

Should modern molecular testing be routinely available?

Musculoskeletal infections are a feared and devastating collection of conditions; presenting clinical challenges at every stage of their pathway^{1–14}, including diagnosis. Multiple attempts have been made to standardise and define the diagnosis of musculoskeletal infections^{15–26}, with bacterial culture remaining central²⁷. However this reliance on culture as the gold standard for diagnosis has led to the clinical conundrum of ‘culture-negative’ infection, in which the clinical parameters of diagnostic criteria are fulfilled but no organisms can be isolated²⁸. The prevalence of culture-negative infections is approximately 10–30%^{23,29,30}. Proposed reasons for negative cultures include fastidious organisms with demanding growth conditions, low number of organisms in a slow growth mode, a patchy distribution of organisms, inadequate sampling, or insufficient resuscitation in the laboratory (enrichment media and prolonged duration of culture³¹). However, the most important cause of culture-negative infection is thought to be antibiotic administration prior to sampling^{32,33}. Sub-therapeutic antimicrobial therapy is known to induce a physiological state in many pathogens known as “viable but non-culturable”^{34–37}, rendering cultures falsely negative^{28,33}. This is a cellular state characterised by low metabolic activity and failure to grow on routine bacteriological media³⁸. A critical feature is that nutritional stimulation can restore metabolic activity and culturability, known as resuscitation^{39,40}.

The first step in optimising microbiological diagnostics is meticulous sampling. This applies to culture and molecular methods. In chronic bone and joint infections samples can be taken pre-operatively (aspirates, biopsies) and/or intra-operatively. For the latter, international recommendations are to take multiple (5) tissue sample for culture from different sites using separate sterile instruments⁴¹. This is in order to optimise sensitivity but also to improve specificity by differentiating true infecting organisms from contaminants. Two or more samples with indistinguishable organisms indicating a positive microbiological diagnosis. Hard parts (devices and hard bone) can also be sonicated with the sonication fluid being cultured quantitatively.

Molecular techniques, targeting bacterial deoxyribonucleic acid (DNA), have been proposed as a sensitive diagnostic modality, particularly in culture-negative cases. Polymerase chain reaction (PCR)-based assays can detect the DNA of specific target organisms and may increase sensitivity, as well as reducing the time to diagnosis^{15,42,43} in comparison to bacterial culture. Multiplexing allows a panel of common pathogens, and also potentially antimicrobial resistance determinants, to be sought simultaneously, however the requirement for specific primers will miss atypical or unexpected pathogens^{44,45} or resistance mechanisms. Sequencing of the 16s rRNA gene in contrast allows a broad range of bacteria to be identified, and in some studies has been found to be more sensitive in the detection of musculoskeletal pathogens than both bacterial culture and PCR-based techniques^{10,15,16}. Primers target highly conserved regions present in almost all bacteria⁴⁶ and variable regions in-between allow bacterial identification. However, no information is provided on antimicrobial resistance and polymicrobial infections can only be detected with high throughput sequencing rather than traditional capillary sequencing-based methods. A further limitation of 16s rRNA sequencing is its poor discriminatory power at the species level and for some genera of bacteria⁴⁶. PCR and 16rRNA methods can be applied to biopsies, aspirates, tissue samples or sonication fluid.

High-throughput metagenomic sequencing potentially overcomes many of the limitations of other molecular techniques, potentially providing pathogen-agnostic detection of full bacterial genomes including unculturable, unsuspected, and non-viable organisms⁴⁷. Sequencing directly from samples can provide more accurate diagnostic information compared to laboratory culture, as well

as detecting additional pathogens^{48–50} and providing additional information such as the presence of antimicrobial resistance genes⁴⁹. Studies so far of metagenomic sequencing have used fluid samples including sonication fluid. Tissue samples are more problematic because of the amount of bacterial DNA that hampers bacterial identification. Further research needs to be done to optimise and validate methods of microbial DNA extraction in such samples⁵¹.

With potentially improved sensitivity from molecular diagnostics, the risk of false positives rises. It is for this reason that, in chronic infections, multiple tissue samples are recommended for culture. Applying this principle, to optimise specificity molecular diagnostics ought also to be done on several samples. The exception might be when seeking a particular pathogen (*e.g. Coxiella burnettii*). In addition to meticulous intra-operative sampling, molecular methods based on amplification of DNA, require specialist laboratory workflows to minimise contamination. Therefore, caution should be exercised when diagnosing infection based on molecular methods alone in the absence of concurrently cultured organisms. Whilst culture-negative infections represent a significant proportion of chronic orthopaedic biofilm-related infections, care must be taken to prevent overtreatment and associated patient morbidity. Furthermore, the clinical relevance of previously unsuspected pathogens, diagnosed by molecular techniques, has yet to be established; with no consensus yet available for clinically-relevant detection thresholds for metagenomic sequencing data. Although proof-of-concept studies have shown that 16s rRNA sequencing can reduce turnaround times to less than 48 hours¹⁶ and metagenomic sequencing to less than three hours (albeit following an initial ~6 hours of sample preparation) whilst maintaining acceptable levels of diagnostic accuracy⁵², further refinements of processing pipelines are required to robustly account for contamination from host genomes, reagents, the laboratory environment, and to prevent bacterial contamination during sample preparation.

High running costs, complex laboratory and bioinformatic workflows, and dependency on batch-based processing remain the primary barriers to widespread adoption of molecular methods. Reliable identification of antimicrobial resistance across a range of pathogens remains a challenge for molecular methods with further research required. Once these techniques become cost effective the speed of detection associated with these technologies has the potential to provide a diagnosis in a more timely manner than traditional microbiological culture and in more infections too. The reduction of diagnostic time would have a significant impact on patient outcome by facilitating earlier commencement of targeted antimicrobial therapy. However, given the high costs and high morbidity associated with misdiagnosis of musculoskeletal infection, it is likely that even with current techniques it would be cost effective for all units treating musculoskeletal infection to use next generation molecular techniques for all cases where no pathogen is identified on initial culture, but infection is strongly suspected by other diagnostic criteria.

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