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MAJOR ARTICLE

Diagnostic Impact of FISHseq as a New Pathologic Criterion for Endocarditis According to the Duke Criteria

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Background. For clinicians treating patients with infective endocarditis (IE), identifying the causative microorganisms poses a critical diagnostic challenge. Standard techniques including blood and heart valve cultures often yield inconclusive results. According to the recent 2023 Duke-ISCVID Criteria, molecular methods represent potent tools to enhance this aspect of IE diagnostics and guide subsequent therapeutic strategies.

Methods. We retrospectively analyzed data from 124 consecutive patients who underwent heart valve surgery due to suspected IE at *München Klinik Bogenhausen*. The standard diagnostic pathway, which included blood culture, valve culture, histopathological analysis, and polymerase chain reaction (PCR)/sequencing, was compared with the enhanced diagnostic pathway, which included fluorescence in situ hybridization + PCR/sequencing (FISHseq) instead of PCR/sequencing alone. The aim of this study was to assess the added value of combining standard diagnostics with molecular methods such as PCR/sequencing or FISHseq for the diagnosis of IE and the potential impact on therapy.

Results. Standard diagnostic methods and PCR/sequencing yielded inconclusive results in 57/124 cases (46.0%). FISHseq provided an added value for diagnostics in 79/124 cases (63.7%) and potentially would have impacted therapy in 95/124 (76.6%) of cases. By adding data through direct visualization and characterization of microorganisms, FISHseq reduced the number of inconclusive cases by 86.0%.

Conclusions. The comparison of 2 molecular diagnostic tools for IE from the same heart valve emphasizes the value of molecular methods including molecular imaging by FISH for IE diagnostics and supports the 2023 Duke-ISCVID Criteria.

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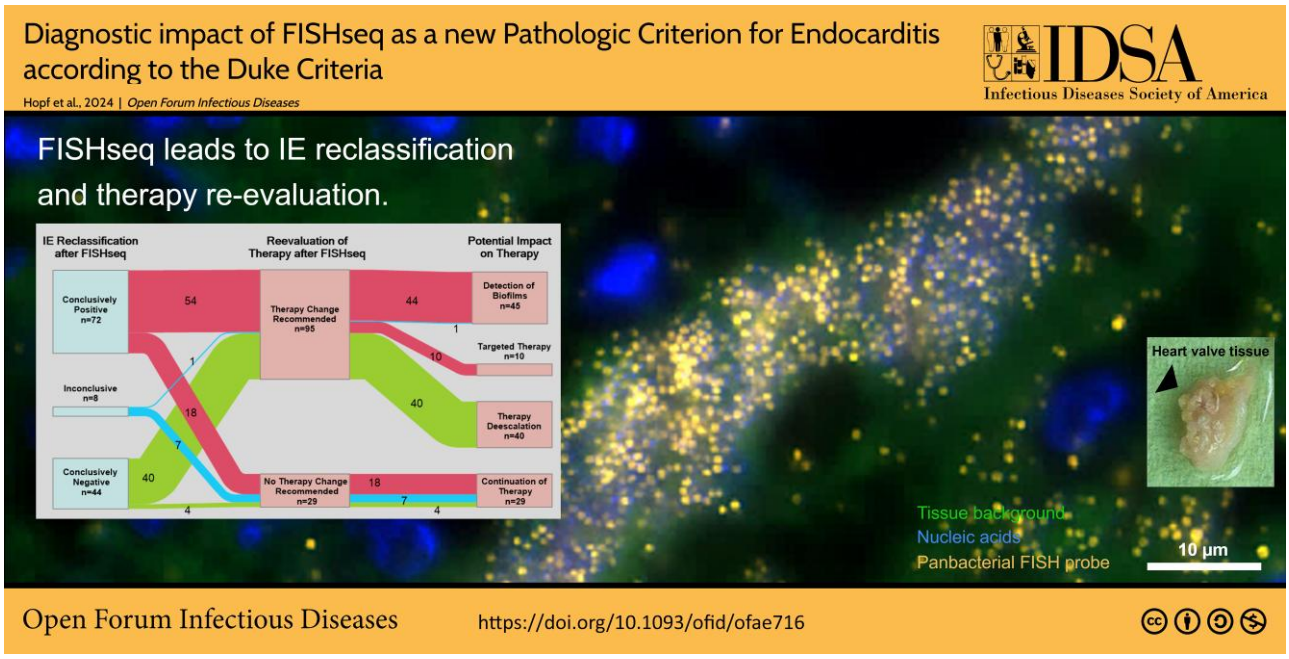
Graphical Abstract



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Keywords. 2023 Duke-ISCVID Criteria; FISHseq; fluorescence in situ hybridization; infective endocarditis; molecular imaging.

Infective endocarditis (IE) is a potentially life-threatening infection of native or prosthetic heart valves, requiring rapid and precise diagnostics followed by an effective antimicrobial therapy [1]. Despite technological advances, mortality rates have remained largely unchanged over the past 2 decades [2]. With global incidences of IE on the rise, identifying the causative microorganisms is crucial for effective antibiotic treatment [3]. As part of initial diagnostics, clinical symptoms such as fever or a cardiac murmur along with blood culture results are evaluated to determine the probability of an IE using the modified Duke Criteria [4] or the currently published 2023 Duke-ISCVID Criteria/European Society of Cardiology (ESC) guidelines [5, 6]. However, with 2.5%–48% of all endocarditis cases remaining blood culture negative, additional diagnostic strategies are of utmost importance [2, 7, 8].

A previous prospective study indicated that 57% of all IE patients required heart valve surgery, allowing subsequent microbiological analysis of resected native or prosthetic valves [9]. Due to low sensitivities for the detection of causative microorganisms in valve culture, the addition of molecular methods to routine diagnostics has been suggested [10]. This is reflected in the 2023 Duke-ISCVID Criteria, which place a stronger emphasis on molecular methods and pathological aspects [5]. 16S rRNA gene polymerase chain reaction (PCR)/sequencing, especially, has been extensively studied and applied in the context of IE diagnostic routines, resulting in additional diagnostic value [11–16]. However, despite the availability of 16S rRNA gene PCR/sequencing, IE diagnostics remain challenging and frequently yield inconclusive results.

Fluorescence in situ hybridization (FISH) + PCR/sequencing (FISHseq) is a molecular technique, combining 16S rRNA gene PCR/sequencing with direct microscopic visualization and characterization of microorganisms in tissue sections through FISH. As part of FISH, fluorescently labeled probes hybridize with the ribosomes within target microorganisms. This enables precise visual identification and morphological classification in their histological context using a fluorescence microscope [17]. In patients in which the clinical relevance of PCR-identified microorganisms is questionable, FISH enables differentiation between active and inactive microorganisms by ribosome content, targeting the 16S ribosomal RNA [17–19]. In addition, information on the spatial arrangement of microorganisms as single microorganisms, microcolonies, or biofilms has the potential to enhance diagnostics and improve treatment strategies [20].

In this study, we performed an in-depth evaluation of previously reported data from 124 patients who underwent cardiac surgery due to suspected IE [21]. In addition to the standard diagnostics including blood culture, heart valve culture, and histopathological analysis, samples were routinely assessed using PCR/sequencing and, in parallel, FISHseq (FISH + PCR/sequencing). Here, we performed a retrospective evaluation of the applied microbiological assays on a case-by-case basis.

The aim of this study was to assess and compare the added value of combining standard procedures with PCR/sequencing or FISHseq for the diagnosis of IE and the potential impact on therapy.

METHODS

Study Characteristics

One hundred twenty-four consecutive, previously reported patients with suspected IE who underwent heart valve surgery at München Klinik Bogenhausen between September 2015 and April 2018 were included in this study [21]. Patients were preoperatively classified based on the modified Duke Criteria using transesophageal echocardiography, blood culture results, and evaluation of the clinical presentation. Postoperative (re-) classification of patients was based on the addition of heart valve culture results and histopathological data (Figure 1*A*).

This study included patients referred to cardiac surgery due to an indicative Duke Criterion or high clinical suspicion of IE, or due to high-grade valve stenosis or regurgitation, initially presenting without any indications of a possible IE. The latter patients, despite being classified as "rejected IE" using the modified Duke Criteria preoperatively, were included due to suspicious intraoperative findings resembling IE-typical deposits or abnormal heart valve morphology.

Diagnostic Algorithm

The diagnostic algorithm was conducted as described previously [21]. Briefly, heart valve tissue was surgically resected, and each resected heart valve was divided intraoperatively by the cardiac surgeon into 4 macroscopically equal parts. These parts were randomly packaged and subsequently sent for histopathological, culture-based microbiological, PCR/sequencing, and FISHseq analysis. Histopathology and microbiological valve culture were performed by the respective departments at München Klinik Bogenhausen using standard techniques. 16S rRNA gene PCR/sequencing of the standard diagnostic pathway was performed at Max von Pettenkofer Institute for Hygiene and Medical Microbiology of the Ludwig-Maximilians-Universität in Munich, Germany. FISHseq samples were immersed in FISHopt fixation solution (MoKi Analytics, Berlin, Germany) in the operating room and sent to Moter Diagnostics in Berlin, Germany, for analysis. Results from the diagnostic tests were used to retrospectively assess the diagnostic impact of FISHseq.

Pre- and Postoperative IE Classification Using the Modified Duke Criteria

Based on the available data, patients were classified as definite IE, possible IE, or rejected IE before and after cardiac surgery based on the modified Duke Criteria (Figure 1*A*) [4]. For the preoperative classification, transesophageal echocardiography, blood culture results, and clinical presentation were considered. For the postoperative reclassification, heart valve cultures and histopathological results were additionally taken into account.

Standard Diagnostic Pathway Including PCR/Sequencing

The standard diagnostic pathway for IE at München Klinik Bogenhausen included blood cultures, histopathology, heart valve cultures, and 16S rRNA gene PCR/sequencing. Based on the modified Duke Criteria [4] and results obtained (positive or negative PCR/sequencing), patients were reclassified into 3 groups: conclusively positive, conclusively negative, and inconclusive (definitions given in Supplementary Data *A*, details on the classification "inconclusive" in Supplementary Data *B*) (Figure 1*B*).

Enhanced Diagnostic Pathway Including FISHseq

As part of the enhanced diagnostic pathway, FISHseq was performed as described previously [17, 22, 23]. Detailed information on FISHseq can be found in Supplementary Data *C*. Figure 2 graphically outlines the FISHseq workflow. Instead of PCR/sequencing results, FISHseq results (active IE, past/treated IE or no IE, definition given in Supplementary Data *D*) were used to reclassify patients into the same 3 groups: conclusively positive, conclusively negative, and inconclusive (Figure 1*C*). Figure 3 provides an overview of the 2 diagnostic pathways.

Case-by-Case Analysis and Added Value

Both diagnostic pathways were blinded to avoid bias between diagnostic laboratories. Results from the diagnostic tests conducted in Munich and Berlin were subsequently used to retrospectively compare both diagnostic pathways on a case-by-case basis with respect to the identification of causative microorganisms, IE reclassification, and possible impact on therapy.

Added value of FISHseq was defined as a change in classification of cases and/or detection of biofilms.

RESULTS

Patient Characteristics and Duke Classification

Patient characteristics are summarized in Table 1. The preoperative and postoperative classification of cases according to the modified Duke Criteria is given in Figure 1*A*.

Before surgery, 67/124 patients (54.0%) presented with positive blood cultures; among those, 43/67 patients were preoperatively classified as definite IE. Negative blood cultures were obtained in 57/124 cases (46.0%); among those, 13/57 cases were preoperatively classified as definite IE.

Heart valve cultures were performed in 122/124 patients and yielded positive results in 35/122 cases (28.7%), whereas 87/122 cases (71.3%) remained negative (Figure 1*A*). Histopathological results indicated active endocarditis in 58/124 patients (46.8%). In 7/124 patients (5.6%), histological examination was not performed. The spectrum of causative microorganisms identified included mostly staphylococci, streptococci, and enterococci, and a few others (Supplementary Data *E*). Figure 4 shows a direct

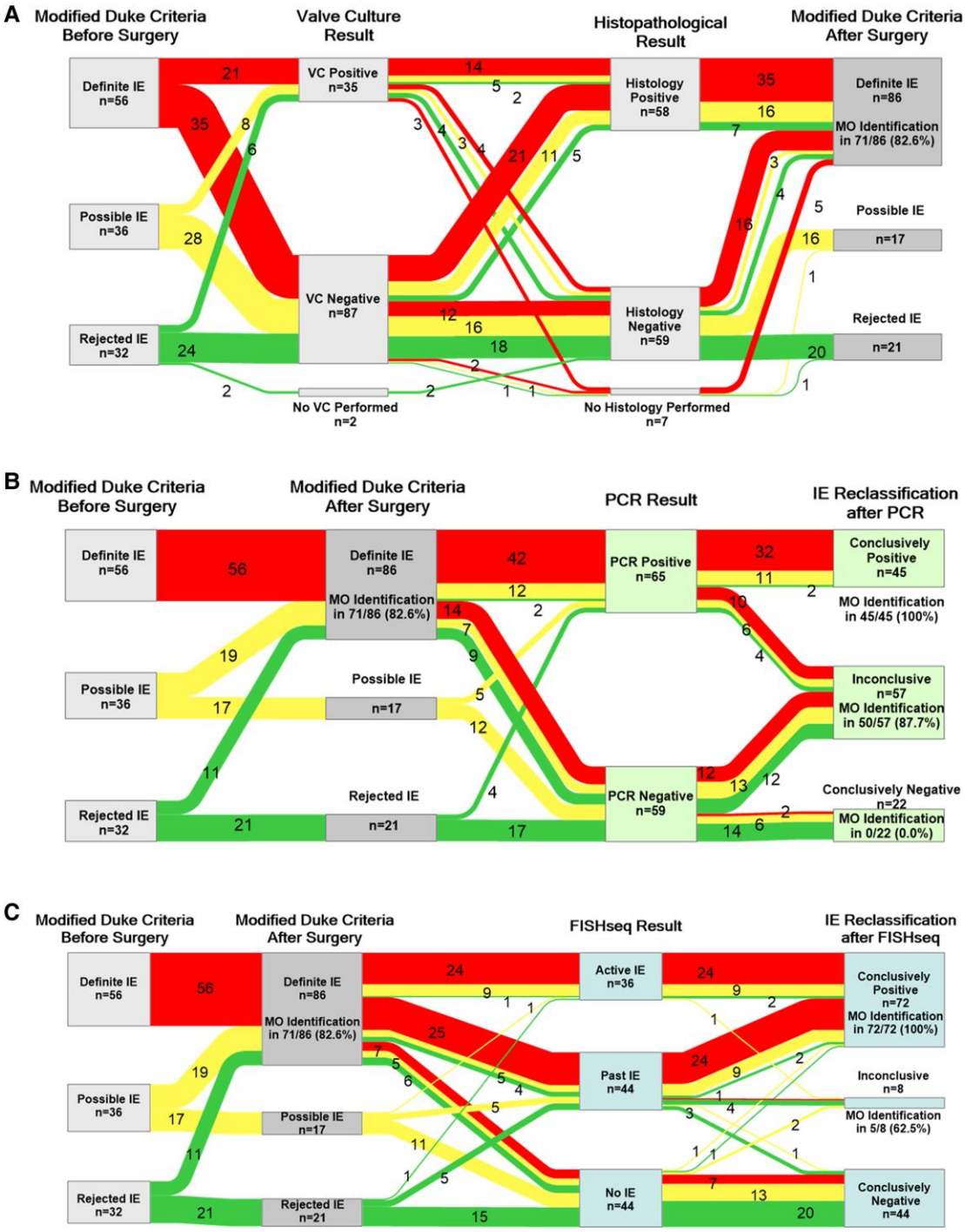

Figure 1. Reclassification of IE cases according to the modified Duke Criteria upon inclusion of postoperative valve culture and histopathology results (*A*), PCR/sequencing results (*B*), or FISHseq results (Enhanced Diagnostic Pathway) (*C*). Red indicates preoperative "definite IE," yellow indicates "possible IE," and green indicates "rejected IE." A, Reclassification of IE cases according to the modified Duke Criteria after inclusion of postoperative VC and histopathology results. B, Reclassification of IE cases after inclusion of PCR/sequencing results (Standard Diagnostic Pathway). C, Reclassification of IE cases after inclusion of FISHseq results (Enhanced Diagnostic Pathway). Abbreviations: FISHseq, fluorescence in situ hybridization + polymerase chain reaction/sequencing; IE, infective endocarditis; MO, microorganism; PCR, polymerase chain reaction; VC, valve culture.

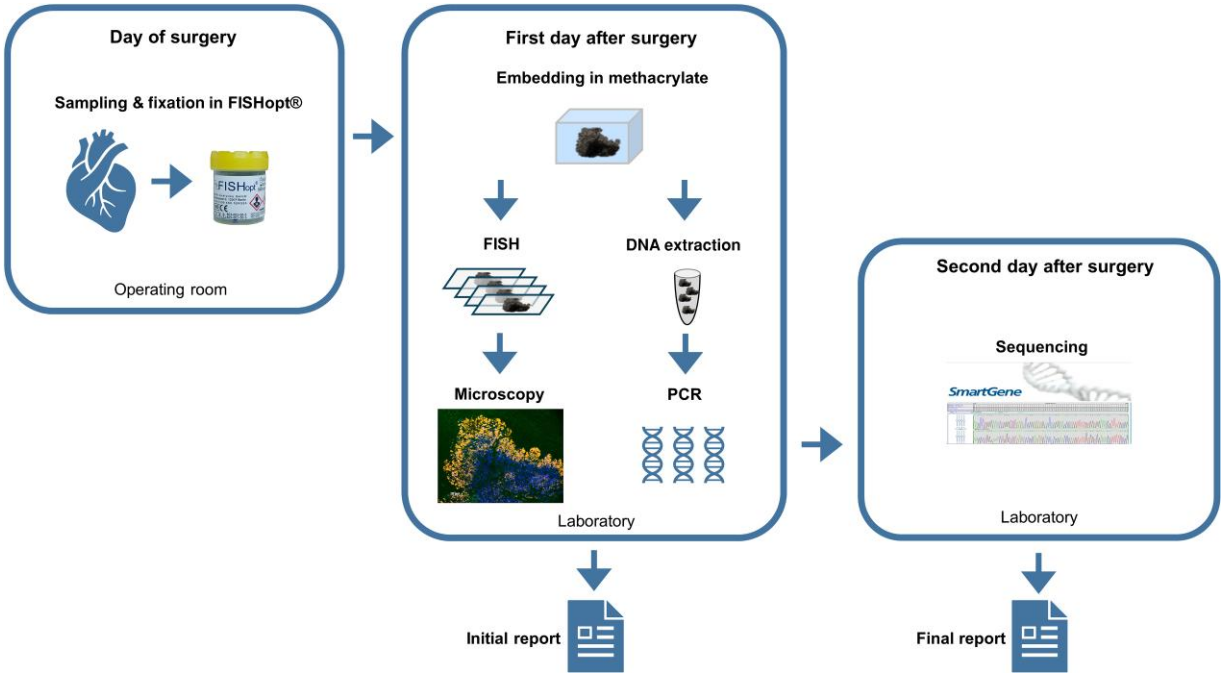

Figure 2. FISHseq workflow. After resection of heart valve tissue and fixation in FISHopt solution, the sample is embedded in cold polymerized resin. Subsequently, FISH is performed as outlined in Supplementary Data *C* to enable an initial FISH report within 24 hours. Furthermore, DNA extraction from consecutive sample sections, as used for FISH, is used for PCR/sequencing. A final FISHseq report is usually available within 36–48 hours after surgery. Abbreviations: FISH, fluorescence in situ hybridization; FISHseq, fluorescence in situ hybridization + polymerase chain reaction/sequencing; PCR, polymerase chain reaction.

comparison of the identified microorganisms using valve culture, PCR/sequencing, and FISHseq.

Impact of the Diagnostic Assays

Standard Diagnostic Pathway Including PCR/Sequencing.

The postoperative classification of patients according to the modified Duke Criteria was reassessed including PCR/sequencing results (Figure 1*B*). PCR/sequencing succeeded in identifying a microbial species from the heart valve material in 65/124 cases (52.4%); among those, 42/65 cases had been preoperatively classified as definite IE. Fifty-nine of 124 cases (47.6%) remained PCR negative.

In the end, upon addition of PCR/sequencing, 45/124 cases (36.3%) were conclusively positive, 22/124 cases (22.6%) were conclusively negative, and 57/124 cases (46.0%) were inconclusive. Species identification was possible in 45/45 cases in the conclusively positive group and in 50/57 cases in the inconclusive group. In the conclusively negative group, PCR was negative; therefore, no species were identified. Without the addition of molecular techniques, valve culture and blood culture identified the same causative microorganism, leading to a conclusively positive result in 14/124 (11.3%) cases.

Enhanced Diagnostic Pathway Including FISHseq. The postoperative classification of patients according to the modified Duke Criteria was reassessed including FISHseq results (Figure 1*C*). FISHseq allowed discrimination of 36/124 cases (29%) with active IE, 44/124 cases (35.5%) with past/treated IE, and 44/124 cases (35.5%) without evidence of IE. Examples of the microscopic presentation of active IE, past/treated IE, and heart valve tissue without evidence of IE are given in Figures 5–7, respectively. FISHseq showed the presence of biofilms in 45/124 cases (36.3%). IE classification in the Enhanced Diagnostic Pathway resulted in 72/124 conclusively positive cases (58%) with microbial species identification, 44/124 conclusively negative cases (35.5%), and 8/124 inconclusive cases (6.5%). Detailed reasons for classification as inconclusive using the Enhanced Diagnostic Pathway are provided in Supplementary Data *F*.

Comparison of Diagnostic Pathways.

Inconclusive Cases. Fifty-seven of 124 cases (46%) remained inconclusive using the Standard Diagnostic Pathway, which included PCR/sequencing. The Enhanced Diagnostic Pathway, which included FISHseq, reduced the number of inconclusive cases by 86.0%, from 57/124 (46.0%) to 8/124 (6.5%) cases, with FISHseq providing an added value for reclassification of IE in 51/124 cases (41.1%).

As an example, a case from this collective is described as follows: A 61-year-old male patient was preoperatively classified as possible IE using the modified Duke Criteria but postoperatively as inconclusive IE using the Standard Diagnostic Pathway because the histopathological examination of his heart valve

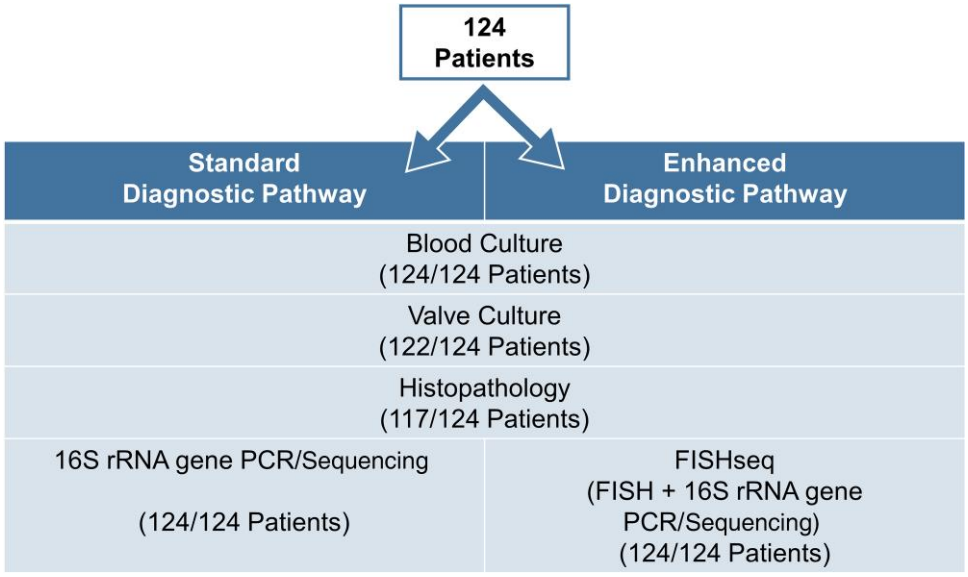

Figure 3. Standard and enhanced diagnostic pathway. Description of the standard and enhanced diagnostic pathway. The key difference of both pathways is that FISHseq is used instead of PCR/sequencing within the Enhanced Diagnostic Pathway. Overall, 124 patients underwent both pathways, but some patient samples did not undergo histopathology or valve culture. Abbreviations: FISHseq, fluorescence in situ hybridization + polymerase chain reaction/sequencing; PCR, polymerase chain reaction.

Table 1. Patient and Clinical Characteristics

Patient characteristics	
Total patients, No.	124
Age, range, mean, y	28–89, 63.4
Male, No.	92
Female, No.	32
Preoperative classification according to modified Duke Criteria, No.	
• Definite infective endocarditis	56
• Possible infective endocarditis	36
• Rejected infective endocarditis	32
Postoperative classification according to modified Duke Criteria, No.	
• Definite infective endocarditis	86
• Possible infective endocarditis	17
• Rejected infective endocarditis	21
Prosthetic valves and clinical characteristics	
Total prosthetic valves, No.	35
Valve type, No.	
• Native valve	89
• Biological prosthesis	20
• Mechanical prosthesis	4
• Mitral annuloplasty ring	11

showed signs of active IE, whereas all other methods collectively yielded no identification of a causative microorganism. In this case, the Enhanced Diagnostic Pathway, or more precisely FISHseq, revealed biofilms containing *Staphylococcus epidermidis* (Figure 8).

Conclusively Positive and Negative Cases. FISHseq confirmed all 45 conclusively positive cases identified by the Standard Diagnostic Pathway. From 22 conclusively negative cases, FISHseq confirmed 21 cases, with 1 case reclassifying as inconclusive because FISHseq revealed DAPI-positive, inactive microorganisms in the investigated tissue, with negative PCR pointing to degraded microbial DNA.

Comparison of PCR/Sequencing (Standard Diagnostic Pathway) With FISHseq (FISH + PCR Sequencing; Enhanced Diagnostic Pathway). FISHseq identified the same microbial species in 58/65 (89.2%) cases that showed a positive PCR/sequencing result within the Standard Diagnostic Pathway. In 7 cases, FISHseq remained negative (including 5 cases with members of the skin flora). Vice versa, FISHseq identified a microbial species in 8 cases, in which PCR/sequencing of the Standard Diagnostic Pathway remained negative (3× *S. epidermidis* with FISH showing the bacteria in the tissue, as shown in Figure 8, 2× *S. aureus*, 2× *S. sanguinis*, 1× *E. faecium*).

Blood Culture–Negative Endocarditis. Fifty-seven of 124 cases had negative blood cultures. Using FISHseq, microorganisms were visualized in 28/57 blood culture–negative endocarditis (BCNE) cases, 11/28 of which had been classified preoperatively as definite IE despite negative blood culture results. Identification of the causative species was successful in 20/28 cases: streptococci (8/20), staphylococci (5/20; *Staphylococcus aureus* [3/5], *Staphylococcus epidermidis* [1/5], and *Staphylococcus haemolyticus* [1/5]), *Cutibacterium acnes* (2/20), *Enterococcus faecalis* (2/20), *Gemella morbillorum* (1/20), *Coxiella burnetii* (1/20), and *Enterococcus* spp. (1/20).

Added Value of FISHseq and Potential Impact on Therapy. FISHseq provided an added value in 79/124 cases (63.7%;

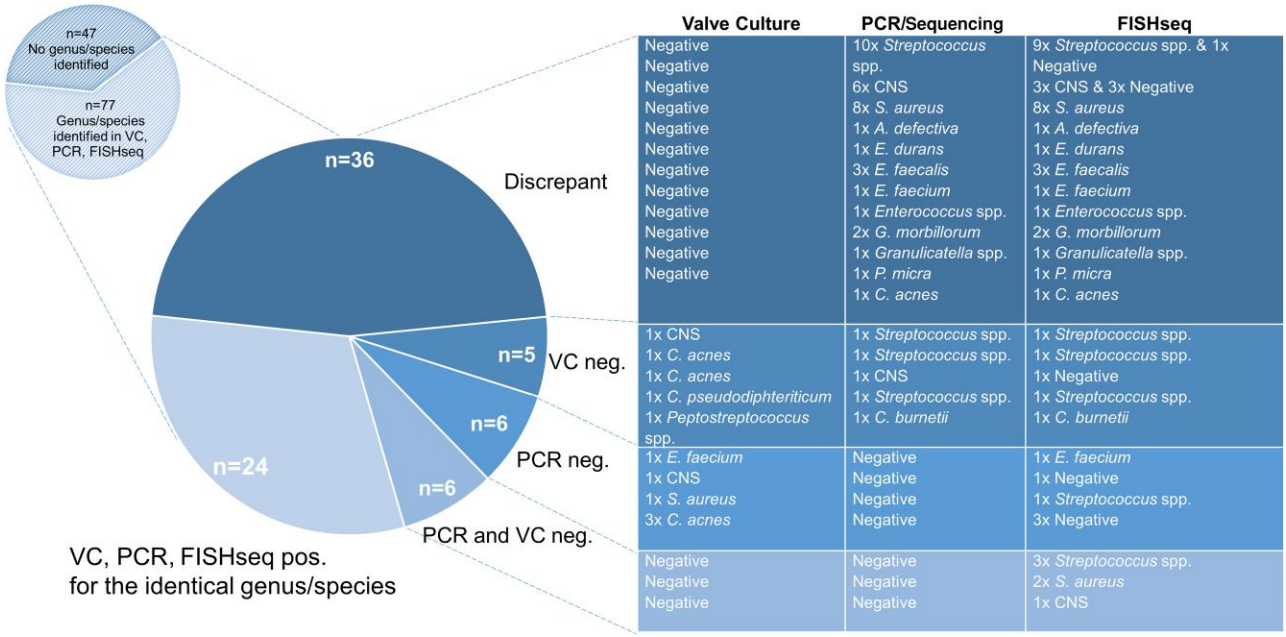

Figure 4. Discrepancy analysis of microbial genus/species identification from heart valve tissue samples using valve culture, PCR/sequencing, and FISHseq. Overall, in 77/124 patients a microbial genus/species was identified from heart valve tissue using valve culture, PCR/sequencing, or FISHseq. In 24/122 cases in which valve culture was performed, VC, PCR/sequencing, and FISHseq identified the same pathogen. In 36/122 cases in which VC was performed, VC failed to identify a pathogen, whereas PCR/sequencing identified a pathogen in 36/36 of these cases and FISHseq identified a pathogen in 32/36 of these cases. In 5/122 cases in which VC was performed, VC identified a different pathogen compared with PCR/sequencing and FISHseq. In 6/122 cases in which VC was performed and identified a pathogen with PCR/sequencing remaining negative, FISHseq identified a pathogen in 5/6 cases. In 6/122 cases in which VC was performed and both VC and PCR remained negative, FISHseq identified a new pathogen. In 2 cases, no VC was performed. In 45/122 cases where VC was performed, neither VC, PCR/sequencing, nor FISHseq identified a pathogen. Abbreviations: FISHseq, fluorescence in situ hybridization + polymerase chain reaction/sequencing; PCR, polymerase chain reaction; VC, valve culture.

51/124 cases due to reclassification with or without detection of biofilms and 28/124 cases solely due to the detection of biofilms without reclassification).

In the next step, we assessed if FISHseq results had a potential impact on therapy (Figure 9). Bacterial biofilms were identified in 45/124 patients (36.3%), for whom a potential therapy escalation with presumably biofilm-active antibiotics may be discussed [24, 25]. In 11/124 patients (8.9%), only FISHseq resulted in the identification of causative microorganisms, allowing for targeted antibiotic therapy. In 40/124 patients (32.3%), a therapy de-escalation was considered, as all results including FISHseq rejected IE. In 22 of those 40 cases (55.0%), FISHseq provided the necessary data for a reclassification. Overall, FISHseq had a potential impact on antibiotic therapy in 95/124 cases (76.7%).

DISCUSSION

Rapid identification of causative microorganisms in IE is of critical importance for effective antimicrobial therapy [2]. Standard diagnostics, including blood and valve culture, remain the cornerstone of diagnostic algorithms but are associated with limitations. The results of this study underline the importance of complementing standard diagnostic algorithms with molecular methods. In this study, we analyzed the impact of 2 molecular methods investigating different parts of the same resected heart valves in parallel in independent laboratories: PCR/sequencing vs FISHseq (FISH + PCR/sequencing).

FISHseq for Diagnosis of IE in the 2023 Duke-ISCVID Criteria and the 2023 ESC Guidelines

FISH for the detection of microorganisms was originally developed in environmental microbiology in the 1990s and has since been further developed for tissue sections and various clinical applications, including IE, since 2008 [17, 19–21, 26–28]. The combination of FISH with 16S rRNA gene PCR and sequencing was termed FISHseq in 2021 [28]. In 2023, Hajduczenia et al. published an assessment of the benefit of FISHseq for the diagnosis of prosthetic valve endocarditis, reporting an improvement compared with conventional culture-based diagnostics in 30% of cases [23]. This study led to the inclusion of FISHseq into the 2023 Duke-ISCVID Criteria as a Pathologic Criterion for Definite IE and as a recommended diagnostic method for diagnosis of BCNE of a prosthetic valve in the 2023 ESC IE Guidelines [5, 6].

So far, FISHseq is only available in a few specialized laboratories, as it uses a labor- and cost-intensive workflow

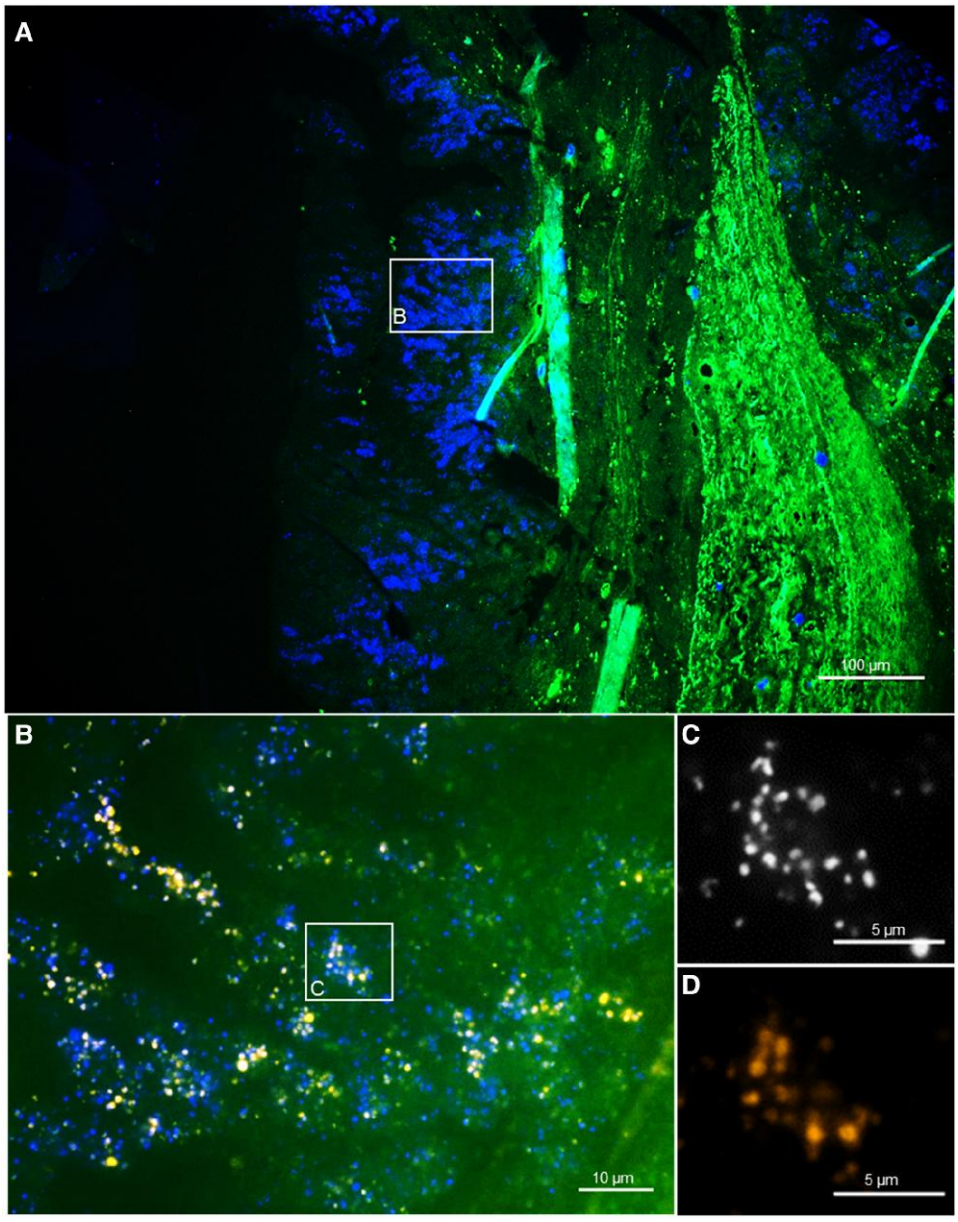

Figure 5. FISHseq detecting active infective endocarditis. FISH of a heart valve section from a 65-year-old male patient with an active IE caused by *Gemella morbillorum*. Blood cultures, PCR/sequencing, and FISHseq identified *G. morbillorum*, whereas the valve culture remained negative. FISHseq visualized active bacteria in biofilm formation. The heart valve section was stained using DAPI for visualization of nucleic acids in host cell nuclei and bacteria. *A*, Overview of the autofluorescent tissue background in green (enhanced for better orientation) with host cell nuclei and bacterial biofilms stained by DAPI in blue. The inset marks a region where bacterial biofilms are visible. *B*, At higher magnification, microorganisms stained with DAPI in blue and partly hybridized with the panbacterial probe $EUB338_{Cy3}$ probe in orange are shown for visualization of active bacteria (based on ribosome content). The hybridization mix also contained NON338, the antisense probe of EUB388 as a nonsense control probe (not shown). The inset (C) shows at even higher magnification single bacteria that are partly FISH-positive and partly only DAPI-positive. *C* & *D*, The same field of view as with single separate microscopic channels for DAPI in black and white, and EUB338 in orange. Abbreviations: FISH, fluorescence in situ hybridization; FISHseq, fluorescence in situ hybridization + polymerase chain reaction/sequencing; PCR, polymerase chain reaction.

combining aspects of microbiology, molecular biology, and pathology that is difficult to integrate into most routine laboratories. However, steps are being taken to simplify access for other laboratories to implement the method [22]; the first accreditation of the method according to DIN EN ISO 157189 was achieved in a laboratory in Berlin in 2024. The turnaround time per sample for an initial FISH result is 1 day, while PCR and sequencing results require 1 more day. This allows for a joint diagnostic interpretation on the second or third day after surgery, which is comparable to culture procedures. The costs for a FISHseq analysis depend on the management of the respective health care systems; in Germany, a FISHseq assay roughly corresponds to the cost of 2 Sanger sequencing analyses.

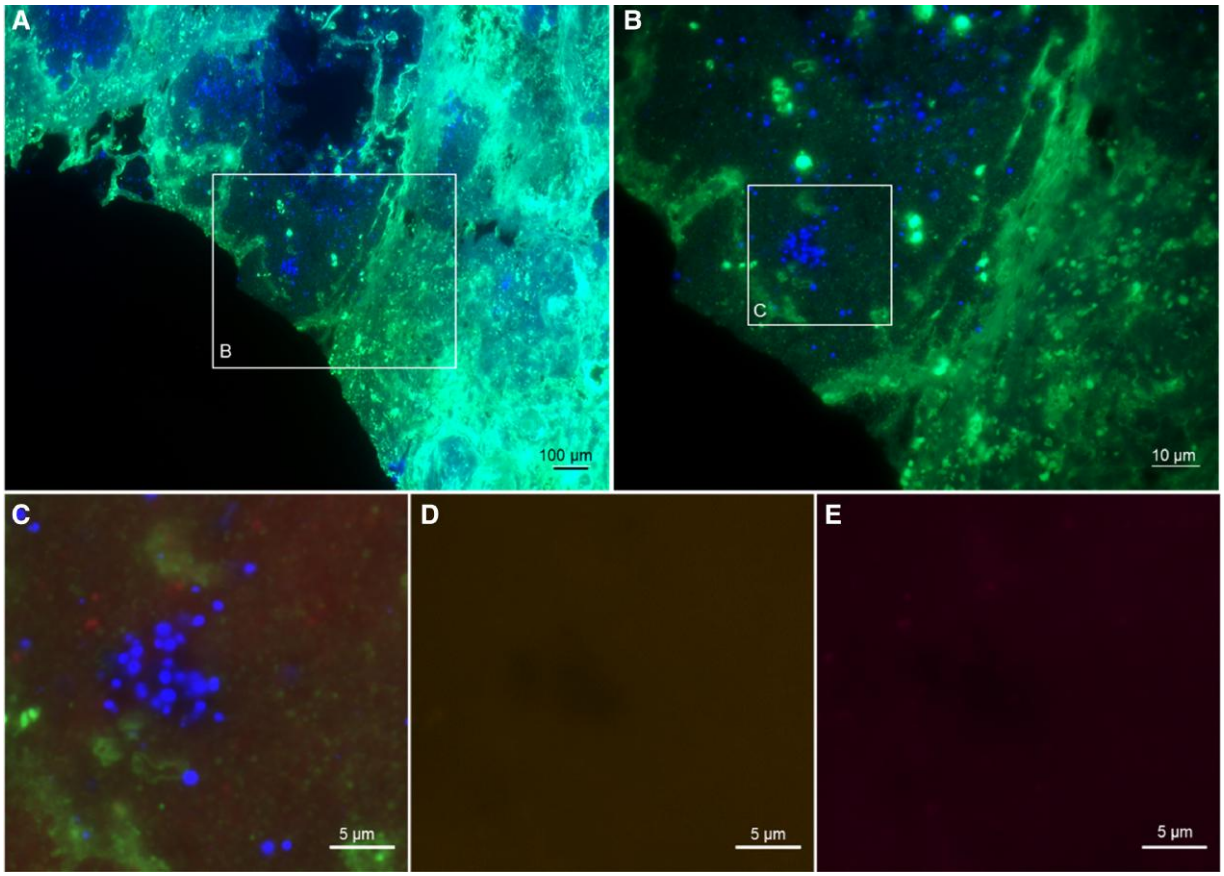

Figure 6. FISHseq showing treated/inactive infective endocarditis. FISH of a native heart valve section from a 64-year-old female patient, classified as "past IE," where FISHseq visualized FISH-negative biofilms and identified *E. faecalis* in sequencing (confirmed by PCR/sequencing alone). Blood cultures had not been taken, and valve cultures remained negative. The section was hybridized with the panbacterial probe $EUB338_{Cy3}$ for visualization of active bacteria (based on ribosome content), the nonsense control probe $NON338_{Cy5}$, and stained with DAPI for visualization of nucleic acids in host cell nuclei and microorganisms. *A*, Overview shows the autofluorescent tissue background in green with DAPI-stained bacterial biofilms in blue. The inset marks the region magnified in (*B*), where DAPI only–positive bacteria are visible (overview of the channels: FITC—tissue background in green, Cy3—EUB338 probe in orange, Cy5—NON338 probe in magenta). The inset marks the region shown at higher magnification in (C–E). C–E, The same microscopic field of view with a merged image of all channels (*C*), the Cy3—EUB338 channel separately without any signal (*D*), and the Cy5—NON338 channel separately also without any signal (*E*). Abbreviations: FISH, fluorescence in situ hybridization; FISHseq, fluorescence in situ hybridization + polymerase chain reaction/sequencing; IE, infective endocarditis.

IE Diagnostics Often Yield Inconclusive Results and Benefit From the Addition of Molecular Methods. Blood culture is an essential part of the diagnostic workup in patients with suspected IE because it is the only routine method for pathogen identification and antibiotic susceptibility testing that does not require cardiac surgery [1, 17]. However, in this study, we found that 46% of all included patients presented with negative blood cultures, reaffirming previous observations [7, 8]. Valve cultures identified microorganisms in 28.2% of all cases in this study, supporting the observation of their overall low sensitivity, ranging from 23% to 46% [11, 12, 16, 29], especially with a previously administered antibiotic treatment [2, 20, 29–31]. However, as valve culture provides the ultimate chance to obtain antibiotic susceptibility testing if positive, it remains an essential part of microbiological diagnostics for IE.

Molecular methods, including cell-free DNA (cfDNA), PCR, and sequencing, have been demonstrated to increase the sensitivity of IE diagnostics [10, 11, 13, 16, 32]. In our study, PCR/sequencing identified microorganisms in 52.4% of all cases (and 75.0% of preoperative definite IE cases), confirming the previously determined sensitivities of 74%–87% [16, 29]. In all cases with species identification in both PCR/sequencing and FISHseq, the results were identical, which underlines the high validity of the results. These results support that molecular methods should be part of routine diagnostics for IE, as also underlined by the 2023 Duke-ISCVID Criteria [2, 4, 5, 30]. However, due to the high sensitivity, results from nucleic acid amplification methods, such as broad-range PCR, should be interpreted with caution [1, 33]. Furthermore, they only provide information about bacterial DNA and do not provide any

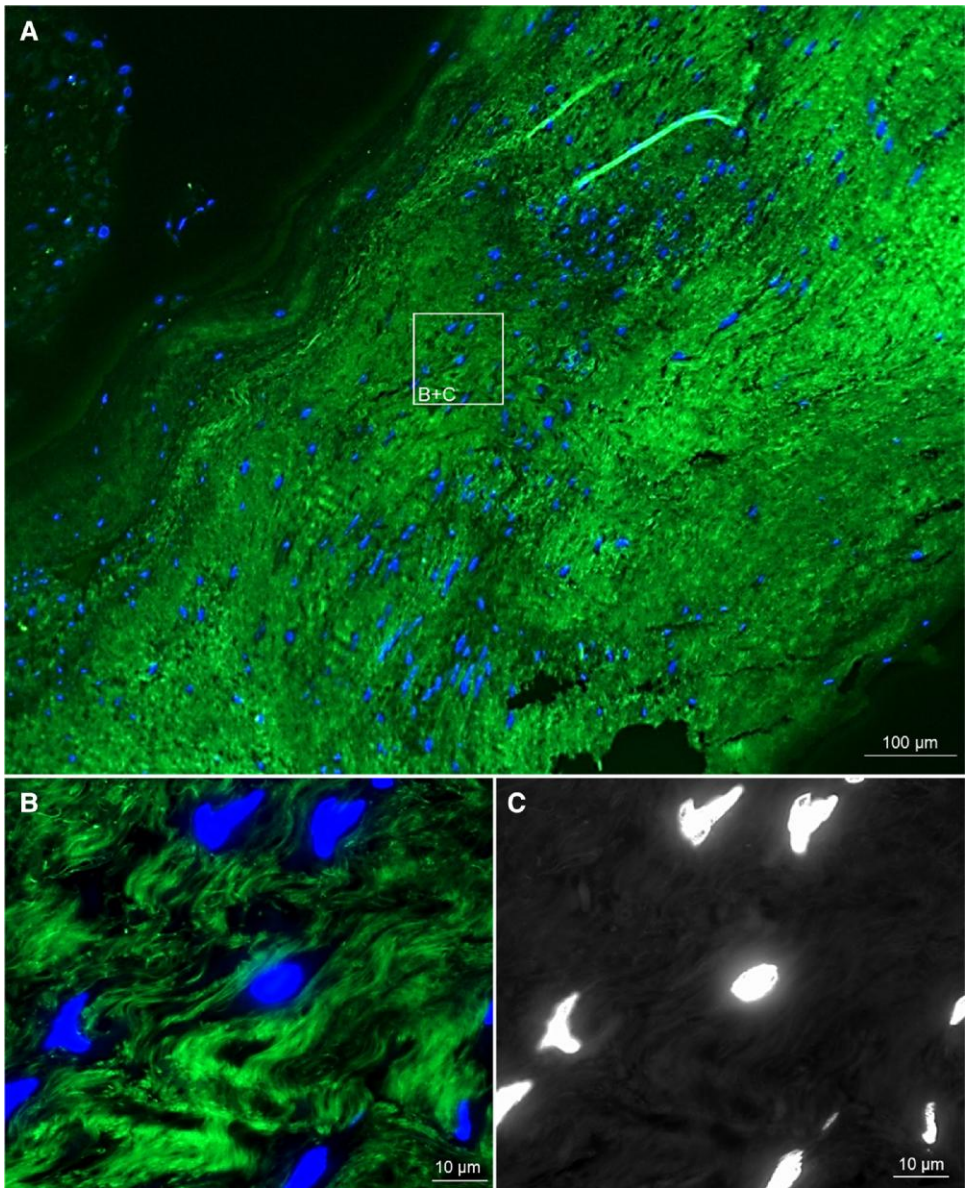

Figure 7. FISHseq showing no signs of infective endocarditis. FISH of a heart valve section from a 55-year-old male patient, initially suspected of IE. Neither the Standard nor Enhanced Diagnostics Pathway resulted in identification of a causative microorganism, with FISHseq visually not detecting any bacteria in the heart valve sample investigated. Hence, this patient was classified as "no IE." The section was hybridized with the panbacterial probe $EUB338_{Cy3}$ for visualization of active bacteria (based on ribosome content), the nonsense control probe $NON338_{Cy5}$, and DAPI for visualization of nucleic acids in host cell nuclei and bacteria. *A*, Overview shows the autofluorescent heart valve tissue in green with host cell nuclei only in blue; no bacteria are visible at higher magnification (B and C). *B*, The merged image of the DAPI channel (blue) and the FITC channel in green. *C*, Greyscale image of the separate DAPI channel. No bacteria are visible. Abbreviations: FISH, fluorescence in situ hybridization; FISHseq, fluorescence in situ hybridization + polymerase chain reaction/sequencing; IE, infective endocarditis.

information about the vitality or activity of the microorganisms at the time the samples were taken.

FISHseq Combines the Advantages of PCR/Sequencing With Direct Visualization and Characterization of Pathogens in Tissue Samples. FISHseq is a molecular technique that combines the results of 16S rRNA gene PCR/sequencing and FISH/microscopy for a joint, comprehensive diagnostic interpretation [20]. As the FISH probes target the microbial rRNA, the fluorescent signal intensity correlates with ribosome content, enabling a differentiation between active and past/treated IE [18, 25]. In addition, especially when standard methods have identified known potential skin flora contaminants such as *C. acnes* or *S. epidermidis*, FISHseq can distinguish between an actual infection and contamination [34]. Of 10 blood culture–positive cases isolating *S. epidermidis* in this study, FISHseq visualized the bacteria in 6 cases, indicating an actual infection, whereas in 3 cases no bacteria were detected

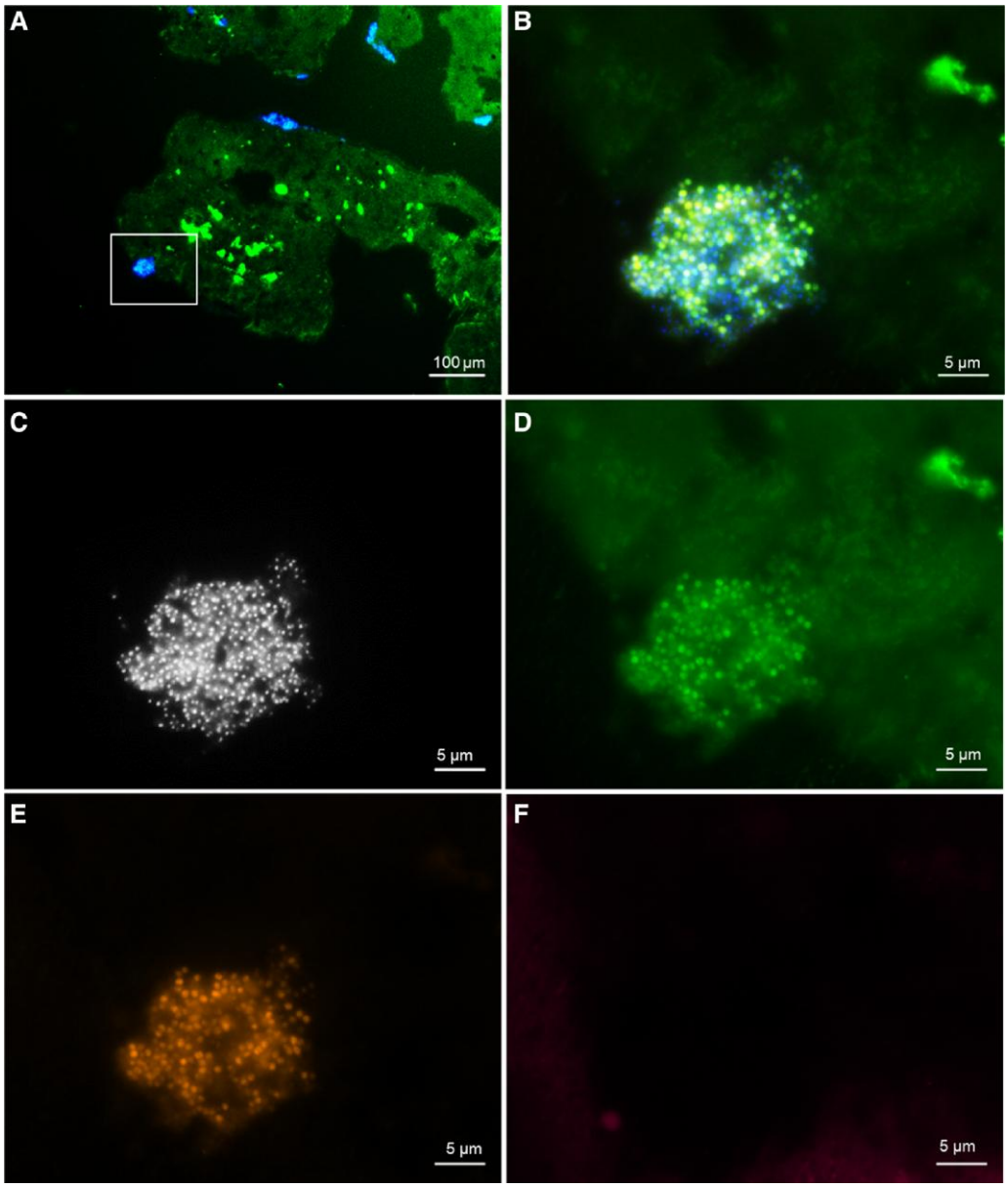

Figure 8. FISHseq proving infection with *Staphylococcus epidermidis*. FISH of a heart valve section from a 61-year-old male patient that was classified as possible IE using the modified Duke Criteria preoperatively but classified as inconclusive by the Standard Diagnostic Pathway because the histological examination of his heart valve showed signs of active IE, whereas all other methods were negative. In this case, FISHseq showed *S. epidermidis* in biofilms. *A*, The overview of the heart valve tissue shows the tissue background in green and bacterial biofilms at different locations within the tissue and at the tissue edges. The inset marked in (A) shows a region magnified in (B–F). The FISH was performed with the pan-bacterial probe $EUB338_{FITC}$ in green, the staphylococci genus–specific probe $STAPHY_{Cy3}$ in orange, the nonsense probe $NON338_{Cy5}$ as negative binding control in magenta, and the nucleic acid stain DAPI. *B*, A merged image of all channels. *C–F*, The same microscopic field of view with separate channels for the nucleic acid stain DAPI in black and white, the EUB338 probe and the tissue background in green, the STAPHY probe in orange, and the negative control probe in magenta, respectively. Visible are active staphylococci visualized by the pan-bacterial FISH probe EUB338 and the staphylococci genus–specific FISH probe STAPHY, whereas no signal is detectable with the nonsense probe NON338. Abbreviations: FISH, fluorescence in situ hybridization; FISHseq, fluorescence in situ hybridization + polymerase chain reaction/sequencing; IE, infective endocarditis.

microscopically, thus indicating presumable contamination. One case remained inconclusive.

Compared With Standard Diagnostics Including PCR/Sequencing, FISHseq Reduced the Number of Inconclusive Cases. In this study, FISHseq was able to reduce the number of inconclusive cases from 57 to 8, indicating its value as a diagnostic routine method. Conclusively positive or negative cases increased from 36.3% to 58.1% and from 17.7% to 35.5%, respectively. FISHseq visually confirmed the presence of microorganisms

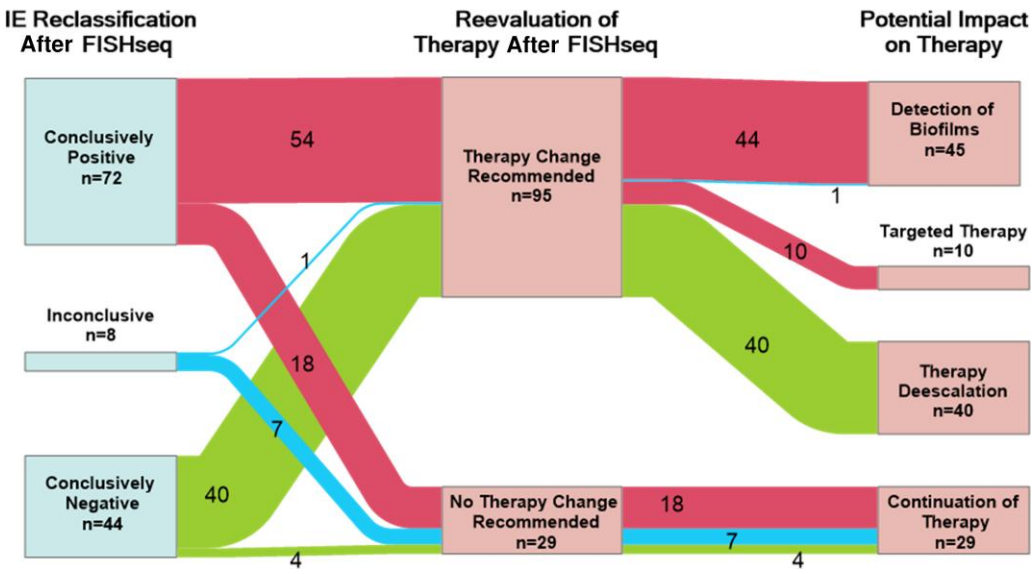

Figure 9. Potential impact of FISHseq results on antibiotic therapy. The figure shows the potential impact of FISHseq results on the antibiotic therapy of the IE patients in this study. The crimson color indicates conclusively positive patients after FISHseq evaluation; blue indicates inconclusive patients, and green indicates conclusively negative patients. The possible impact on antibiotic therapy is based on current guidelines and selected publications and refers to a potential therapy escalation because of microbial biofilms in the heart valve, a targeted antibiotic therapy upon causative pathogen identification, or therapy de-escalation because of inactive bacteria/absence of bacteria. Abbreviations: FISHseq, fluorescence in situ hybridization + polymerase chain reaction/sequencing; IE, infective endocarditis.

in all BCNE cases, where PCR/sequencing alone was also successful. These results correspond with those of a study on FISHseq by Hajduczenia et al., in which FISHseq provided a diagnostic benefit as compared with conventional cultural diagnostics in 30% of cases [23]. Compared with the results of the study by Hajduczenia et al., FISHseq in our study enabled the reclassification of Duke Criteria in 51/124 (41.1%) cases, indicating a similar improvement in diagnostics as previously observed not only for prosthetic valve endocarditis but in endocarditis in general.

Overall, in this study the results of the Enhanced Diagnostic Pathway support the intraoperative findings and therefore the inclusion of the surgical evidence as a new major diagnostic criterion [5]. However, this does not mean that microbiological and histopathological diagnostics should be omitted.

The spatial analysis of microorganisms through FISHseq enables the differentiation between single bacteria, microcolonies, or biofilms [18]. Indeed, in this study, in 36.3% of all cases and 50.0% of preoperative definite IE cases, FISHseq revealed the presence of bacterial biofilms. Despite their accepted clinical importance, standard diagnostics are currently unable to detect biofilms. Enhancing the understanding of pathogen localization in IE, including predilection sites on prosthetic valves, is a key element for comprehensive diagnostics.

FISHseq Contributes to Unambiguous Results in IE Diagnostics, Contributing to Personalized Therapeutic Strategies. Collectively, the results of this study indicate that FISHseq improves the diagnostics of IE and can contribute to the establishment of personalized therapeutic concepts for patients with IE. As various studies have previously highlighted, selecting appropriate antimicrobial strategies is not only highly dependent on the diagnostic results obtained, but presents a unique challenge itself [2, 7, 35]. Based on the FISHseq data, in combination with routine diagnostic data, a therapy change would have been recommended in 76.6% of cases investigated here. However, due to the retrospective study design, the actual therapy change could not be analyzed in this study.

The POET study concluded that partial oral antibiotic treatment is noninferior to solely performing intravenous treatments in patients with left-sided IE [36]. As indicated by Spellberg et al., this "step-down" therapy should be incorporated into clinical practice [37]. However, selecting the appropriate patient collective for this de-escalation of therapy is essential for patient safety. In this study, standard diagnostics including PCR classified 22 patients as conclusively negative, while FISHseq classified 44 patients as conclusively negative. Besides the high importance of surgical expertise when evaluating suspicious heart valve lesions intraoperatively, these findings reiterate the value of FISHseq for patient safety and personalized therapies, especially when considering prospective studies, such as the POET trial.

Limitations

The limitations of our study include a possible sampling error when dividing the resected heart valve tissue, as some diagnostic

tests potentially received nonendocarditic tissue. However, the high agreement between PCR/sequencing and FISHseq results indicates that the endocarditic samples were divided equally, minimizing the impact of this error. In addition, the time from IE diagnosis to surgery was not considered in this study, nor was the duration of preoperative exposure to antibiotics, which may have influenced FISHseq results as well as the results of other diagnostic methods. Results of potential therapy changes were based on 2015 ESC Guidelines and selected publications [24, 36, 38]. Due to the retrospective nature of this study, however, actual therapy changes and outcomes were not assessed, and the data were partially incomplete. This clearly highlights the need for further prospective studies on the optimal therapy for patients with IE, including FISHseq, in routine diagnostics.

CONCLUSIONS

This study reaffirms the value of molecular methods within routine diagnostic algorithms for patients with IE. This study indicates that FISHseq is a potent technique to improve diagnostics for patients with IE. Through rapid, direct visualization and characterization of microorganisms, FISHseq could reduce the number of inconclusive cases by 86.0%, and thereby increase the number of conclusively positive or negative cases substantially. FISHseq provided an added value in 79/124 cases (63.7%) and potentially would have impacted therapy in 95/124 cases (76.6%), taking not only species identification but also activity, localization, and spatial architecture into account. Future prospective studies assessing therapeutic strategies for IE patients should consider the addition of FISHseq diagnostics due to its unambiguous results, contributing to safe, personalized treatment.

Supplementary Data

Supplementary materials are available at *Open Forum Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Acknowledgments

Author contributions. A.G.M.H., F.R.K., A.M., W.E., and J.K. have conceptualized the study and analyzed and interpreted the data. A.G.M.H., A.M., and J.K. wrote the original draft with input from all authors. All authors critically reviewed and approved the final version of the manuscript.

Ethics statement. The study was performed in accordance with the ethical guidelines of the 1964 Declaration of Helsinki and was approved by the local ethics committee (EA4/120/21) of the Charité–Universitätsmedizin Berlin, Germany. As the study did not modify the sampling during surgery, FISHseq is a standard routine diagnostic technique established in the Biofilmcenter, and the comparison of results was performed retrospectively, the need for informed consent was waived according to the ethics committee approval. All authors vouch for the completeness and accuracy of the data and analyses.

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Potential conflicts of interest. J.K. is CEO of MoKi Analytics GmbH. A.M. and J.K. are shareholders of MoKi Analytics GmbH. A.M. is the owner of the private practice Moter Diagnostics. All other authors report no potential conflicts.

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