for diagnosis, or even concerns about false positives. Recently, a commercial test was especially designed for the diagnosis of bone and joint infections (Unyvero i60i™, Curetis AG, Germany). The test not only detects microorganisms, but also resistance markers, which would represent a kind of ‘molecular anti-biogram’. The test (a cartridge easy-to-use methodology) was designed to avoid the main problem of molecular biology in this setting: the potential contamination with skin microorganisms that may also cause PJI. To reach

this objective, a high-specificity technique was designed, although with low sensitivity,<sup>44,46–54,56–58</sup> meaning that a positive result is indeed a true positive in most cases, but a negative result does not exclude infection. This is a very important issue to be integrated into the evaluation of the results, because only a positive result can be considered trustworthy. Furthermore, the method takes at least five hours to be completed, so it would not be valid for intraoperative diagnosis. Considering these issues, the kit could be used as a complement to conventional methodology that improves the overall specificity and sensitivity of all techniques taken together.

### Next-generation sequencing

New molecular methods based on metagenomics offer an interesting approach that will probably transform our knowledge on the pathogenesis and evolution of implant-related infections.<sup>59–69</sup> Next-generation sequencing has recently appeared as a method that theoretically avoids the problem of contamination, because no PCR is required. The technique detects the entire DNA present in the sample, and with the help of bioinformatics, it shows the detected sequences in a quantitative manner, and identifies them as different microbial species. The method, compared with conventional culture methods, including sonication,<sup>69</sup> allowed the authors to establish a threshold and differentiate between contaminants and true pathogens. This is a required step, because negative controls can give a low number of reads.<sup>69</sup> This group also reported a study where real-time results could be obtained using a specific platform.<sup>68</sup> When specifically

employed on periprosthetic tissue,<sup>70</sup> this method has shown higher sensitivity and specificity than microbial cultures (95% to 72%, and 90% to 77%). However, optimization of the whole process is necessary to implement its use, and would be of great interest if the process could be shortened to just some minutes after starting sequencing. In another study,<sup>71</sup> shotgun metagenomics could diagnose usual pathogens in 43% of culture-negative PJI, with a percentage of positive samples in non-infected patients of 3.6%, even with the use of a threshold. Finally, this technology has been employed in shoulder surgery,<sup>60</sup> where infections usually have a different pattern than in knee or hip prosthesis. They obtained also good results, but detected a higher number of polymicrobial infections whose clinical meaning needs further evaluation.

The authors of these studies expressed their concerns about the potential contaminants, because even unculturable, unviable pathogens may be detected,<sup>72</sup> and a strict methodology is recommended to avoid these undesired results.<sup>69</sup> However, the detection of these pathogens cannot be considered automatically as a contaminant, and the potential existence of a 'synovial microbiome' opens many questions that need further research. In this sense, a recent study<sup>73</sup> showed that antibiotic therapy guided by the results of metagenomic next-generation sequencing was associated with a similar outcome to empirical therapy, with fewer undesired side effects.

### Implementing new diagnostic techniques in clinical practice

Only limited evidence is available from level I and II diagnostic clinical studies regarding novel techniques to diagnose PJI. This is why only moderate-strength recommendation can be placed on these techniques to diagnose prosthetic joint biofilm infection. The use of PCR-based molecular biology methods poses questions regarding not only how many samples but also what an appropriate sample is. This remains to be clarified, as samples are obtained usually from synovial fluid, sonicate fluid after implant removal, and tissues where microorganisms are suspected to be present. Considering the high cost of PCR and the required time to deliver results for a large battery of potential pathogens, sample and patient selection is essential. Patients with negative cultures are those who immediately benefit from PCR, but the high specificity of PCR may also enable diagnosis from isolates with doubtful significance. The new metagenomics methodology is a step forward, so far experimental, that still requires understanding the relevance of all the microorganisms detected in a single sample. But a definite gap in these techniques is the time to obtain accurate information, currently unavailable within operative time. Technical progress will hopefully solve these issues.

As identification of microorganisms in periprosthetic tissue samples is enhanced through molecular biology techniques, despite potential low bacterial load, different techniques are progressing towards higher sensitivity and specificity. Intraoperative samples inoculated in blood culture bottles allowed an increased identification of bacterial reads,<sup>74</sup> although the need for a positive blood culture may slow the diagnosis. Of considerable interest is the clinically relevant preoperative diagnosis. Some techniques proved high sensitivity and specificity not only in periprosthetic tissue samples<sup>70</sup> but also in synovial fluids that can be preoperatively analysed.<sup>75</sup> This may open an interesting approach for a presurgical diagnosis of these patients.

Many questions still need to be solved in clinical practice. First of all, how to obtain the sample is key. Clinical suspicion during surgery leading to adequate sample collection may be determinant. Increased accuracy in synovial fluid sampling has been obtained when guided through computerized tomography (CT),<sup>76</sup> particularly when combined with image findings.<sup>77</sup> Percutaneous synovial biopsy is a good alternative to synovial fluid samples, although its real value is still debated.<sup>78,79</sup> Periprosthetic surgical biopsies prior to index surgery<sup>80</sup> appear a reasonable alternative to improve sensitivity, although this supplementary surgical procedure adds considerable burden to the case, because a revision procedure will be required anyhow.

Other problems about molecular diagnosis still to be solved include the meaning of all detected organisms, the necessity to treat and what organisms must be treated. Moreover, this diagnosis requires standardization, highly prepared laboratories, specialized personnel, adequate surgical sampling and planned workflows for samples from the operating rooms. All these issues may be difficult to implement in the current routine of the hospital, and specifically in a clinical microbiology laboratory.<sup>81</sup> Cost containment, above the costs of unsolved prosthetic joint infection, may not justify the barriers to spread the technology. However, confidence in the techniques and adequate training may be required to spread in clinical diagnosis. In the near future, probably all these questions can be answered and molecular-biology-based technologies may be added to the available microbiological tools for the diagnosis of PJI. Further research, especially aimed to avoid the contamination of samples and the implementation of standardized thresholds, will be necessary prior to its wide use in clinical laboratories. The evaluation of this methodology under routine conditions will be also of extreme importance.

Variability of the microorganisms and patients are difficult barriers in the precise diagnosis of PJI. Multicentric studies are probably required to standardize new techniques and diagnostic protocols in clinical routine.

Sampling protocols, understanding the best sensitivity for each technique, may reinforce the need of surgical standardization in the microbiological sampling. Comparing the effectiveness of each sampling (fluid or solid, from implant or tissue, preoperative and intraoperative) may help to establish these protocols. The specific request of a technique for a specific sample may expedite intraoperative diagnosis, while other samples (including the retrieved implant) may help to confirm, validate or reorient the associated treatment.

## Future research

Classic microbiological culture alone has probably reached its maximum effectiveness. An adequate combination of available technologies, implemented in a high number of hospitals, will increase our protocol experience to complete inter-centre comparisons and to develop multicentric studies. This evidence-based methodology is obviously slow, but will develop the next gold standard.

The combination of adequately prioritized and evaluated novel techniques will improve PJI diagnosis in the next five years. Both biofilm models and surgical sampling studies on biofilm developed on prosthesis will facilitate the earlier isolation of biofilm-forming microorganisms, guiding new and established treatment options. Molecular biology seems well placed for the future. Its high specificity unfortunately involves high costs and time, barriers to clinical routine and intraoperative use. But this technology is rapidly evolving. A new multiplex PCR assay under evaluation, based on the cartridge technology, may give results in one hour (within intraoperative time frame). Good preliminary results for the microorganisms included in the kit are already being shown.<sup>82</sup> Moreover, shotgun metagenomics are experiencing impressive advances and probably could be the next tool to be added to the microbiology lab. Other experimental molecular tools, such as electrospray ionization time of flight mass spectrometry (ESI-TOF) or Fourier transformed near infrared (FT-NIR) spectroscopy have been also used for the experimental diagnosis of these infections,<sup>30,31,35</sup> although these are still not ready to access the clinical diagnosis. On the contrary, matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF) has become an essential tool for bacterial and fungal identification in isolates from cultures, but not from direct clinical samples.<sup>32,36</sup> Expected technical advances, particularly when PCR has become a popular word outside laboratories, may offer significant opportunities in these next years.

However, with the increasing number of techniques, a closer relationship between clinicians and microbiologists is still the best approach in the final aim that has not changed for decades: to cure orthopaedic patients with prosthetic infections.

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