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## Molecular Methods for Diagnosis of Infective Endocarditis

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### Abstract

Infective endocarditis (IE) is a life-threatening disease associated with high mortality. Conventional microbiologic diagnosis is based mainly on culture-dependent methods that often fail because of previous antibiotic therapy or the involvement of fastidious or slowly growing microorganisms. In recent years, molecular techniques entered the field of routine diagnostics. Amplification-based methods proved useful for detection of microorganisms in heart valve tissue. More recently, they were applied to blood samples from patients with IE. Direct detection of microorganisms in valve specimens by fluorescence in situ hybridization allowed identification of the causative agent and simultaneous visualization of complex microbial communities. These techniques will gain more importance in the near future, provided that procedures are standardized and results are interpreted with caution. With this review, we intend to give an overview of the impact and limitations of molecular techniques for the diagnosis of IE, including a focus on recent developments.

### Keywords

Endocarditis; Molecular diagnostics; Blood culture; Heart valves; Fluorescence in situ hybridization; PCR; Culture-negative infectious endocarditis; Duke classification; Duke criteria; Bloodstream infections; 16S rRNA Amplification-based methods; Autoimmunohistochemistry; Serology Culture techniques; *Abiotrophia*; *Brucella*; *Bartonella*; *Coxiella*; HACEK group; *Tropheryma whippelii*

### Introduction

Infective endocarditis (IE) remains a major medical concern with an unchanged incidence and mortality over the past 30 years [1]. In contrast, the underlying pathology, predisposing factors, spectrum of species involved, and consequently the epidemiology and patient characteristics have changed considerably during the past decade [1–4]. Whereas IE used to

affect young adults with rheumatic valve disease, it has become more common in the elderly in recent years and associated with underlying diseases and health care procedures. Accordingly, the diagnostic tools, antibiotic therapy, and patient management need to be adapted; comprehensive guidelines and recommendations have been compiled by the respective national and international committees [5•,6–8].

The timely diagnosis of IE is crucial for the patient, but remains a challenge [9]. Its wide variety in symptoms and systemic signs result in late or misdiagnosed cases. The modified Duke criteria propose diagnostic algorithms that help to standardize and classify the diagnosis of IE [10,11]. Besides clinical and laboratory parameters, the main pillars of the Duke criteria are vegetations on the heart valves detected by echocardiography, and repetitive positive blood cultures. Unfortunately, in a substantial number of IE cases (2.5%–31%), cultures fail to grow the bacteria and the infectious agent remains undiagnosed [12•]. Two reasons account for blood culture–negative endocarditis (BCNE). First, in many critical clinical situations, empirical antibiotic therapy is administered prior to diagnosis or even before the patient is suspect of having IE. Blood cultures drawn under antibiotic treatment have a significantly lower sensitivity [13]. Second, fastidious or yet uncultured microorganisms that are regularly missed by our routine culture methods may be involved in BCNE cases. These comprise typically *Abiotrophia* spp, *Bartonella* spp, *Brucella* spp, *Coxiella burnetii*, *Listeria monocytogenes*, *Mycoplasma* spp, *Legionella* spp, *Tropheryma whippelii* and members of the HACEK group (*Aggregatibacter* spp [comprising *Haemophilus aphrophilus*, *Haemophilus paraphrophilus*, and *Actinobacillus actinomycetemcomitans*], *Cardiobacterium* spp, *Eikenella corrodens* and *Kingella* spp).

If the IE fails to resolve under antibiotic therapy, or if the hemodynamic complications require surgical intervention, the infected heart valves are removed and replaced by biologic or mechanical prostheses. Often in BCNE, the histologic examination of the resected heart valve may prove bacterial colonization, allowing the definite diagnosis of IE, but without revealing the identity of the microorganisms. This situation is a major problem for the choice of the optimal antibiotic regimen, which should be as specific and efficient as possible for this life-threatening disease. In view of a changing epidemiology and a growing number of resistant strains, the correct diagnosis of IE-causing pathogens has become even more important. Furthermore inadequate therapy carries the risk of relapse or re-infection after heart valve replacement, with a poor prognosis [14,15].

With the advent of molecular methods for the detection and identification of microorganisms, culture-independent techniques like nucleic acid amplification techniques have emerged into routine diagnostics.

An elaborate review of the advantages and technical challenges of molecular diagnostics of IE using the 16S rRNA gene was published previously in this journal [16•]. Therefore, we survey the potential and the limitations of the different diagnostic approaches with a focus on recent developments in the molecular in situ detection of bacteria.

## Conventional Microbiologic Diagnosis of Endocarditis

### Blood Cultures

Positive blood cultures are the only possibility to date that allows identification and antimicrobial susceptibility testing of the pathogen in IE without heart valve surgery. Therefore, withdrawal of three blood culture sets (each containing one aerobic and one anaerobic bottle) at several time points and, if possible, prior to antibiotic therapy is of utmost importance. Common problems of blood culture diagnostics are the risk of contamination and resulting lack of specificity. In particular, growth of skin flora, like

coagulase-negative staphylococci or *Propionibacterium* spp, might be difficult to interpret. The number of positive blood cultures with a respective pathogen can help with the decision, but results in a time-consuming diagnostic procedure. Because IE is often associated with low numbers of bacteria in the blood [12,17], this procedure may require additional days until growth is detected by the blood culture system, or may be not successful at all. As mentioned above, culture techniques fail because of the limited sensitivity of blood cultures from concomitant antibiotic therapy or for cases with fastidious microorganisms.

### Valve Culture

Obviously, resected heart valve tissue is available for diagnosis only if the patient undergoes heart valve replacement. This material is the most valuable because it is the focus of the infection and contains the highest number of bacteria. Although heart valve culture belongs to the major Duke criteria, its diagnostic value for isolation of the pathogens is limited, because culture of valves often fails. In a recent study [18], the results of heart valve cultures from patients with and without IE according to the Duke criteria were compared. Interestingly, 28.4% of the valve cultures from non-IE cases were positive. The sensitivity of heart valve cultures for the diagnosis of IE was 25.4% with a specificity of 71.6%. The authors concluded that routine culture of unselected patients should be avoided and culture results even of IE patients should be interpreted with caution.

Again, antibiotic therapy administered before surgery might account for failure of valve cultures. Also, the fact that the bacteria within the vegetations are embedded in organized communities, so-called microbial biofilms, can result in metabolically less active but viable bacterial populations that are missed in culture.

### Serology

The complement of culture-dependent methods by serologic techniques was shown to be useful for diagnosis of IE in cases of BCNE and for selected organisms such as *Coxiella* spp, *Bartonella* spp, *Brucella* spp, *Legionella* spp, and *Aspergillus* spp [19]. However, the tests differ in sensitivity and specificity, and the impact of serologic assays depends on the regional epidemiology of the causative agents. The introduction of automated systems in routine application for cases of BCNE has the potential to substantially improve diagnostic procedures and is being evaluated [20].

### Molecular Diagnostics

Since their advent, molecular techniques for the detection and identification of microorganisms have been increasingly used for diagnostics in clinical microbiology. The nucleic acid amplification techniques have a theoretical detection limit of as few as 1 to 10 microorganisms. However, the diagnostic sensitivity in clinical specimens is considerably lower and is difficult to determine. Critical points leading to false-negative results are the DNA extraction, which might have a different efficiency depending on the technique and cell wall of the bacteria, the amount of background DNA from bacteria or eukaryotic DNA from the patient, the size and abundance of the amplified fragment, and the presence of inhibitors. On the other hand, nucleic acid amplification techniques are prone to contamination because of the ubiquitous presence of contaminating bacterial DNA not only from the clinical specimen itself, but also from handling, reagents, and even plastic materials like tubes and pipette tips. Therefore, thorough controls included in every step of the diagnostic procedure, specific requirements regarding room separation, and trained personnel make amplification-based methods rather expensive and time consuming.

Among the different nucleic acid amplification techniques developed for sensitive detection of bacterial DNA, the polymerase chain reaction (PCR) is most commonly used and is

referred to as “PCR-based methods” in the following. A useful target gene is the 16S ribosomal DNA, which is composed of highly conserved regions and variable regions that allow for design of either broad-range PCR primers or genus- or species-specific primers. Sequencing of the 16S rRNA gene allows culture-independent phylogenetic classification of many bacteria, and to date it is the bacterial gene with the most sequences available in public databases. For a comprehensive survey of the potential and limitations of the use of the 16S rRNA gene for diagnosis of culture-negative endocarditis, see Madico et al. [16••]. However, other genes, such as the 23S rRNA gene, 16S–23S intergenic spacer, *rpoB*-gene, or other species-specific genes, have been successfully used for sensitive detection of bacteria in clinical specimens.

The fact that PCR-based methods amplify DNA implies that it does not differentiate between viable or dead bacteria as well as contaminating free bacterial DNA. Taken together, the results of any PCR-based method must be interpreted with caution and within the context of all diagnostic procedures and clinical data. Nevertheless, they can be of great value and are particularly promising for patients with IE. In 2001, it was proposed to add molecular-based diagnosis of nonculturable species to the Duke classification scheme as major criterion [21].

### **PCR-based Techniques for Detection of Microorganisms in Blood Specimens of IE Patients**

Standard diagnostic techniques for microbiologic diagnosis of sepsis syndrome include incubation of blood culture bottles, subculture, and biochemical identification. These procedures are time-consuming and contrast with the need for a rapid and exact identification of the pathogen to optimize the antimicrobial treatment. To speed up this process, highly sensitive PCR-based methods have been developed to identify microorganisms directly from blood samples without previous incubation. The first commercial assays are currently being evaluated for diagnosis of sepsis syndrome [22]. Results are discussed controversially, because the high level of sensitivity implies the risk to detect contaminations. In addition, PCR does not provide information about viability and may report detection of bacterial DNA as a positive result.

Because the level of bacteremia associated with IE is low and growth inhibition by antibiotic treatment occurs frequently, the application of PCR-based methods to detect microorganisms in blood samples may be advantageous. In a study conducted by Casalta et al. [23], bacteria were successfully identified directly in blood samples in BCNE cases using the SeptiFast test (Roche Diagnostics GmbH, Mannheim, Germany), a commercially available, broad-range, real-time PCR assay. Among 20 IE patients with negative blood cultures caused by previous antibiotic therapy, the assay detected three cases with *Streptococcus gallolyticus*, *Staphylococcus aureus*, and *Enterococcus faecalis*, respectively. The analytical specificity of the test was 100%. However, the test does not include the organisms of the HACEK group. Furthermore, the assay was less sensitive in cases of blood culture–positive endocarditis (BCPE) compared to routine blood cultures. Further molecular techniques and commercial assays have to be developed to account for the spectrum of causative agents and the low level of bacteremia that is characteristic of IE.

### **PCR-based Methods for Diagnosis of IE Using Heart Valve Tissue**

In a survey modified and supplemented from Baty et al. 2009 [24], we compiled exemplary studies investigating the impact of PCR-based methods using heart valve specimens for microbiologic diagnosis of IE (Table 1). In these studies, resected heart valves from patients with definite or suspected IE were subjected to broad-range PCR of the 16S rRNA genes and sequencing; in some studies, this was followed by more specific PCR for certain

species. Results were compared to data obtained by routine blood culture procedures. In total, 481 IE cases were investigated, including 110 (22.9%) cases of BCNE. In the group of BCNE, PCR identified microorganisms in 47 cases, corresponding to 42.7% of BCNE and 9.8% of total investigated cases. In the group of BCPE, ameliorative information was obtained by PCR-based methods in 47 cases (9.8% of IE cases), resulting in a reclassification in the Duke criteria scheme, specification of the causative agent, or resolution of disagreement within the standard diagnostic procedures. PCR-based methods yielded false-negative results compared to blood cultures in 55 cases (11.4% of IE cases).

In summary, all studies unanimously recommended the application of molecular techniques for microbiologic diagnosis of BCNE and questionable IE, such as cases classified as possible IE according to the Duke criteria. The use of molecular techniques could also clarify the diagnosis when contradictory data resulted from routine diagnostic procedures, when blood cultures were positive only once, or when species identification was unclear [25]. Especially fastidious, difficult-to-culture, or slowly growing microorganisms that were missed by routine culture-based methods could be detected by PCR-based methods. In the studies included in Table 1, *Tropheryma whippelii* (n=5), *Bartonella* spp (n=10), and *Coxiella* spp (n=4) were identified by PCR and sequencing in 19 cases of BCNE. Interestingly, in a recent study investigating BCNE cases of prosthetic valves, molecular and serologic methods revealed high prevalence of fungi that are missed by 16S rRNA-gene analysis [26]. Thus, the use of molecular techniques may also complement the hitherto incomplete information about the epidemiology and spectrum of causative agents of IE. For detection of easy-to-culture pathogens such as streptococci and staphylococci, PCR-based methods can be useful when growth is hampered by antibiotic treatment [27].

However, it must be emphasized that the data of the studies presented are complex and require careful interpretation. Several other studies point in the same direction, underlining the benefit of PCR-based diagnostics of valve tissue. However, they were not included in Table 1 because of differences in study design. Direct comparison may be misleading. Results are influenced by differences in regional epidemiology and discrepant inclusion criteria for IE cases, such as blood culture status, length of antibiotic therapy, or specimen characteristics (ie, native vs prosthetic heart valves). Techniques for broad-range PCR and sequencing differ in terms of amplification protocols, target regions, and length of the amplified fragment [16••]. In general, inter-laboratory differences and lack of standardization in PCR-based methods complicate interpretation of results and is an important concern when discussing the inclusion of molecular techniques into the Duke criteria [28].

Another challenging aspect in the evaluation of PCR-based methods for microbiologic diagnosis of IE is the identification of false-positive results. It has been shown that bacterial DNA can persist in heart valves under antibiotic therapy and after IE is cured [29]. The viability of these microorganisms is unclear, and the detection of bacterial DNA should be interpreted with caution, especially in heart valves from patients with a past history of treated IE [30]. On the other hand, metabolically inactive bacteria may reside in IE valve tissue and biofilms, causing relapse after weeks and months [31]. As stated above, broad-spectrum PCR is an extremely sensitive method that is principally prone to contamination. In studies included in Table 1, the specificity of the PCR assays varied from 95.3% to 100%, depending on the size of the control groups and the interpretation of inhibited or unreliable PCR results. Nomura et al. [32] investigated heart valves from IE patients using 16S rRNA broad-range PCR followed by cloning and sequencing, and detected multiple species in single specimens. Results were interpreted as microorganisms incidentally disseminated into the blood stream. Real-time PCR techniques, including crossing point and melting

temperature analysis, might have the potential to facilitate the interpretation of the data obtained [33,34].

In conclusion, PCR-based methods using heart valve tissue are of great value for diagnosis of the infectious agents in BCNE. Furthermore, they can clarify questionable cases of BCPE. We should make an effort to standardize the diagnostic procedure to make it available for more routine diagnostic laboratories. PCR-based methods cannot differentiate between viable and dead microorganisms and therefore are not suitable for assessment of treatment success. Presently, PCR-based methods cannot replace culture techniques, and must be interpreted with caution and in the context of traditional histology and culture.

### **Autoimmunohistochemistry**

For the interpretation of putative contaminations, further information can be provided by in situ visualization of the causative agents in resected heart valves.

Autoimmunohistochemistry is an immunohistochemical peroxidase-based method that uses the patient's own serum as source of antibodies against the colonizing microorganisms in the heart valve tissue. This sophisticated approach was developed and evaluated by Lepidi et al. [35] to link pathologic and serologic techniques for diagnosis of IE. It permits direct detection of microorganisms in heart valve specimens and was successfully applied in 15 cases of BCNE. Although the method seems useful to verify the diagnosis of IE, it does not provide identification of the species involved.

### **Fluorescence in Situ Hybridization**

Fluorescence in situ hybridization (FISH) is a molecular technique that uses fluorescently labelled probes to detect RNA or DNA. The most commonly used target in FISH for microbiologic applications is the 16S ribosomal RNA because it allows design of specific oligonucleotide probes for most bacteria, as well as genus-specific or species-specific probes. The fluorescent probes are applied to fixed samples, either smears or tissue sections on slides. They penetrate the morphologically intact microorganisms and hybridize specifically to their target sites in the highly abundant ribosomes. Therefore, FISH allows culture-independent identification and simultaneously visualization of bacteria.

Consequently, FISH has been established as an invaluable tool to investigate complex microbial communities in environmental microbiology allowing rapid detection, and visualization of the spatial arrangement in their natural environment [36,37]. In the past years, it has emerged as an important instrument in analysis of microbial biofilms, medical research, and lately also in microbiologic diagnostics.

### **FISH for Identification of Bacteria in Positive Blood Cultures**

Standard laboratory procedure of blood cultures comprises incubation of the bottles with continuous, automated growth detection. If a bottle is reported positive, Gram staining is performed, which allows a first rough classification of the microorganisms. Using the well-established routine methods, subcultures and 1 to 2 more days are needed for exact identification. As a culture-independent method, FISH can speed up this process, allowing identification within 2 to 3 h after growth detection in the automated blood culture system. Whereas most FISH assays are in-house techniques and lack standardization, commercial probes are available for rapid identification of gram-positive cocci or fungi in positive blood cultures. These FISH kits use fluorescent peptide nucleic acid (PNA) FISH probes. Taking advantage of their noncharged peptide backbone, PNA probes penetrate unhindered into gram-positive bacteria. Recent publications show a significant impact of the PNA FISH on time to diagnosis, change of therapy regimen, costs, and patient outcome [38,39].

However, only a limited selection of PNA probes is available, and those are more costly than oligonucleotide probes. We recently developed and evaluated a more comprehensive panel of FISH probes for the identification of gram-positive cocci in positive blood cultures. These also include probes for the typical microorganisms involved in IE and some of the fastidious species [40].

### FISH for Identification of Bacteria in Heart Valve Sections

A favorable feature of FISH is information about spatial resolution when applied to intact biofilms or tissue sections [41], showing microorganisms within their histologic context. Therefore, FISH is a bridging technique between microbiology, pathology, and molecular diagnostics. We took advantage of this fact and applied FISH on tissue sections of 54 heart valves from suspected endocarditis patients [42]. Specimens were screened for bacteria using a probe detecting most bacteria along with a probe panel of genus- or species-specific probes for identification of streptococci, staphylococci, enterococci, *Bartonella quintana*, or *Tropheryma whippelii*. Results were compared with those of culture-based diagnostics and clinical data. Discrepant results were subjected to comparative sequence analysis of PCR-amplified 16S rRNA genes. Bacteria were identified in 26 heart valves; 8 of 18 cases with possible or rejected IE could have been reclassified as definite IE if FISH was added as a criterion to the Duke classification scheme. In 5 of 13 BCNE cases, microorganisms could be visualized in situ by FISH (Fig. 1), revealing *Bartonella quintana* [43] and *Tropheryma whippelii* in one case each. However, FISH was false negative in two cases compared to valve culture, blood culture, and PCR results, possibly indicating sampling errors or too low sensitivity.

In contrast to other molecular techniques, FISH allows simultaneous detection and identification of microorganisms within the tissue and thereby has the potential to discriminate between causative infectious agents and contaminations. This aspect is crucial for microbiologic diagnosis of IE, because bacteria of the skin flora (eg, coagulase-negative staphylococci, *Propionibacterium* spp, or *Corynebacterium* spp) are known causes of IE and are difficult to differentiate from putative contaminants when detected in blood cultures. In the presented pilot study, coagulase-negative staphylococci were visualized in five cases (Fig. 2). FISH was the only technique that could unequivocally prove the diagnosis of IE in these cases. Targeting the 16S rRNA, FISH provides evidence about ribosome content and presumably the metabolic status of the microorganisms. It therefore invites speculation about their viability. Besides bacteria with a strong FISH signal indicating a high ribosomal content and metabolic activity at time of sampling, we found numerous probably dead cells, staining only with a nonspecific nucleic acid stain, 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI) (Fig. 3). Furthermore, FISH allows the visualization of structured bacterial communities embedded in the surrounding host tissue (Fig. 4). The features of these microbial complexes and the distinct and complex architectures of these biofilms might further our understanding of their growth mechanisms and survival despite antibiotic therapy and might enable us to develop new therapeutic or preventive approaches.

However, it must be emphasized that FISH cannot substitute for routine diagnostic methods, because culture is indispensable for antibiotic susceptibility testing. Because FISH is a microscopic method, sensitivity is low compared to other molecular techniques and depends on sampling accuracy. Like valve culture and valve PCR, FISH can only be performed on resected heart valves. The diagnostic impact is limited in cases when microorganisms are detected by an unspecific nucleic acid stain, but hybridization with a FISH probe fails.

In summary, FISH is a fast, inexpensive, and culture-independent technique that allows simultaneous visualization and identification of microorganisms in infected heart tissues. Thus, it has the potential to be used in routine diagnostic procedures for diagnosis of IE,

especially in culture-negative cases and questionable cases. FISH does not represent a stand-alone technique, but can complement other molecular and culture-based methods. As for the PCR-based diagnostics of heart valve tissues, standardized protocols or commercial tests for FISH on heart valves are not available, and the technique so far remains restricted to laboratories with the respective expertise. Because FISH has the potential to measure ribosome content of the bacteria in situ, it might help to assess the efficiency of antibiotic treatment on biofilms in vivo.

## Conclusions

Facing the diagnostic and therapeutic difficulties caused by IE, it is evident that the present conventional culture-based microbiologic methods are unsatisfactory. Molecular techniques such as PCR-based methods have proven to be a useful complementary tool, especially in culture-negative and questionable IE cases. Further investigations are needed to accurately define the group of patients who benefit from the integration of molecular techniques into the diagnostic workflow. The examination of heart valve tissue using FISH can help the definite diagnosis of IE by visualizing the microorganisms directly in situ. Furthermore, the analysis of microbial communities or biofilms might elucidate the pathogenesis of IE and help to develop new prevention strategies or therapeutic targets. It should be emphasized that molecular techniques cannot replace conventional diagnostic procedures, but represent an additional instrument to improve the diagnosis of IE on the basis of interdisciplinary teamwork between clinicians, microbiologists, and pathologists. Standardization of procedures is required to open these sophisticated techniques to a broader application, to be of benefit to more patients.

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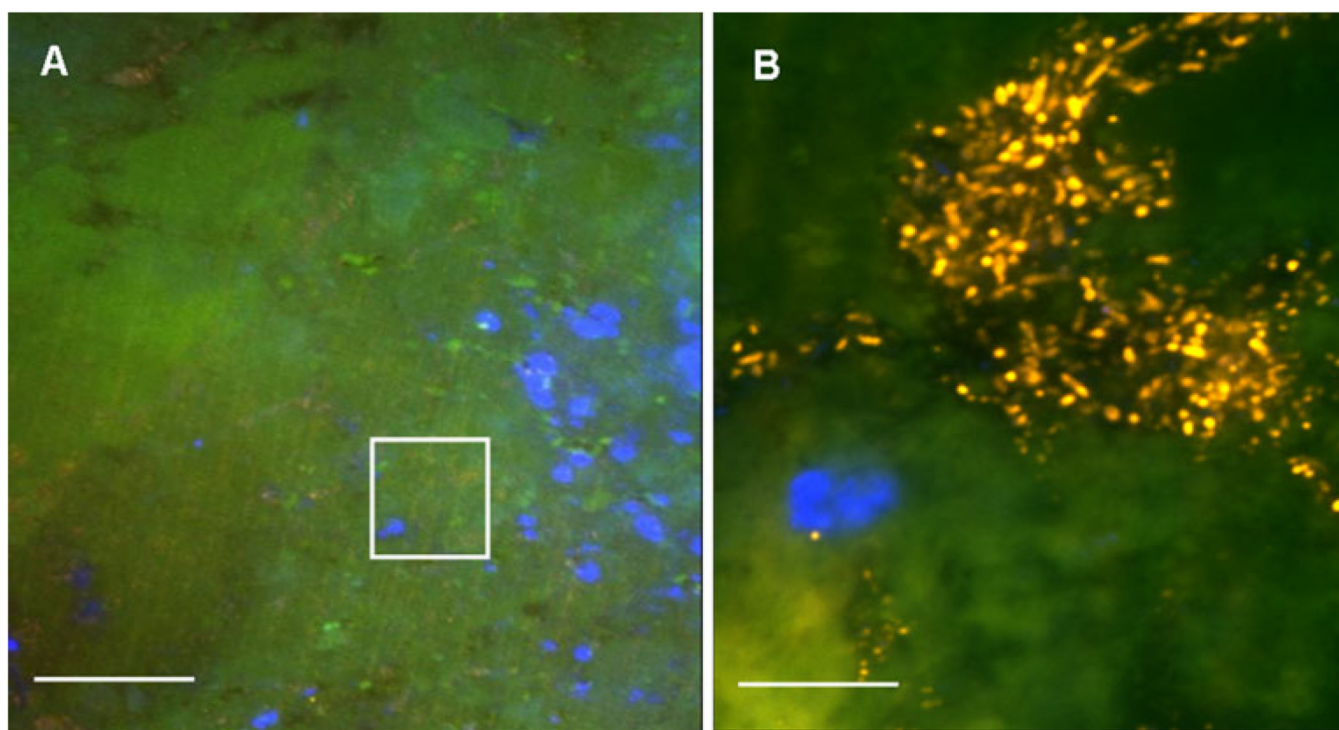
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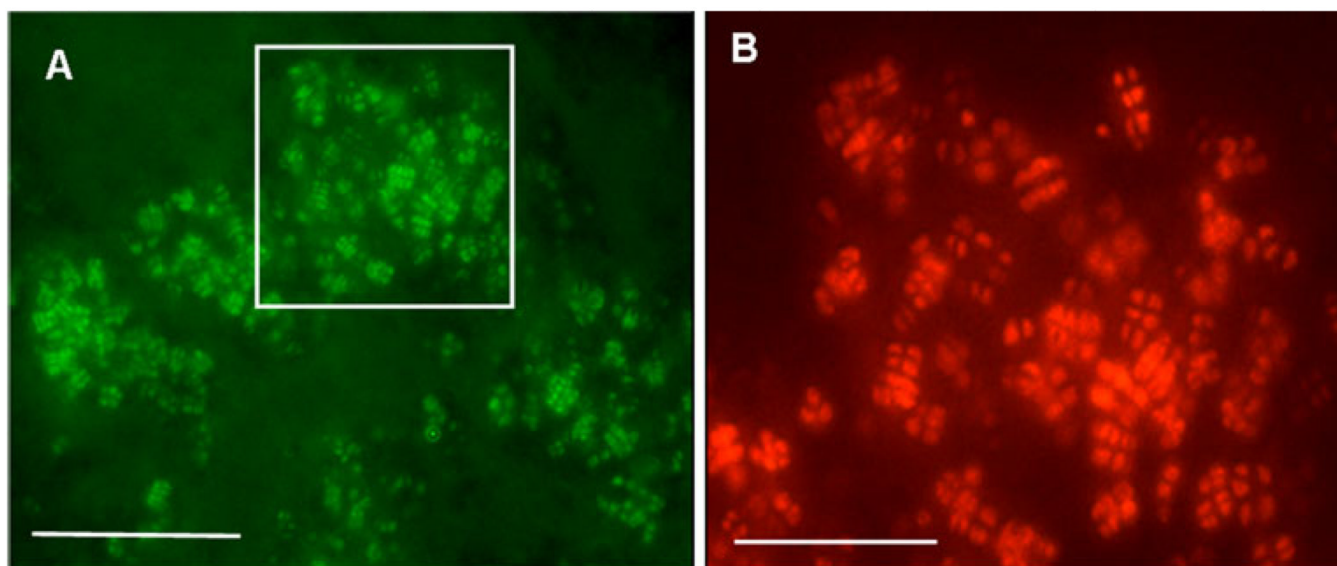
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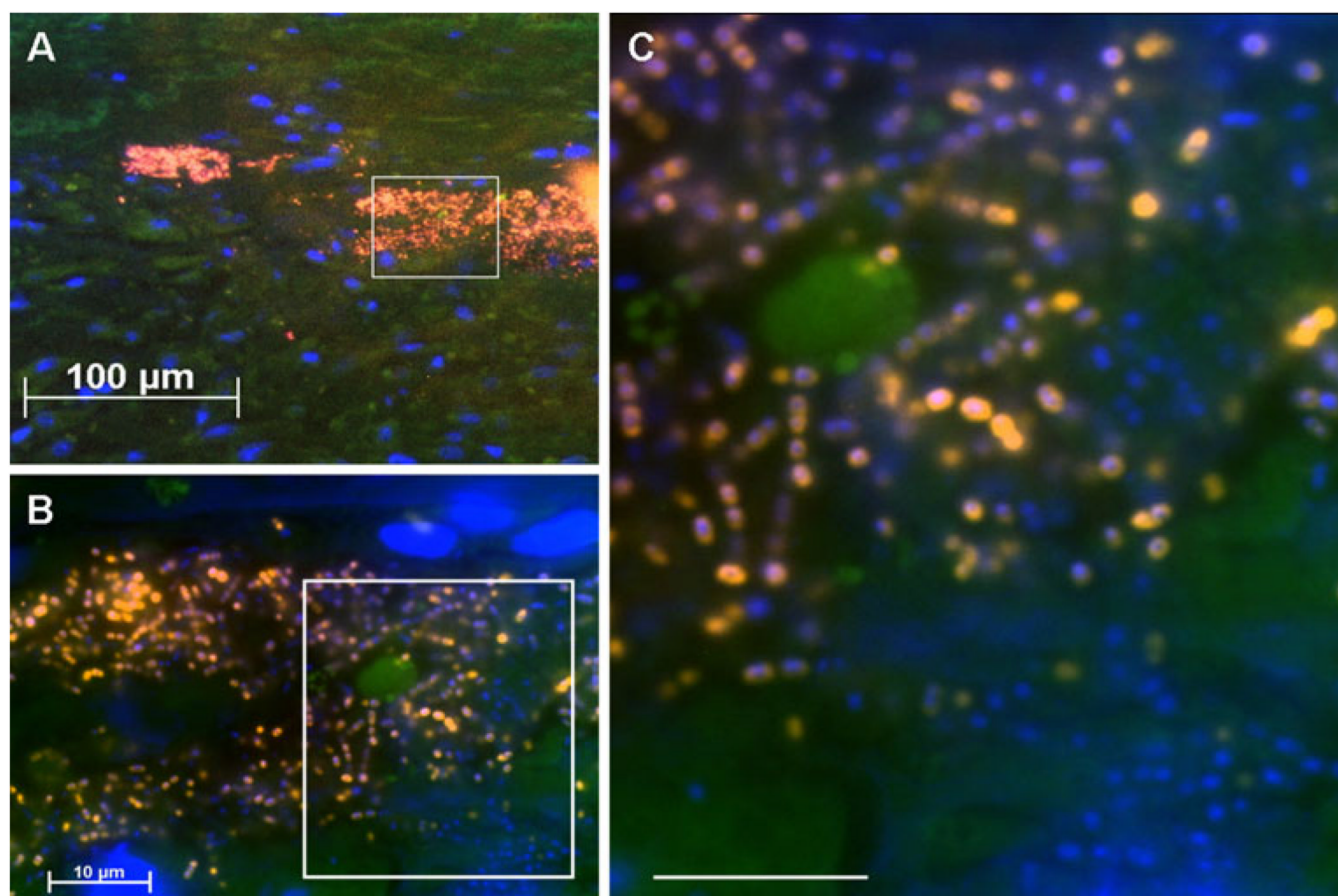


**Fig. 1.**

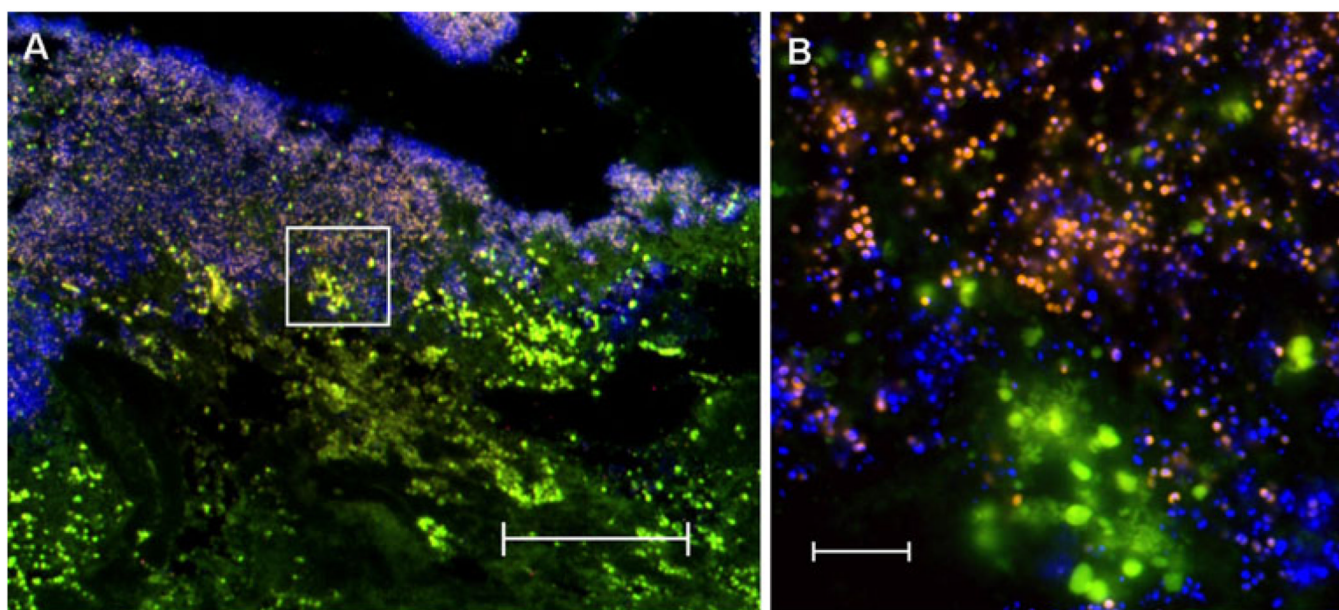
Fluorescence in situ hybridization of a heart valve section showing streptococci in a culture-negative case of infective endocarditis. Nucleic acids were nonspecifically stained with the fluorochrome 4',6--diamidino-2-phenylindole dihydrochloride (*blue*) showing some leukocytes. **a**, Overview (*bar*=50  $\mu$ m). *Insert*, **b**, Hybridization with a *Streptococcus* genus-specific probe (*orange*) shows single streptococci scattered within the heart valve tissue. (*bar*=10  $\mu$ m)



**Fig. 2.** Visualization of coagulase negative staphylococci in mitral valve tissue. Fluorescence in situ hybridization allows definite diagnosis of infective endocarditis in this questionable case with coagulase-negative staphylococci grown in blood culture. **a** (*bar*=25  $\mu$ m), Bacterial probe (*green*) shows multiple tetrads of cocci in the tissue. *Insert*, **b**, All bacteria stain positive with the *Staphylococcus* genus-specific probe as well (*red*, *bar*=10  $\mu$ m)



**Fig. 3.** Fluorescence in situ hybridization of a heart valve section of a patient with *Streptococcus* endocarditis. **a**, Overview shows a field of bacteria stained with the *Staphylococcus* genus-specific probe (orange) and blue host cell nuclei stained with 4',6-diamidino-2-phenylindole dihydrochloride. **Insert, b**, At higher magnification, short chains are visible. **Insert, c**, (bar=10 µm), Note the discriminative fluorescence intensity indicating differential ribosomal content of the cells



**Fig. 4.** Fluorescence in situ hybridization (FISH) of a heart valve colonized by *Enterococcus faecalis* showing a mature biofilm. **a**, Overview (*bar*=100 μm) shows a structured biofilm with a superficial layer of 4',6-diamidino-2-phenylindole dihydrochloride (DAPI)-positive cells. *Insert*, **b**, At a higher resolution, bacteria are identified by the *E. faecalis*-specific FISH probe among many bacteria stained with DAPI only (*bar*=10 μm)

**Table 1**

Studies analyzing resected heart valves by broad-range PCR and sequencing

Study	Suspected IE cases, N	BCPE respecified by PCR <sup>a</sup>	BCNE with positive PCR result (total BCNE cases)	False-negative PCR <sup>b</sup>	Conclusion
Goldenberger et al. [44]	18	0	2 (4)	2	PCR recommended for BCNE
Bosshard et al. [45]	36	6	3 (13)	3	PCR recommended for BCNE and questionable cases; molecular techniques should be added to the Duke criteria
Podglajen et al. [46]	46	10	5 (6)	3	PCR recommended for BCNE and questionable cases; improved patient management through species identification by sequencing
Gauduchon et al. [25]	29	3	3 (4)	1	PCR recommended for BCNE and questionable cases
Lang et al. [47]	37	1	1 (8)	6	PCR recommended for BCNE and questionable cases, bacterial DNA may persist within valve tissue
Breitkopf et al. [27]	51	1	12 (26)	10	PCR improves detection of fastidious and easy-to-culture pathogens
Greub et al. [48]	127	15	11 (31)	ND	PCR recommended for BCNE and questionable cases
Kotilainen et al. [49]	38	1	4 (8)	13	PCR recommended for BCNE; bacterial DNA may persist during treatment
Marin et al. [33]	35	7	3 (3)	1	PCR recommended for BCNE, should be included as a new major Duke criterion
Voldstedlund et al. [50]	64	3	3 (7)	16	PCR recommended for BCNE
Total	481	47	47 (110)	55	

BCNE blood culture–negative endocarditis; BCPE blood culture–positive endocarditis; IE infective endocarditis; ND no data available; PCR polymerase chain reaction (*Modified from* Batty et al. [24].)

<sup>a</sup> Means reclassification in Duke criteria, resolution of disagreement, or specification of the causative agent

<sup>b</sup> Compared to blood culture results, excluding inhibited PCR assays