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Molecular Analysis With 16S rRNA PCR/Sanger Sequencing and Molecular Antibiogram Performed on DNA Extracted From Valve Improve Diagnosis and Targeted Therapy of Infective Endocarditis: A Prospective Study

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Background. Molecular analysis (MA) on heart valve (HV) improves the microbiologic diagnosis of infectious endocarditis (IE). The main drawback of MA is the lack of antimicrobial susceptibility information.

Methods. We conducted a prospective cohort observational study of consecutive adult patients from April 2012 to May 2021 who underwent valve surgery at our hospital. The performance of MA, blood cultures (BC) and valve cultures (VC), and the diagnostic and therapeutic impact of MA were evaluated. Molecular antibiogram results were compared to culture-based antimicrobial susceptibility testing (AST).

Results. A total of 137 patients with definite IE and 52 patients with no IE were enrolled in the study. Among IE cases BC, VC, and MA were positive in 75 (55%), 30 (22%), and 120 (88%) of IE cases, respectively. Among 62 cases of BC-negative IE (BCNE), 57 achieved diagnosis with MA. MA led to a change of antimicrobial therapy in 92% of BCNE. MA was negative in 100% of patients with no IE. Molecular antibiogram performed on 17 valve specimens that resulted positive for pathogens potential carrier of genes encoding for multidrug resistant mechanisms showed 100% concordance with AST.

Conclusions. MA showed a high specificity and sensitivity in etiological diagnosis of IE. Molecular antibiogram could overcome the major limitation of MA that is the lack of susceptibility testing. We advocate for the inclusion of MA among diagnostic criteria for IE and for a more extensive use of molecular antibiogram when the culture result is negative, and MA is the only positive test.

Keywords. infective endocarditis; blood culture negative endocarditis; molecular analysis; 16S and 18S rRNA; molecular antibiogram.

Despite diagnostic and therapeutic advances in its management, infective endocarditis (IE) is still associated with high morbidity and mortality mainly due to its complications [1]. Prompt diagnosis, targeted antibiotic and surgical therapy are crucial for its prognosis [1]. Nevertheless, blood cultures (BC), the gold standard for the etiological diagnosis, are negative in up to 70% of cases [2, 3]. BC negativity is due to 3 major reasons: previous administration of antimicrobial agents, inadequate microbiological techniques, and infections caused by highly fastidious bacteria or non-culturable pathogens [2–5].

Up to 42% of cases of IE require surgical repair or replacement of the affected valve [1, 6–8], but when fastidious microorganisms are involved, the rate is higher, reaching 80%, probably due to delay in diagnosis and in starting an appropriate treatment [3, 9]. Heart valve (HV) repair or replacement offers the opportunity for the microbiology to provide an

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etiological diagnosis, but valve cultures (VC) have shown suboptimal diagnostic performance [6, 10–14].

Since the beginning of the 1990s, molecular analysis (MA) based on amplification and sequencing of ribosomal RNA (rRNA) genes (16S rRNA for bacteria and 18S rRNA for fungi) has been used on valve tissue to identify causative microorganisms [15]. Several studies demonstrated the diagnostic value of MA on HV for etiological diagnosis of IE [13–20] and its impact on antimicrobial therapy [21, 22]. For these reasons, MA has been proposed for inclusion into Duke's Criteria [14, 23, 24]. A common limitation reported from MA studies is the lack of antimicrobial susceptibility information. Recently, the possibility of detection of some resistance mechanisms at the molecular level has become available in diagnostic practice. This approach has become very attractive to deliver faster results on antimicrobial susceptibility, with a potentially remarkable impact on antimicrobial stewardship and clinical outcomes [25].

In our tertiary level hospital, where patients are referred for cardiac surgery and have received previous antibiotic therapy, percentage of IE lacking microbiological diagnosis was very high until 2011. Therefore, we added to our diagnostic workflow of IE undergoing HV surgery the 16S–18S rRNA polymerase chain reaction (PCR) MA on HV to reduce the rate of blood culture-negative endocarditis (BCNE). Although the diagnostic value of MA is well described in literature, less is known on the added value of molecular antibiogram for susceptibility data in order to guide antimicrobial therapy. In our opinion, this would overcome the main limitation of MA and help to avoid unnecessary broad-spectrum antibiotics promoting antimicrobial stewardship principles.

Objectives

Our aim for current study was to assess diagnostic performance of 16S–18S rRNA PCR/Sanger sequencing (MA) for IE diagnosis in patients with HV infection, evaluate the impact of MA on microbiological diagnosis and therapeutic management of IE, in particular for BCNE, and to assess the performance of molecular antibiogram.

METHODS

The Mediterranean Institute for Transplantation and Highly Specialized Therapies (ISMETT) is a tertiary care hospital where patients with IE are referred for HV cardiac surgery from hospitals without cardiac surgery unit. At the time of the admission patients are often already on antibiotic treatment, but without a microbiological diagnosis. We performed a prospective cohort study between April 2012 through May 2021 of all consecutive patients who underwent HV surgery (repair or replacement) at our center either with definite IE or with rejected IE diagnosis. The study was approved by our Institutional Research Review Board (IRRB/21/12). All patients gave their written informed consent for research.

MA and culture were performed on every valve specimen, whereas BC were performed only for patients with definite IE, diagnosed according to modified Duke's criteria [26]. In all patients with definite IE and negative blood cultures, serologies for *Mycoplasma*, *Legionella*, *Brucella*, *Bartonella,* and *Coxiella* were performed. Molecular antibiogram was added into the study protocol in 2019 and was performed for IE caused by selected isolates based on 3 criteria: (1) availability of the result both in MA and in culture, with culture-based antimicrobial susceptibility testing (AST) available, in order to phenotypically validate molecularly determined resistance genotypes; (2) bacteria in which susceptibility testing results could have modified the treatment, that is, in pathogens that could potentially carry resistance genes. Streptococci were excluded from molecular antibiogram testing because our molecular panel included only genes conferring resistance to macrolides, tetracycline, and erythromycin, which are not used in treatment of IE. Regarding *Enterococcus faecalis*, the molecular antibiogram offers the opportunity to detect gene encoding high-level resistance to gentamicin, but in the therapy in enterococcal IE the combination of double beta-lactam is used more often in our center, rather than beta-lactam plus gentamicin. Moreover, resistance to vancomycin is infrequent among *E. faecalis*, and these strains usually retain susceptibility to beta-lactams; therefore, obtaining the molecular antibiogram for *E. faecalis* was considered not very useful for therapeutic purposes in our study. Cost-effectiveness of including these analyses might be different in vary in settings with different pattern of resistance or in selected cases; (3) availability of the stored strain and HV specimen in our laboratory.

Microbiological Analysis

Native or prosthetic valves were entirely immersed in liquid culture medium, where it remained for 24 hours before proceeding with the microbiological and molecular analysis. When perivalvular tissue was sent for analysis, it was processed together with the prosthetic valve.

Microbiological workflow is shown in Figure 1.

Blood and valve cultures: standard of care as elsewhere described [27]. Briefly, BC were incubated for 5 days in bioMerieux Bact/Alert 3D platform, VC were homogenized by a rotor-stator unit (IKA Ultra-Turrax) and incubated for 72 hours. In presence of microbial growth, for both BC and VC, the microorganisms were identified by MALDI-TOF using the Vitek MS instrument (bioMerieux), antimicrobial susceptibilities were determined using broth microdilution Vitek 2 Compact (bioMerieux), SensititreTM microdilution plates and ETEST strip (tigecycline only, bioMerieux), and interpreted according to EUCAST guidelines.

Molecular analyses. Total gDNA was extracted from homogenized tissue with QIAamp UCP Pathogen Mini kit

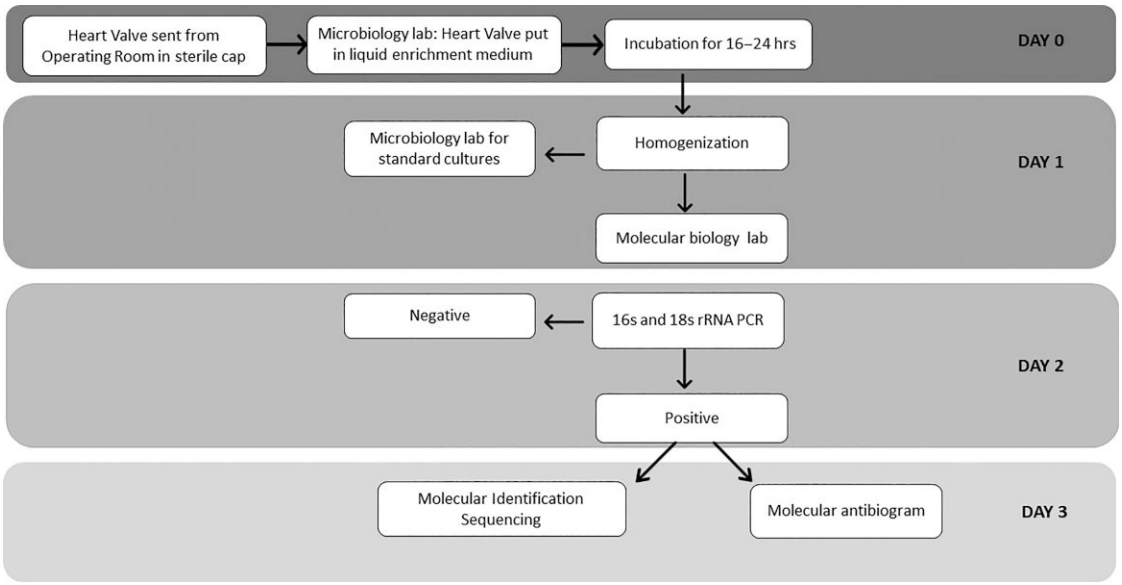

Figure 1. Microbiological workflow. The 18S rRNA was negative in all but 3 samples where *Candida* spp. were identified. Abbreviations: PCR, polymerase chain reaction; rRNA, ribosomal RNA.

(Qiagen), amplified and sequenced using MicroSEQ 500 16S rDNA Bacterial and D2 LSU rDNA Fungal Identification kit (Life Technologies), and analyzed using MicroSEQ ID Analysis software (Life Technologies).

Serology. Indirect immunofluorescence assays to detect significant levels of antibodies to *Coxiella burnetii*, *Bartonella quintana*, *Bartonella henselae*, and *Legionella pneumophila* were performed as elsewhere described [5]. Enzyme-linked immunosorbent assays were performed for *Brucella* spp. and *Mycoplasma pneumoniae*. *Coxiella burnetii* specific PCR targeting htpB gene was performed in cases of *Coxiella* positive serology [28].

The total gDNA extracted from homogenized tissue was used for the detection of antibiotic resistance genes performed by Antibiotic Resistance Genes Microbial DNA qPCR Array (Qiagen). The assay detects the presence of 87 antibiotic resistance gene belonging to aminoglycoside, beta-lactam, erythromycin, fluoroquinolone, macrolide-lincosamide-streptogramin b, tetracycline, vancomycin, and multidrug resistance classifications. In case of *Staphylococcus aureus*, *S. aureus* Microbial DNA qPCR Multi-Assay Kit was used (Qiagen) for the detection of mecA resistance gene (encoding penicillin-binding protein 2a, which confers resistance to beta-lactams, and considered the reference standard for the detection of methicillin-resistant *S. aureus* [MRSA]) and 2 virulence factor genes: lukF (Panton-Valentine leukocidin chain F precursor, encoding an exotoxin of *S. aureus*) and spa (*S. aureus* protein A, an important virulence factor that allows *S. aureus* to escape innate and adaptive immune responses).

Statistics

In order to assess the diagnostic accuracy of the MA, sensitivity, specificity, as well as positive and negative predictive values with their 95% confidence intervals, were calculated, as previously described [29]. All statistical analyses were performed with SAS 9.4 and R 4.0.5.

RESULTS

During the study period we included 137 consecutive patients with definite IE (cases) and 52 consecutive patients with rejected IE diagnosis (control group) who underwent HV surgery. Baseline characteristics of the population with IE are reported in Table 1. Overall, the median patient age was 60 years (interquartile range [IQR] 25–85), and 73.7% of patients were male. The more affected valves were aortic (56.9%) and native valves (70%). The most frequent risk factor was the presence of a preexistent cardiac device.

Etiological Diagnosis

BC were positive in 75/137 (55%) of IE cases. VC were positive in 30 (22%) cases, but in 3 cases, as BC and MA were concordant in identifying another causative agent, they were considered a contamination and false positive result. MA was positive in 120/137 (88%) cases (Table 2). In 63 cases MA confirmed the result provided by BC; in 57 cases it was the only positive test and allowed etiological diagnosis (Table 3).

In the group of 119 IE cases caused by non-fastidious pathogens, MA provided microbiological diagnosis in 47 BC negative cases (39.5%). MA identified 27 cases of *Streptococcus* spp., 12 cases of coagulase-negative Staphylococci (CoNS), 3 cases of

Table 1. Demographics Characteristics, Risk Factors for Infective Endocarditis and Outcome

Characteristics	Total Number, n = 137 (100%)
Age, years, median (range)	60 (IQR 25–85)
Gender, male	101 (73.7%)
Affected valve	
Aortic	78[a] (56.9%)
Mitral	56[a] (40.8%)
Pulmonary	3[a] (2.2%)
Tricuspid	3 (2.2%)
PM	1 (0.73%)
Valve material	
Native valve	97 (70.8%)
Biologic valve device	24 (17.5%)
Mechanical valve device	16 (8.5%)
Risk factors[b]	
Preexisting cardiac device	40 (29%)
None	34 (24.8%)
Gastrointestinal source	23 (16.8%)
Valvulopathy	14 (10.2%)
Odontogenic source	12 (8.8%)
IVDU	3 (2.2%)
Others[c]	15 (10.9%)
Outcome	
Abdominal septic emboli	35/89 (39.3%)
CNS septic emboli	31/49 (63.2%)
Pulmonary septic emboli	9/50 (18%)
In-hospital mortality	16 (11.6%)

Abbreviations: CNS, central nervous system; IQR, interquartile range; IVDU, intravenous drug user; PM, pacemaker.

[a]Two cases of mitro-aortic endocarditis, and 1 case of endocarditis involving the aortic, mitral, and tricuspid valves.

[b]Seven patients had more than 1 risk factors.

[c]Others: Risk factors that were likely responsible for bacteremia included previous invasive procedures, recurrent urinary tract infection (UTI), trauma, hemodialysis.

S. aureus, 3 of *Enterococcus*: in these 45 cases MA allowed to narrow the antimicrobial spectrum of provided treatment. MA also identified 2 gram-negative bacteria in BCNE allowing the change of antimicrobial treatment (Table 4).

In the group of 15 cases due to nonculturable/fastidious bacteria, the diagnosis was resolved by MA in 10 cases. As such MA allowed to provide targeted antibiotic therapy in 67% of cases in this group of difficult to diagnose BCNE (Tables 3 and 4).

Among the 5 cases of IE with negative culture and negative MA, 2 cases were diagnosed as *Coxiella burnetii* infection with serology and confirmed with specific PCR performed on HV. In 3 cases of IE no microbiological diagnosis was obtained.

In the control group, valve culture was positive in 1 case for *P. aeruginosa* (this patient had no sign of active or past infection and is alive despite no specific treatment provided, thus considered as a false positive result), whereas MA was negative in all control patients, thus showing a specificity of 100%.

Serology for *Brucella* was positive in 2/2 *Brucella* IE cases, supporting cultural and molecular data. Serology for *Bartonella* was positive in the single *Bartonella* IE case, supporting MA diagnosis. Serology for *Mycoplasma* and *Legionella* were negative in all tested patients. In our cohort etiologic diagnosis was obtained in 98% of patients (134/137), and 59 (95%) of 62 patients with BCNE received a targeted antimicrobial therapy thanks to the addition of MA to our diagnostic workflow (Tables 3 and 4).

Molecular Resistance Testing

In order to evaluate concordance of molecular antibiogram and culture-based AST, we identified 19 microorganisms, diagnosed with both MA and culture, that could potentially carry resistance genes (13 *S. aureus*, 1 *Enterococcus faecium*, 1 *Pseudomonas aeruginosa*, 1 *Proteus mirabilis*, 2 *Klebsiella pneumoniae*, 1 *Serratia* spp.). Seventeen of these 19 strains (89%) were stored in our laboratory and comparison of molecular antibiogram for detection of resistance genes and standard AST was carried out (Table 5).

In 13 cases of *Staphylococcus aureus* there was 100% concordance between molecular test and AST (all samples were methicillin susceptible on the microdilution test and did not carry the mecA gene at the molecular antibiogram). In 2 cases of *Klebsiella pneumoniae* KPC, 1 *Proteus* spp. ESBL and 1 *E. faecium* we found again a 100% concordance between the two methods.

In additional 3 cases of *S. aureus* IE the diagnosis was made only with MA. Using the MRSA qPCR assay, we were able to exclude the presence of the mecA gene and to overcome the absence of a culture-based AST.

DISCUSSION

In this prospective observational study on IE we reached etiological diagnosis in 98% of cases of IE using molecular analysis coupled with blood cultures and serology. Addition of MA to

Table 2. Diagnostic Performance of Blood Culture, Valve Culture, and MA on Valve

	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
Blood culture	55%	…	…	…
Valve culture	22% (15%–30%)	99% (90%–100%)	97% (83%–100%)	32% (25%–40%)
Molecular analysis	88% (18%–93%)	100% (93%–100%)	100% (97%–100%)	75% (64%–85%)

Abbreviations: CI, confidence interval; MA, molecular analysis.

Table 3. Aetiology of Endocarditis in Our Cohort, Positivity Rate of Blood Culture and MA on Valve in IE Cases Due to Easy Growing and Fastidious Microorganisms and MA Role (Diagnosis, Confirmation of Blood Culture Results, or None) in IE Diagnosis

Agents of IE, 134/137		Blood Culture Positive N = 75 (%)	Molecular Analysis Positive N = 120 (%)	MA Contribution		
Pathogen Identified (N = 134; 98%)				Diagnosis	Confirmation of Blood Culture	None
Non-fastidious, N = 119	*Streptococcus* spp. (52; 37.9%)	25 (48%)	49 (94%)	27 (52%)	22 (42%)	3 (6%)
	Enterococcus spp. (20; 14.6%)	17 (85%)	18 (90%)	3 (15%)	15 (75%)	2 (10%)
	Staphylococcus aureus (19; 13.9%)	16 (82%)	16 (82%)	3 (16%)	13 (68%)	3 (16%)
	Coagulase-negative *Staphylococcus* (18; 13.2%)	6 (33%)	15 (83%)	12 (66%)	3 (17%)	3 (17%)
	Escherichia coli (2; 1.7%)	1 (50%)	1 (50%)	1 (50%)	0	1 (50%)
	Klebsiella pneumoniae (2; 1.7%)	1 (50%)	2 (100%)	1 (50%)	1 (50%)	0
	Pseudomonas aeruginosa (1; 0.8%)	1 (100%)	1 (100%)	0	1	0
	Proteus mirabilis (1; 0.8%)	1 (100%)	1 (100%)	0	1 (100%)	0
	Serratia spp. (1; 0.8%)	1 (100%)	1 (100%)	0	1 (100%)	0
	Candida spp. (3; 2.2%)	3 (100%)	3 (100%)	0	3 (100%)	0
Fastidious, N = 15	*Brucella melitensis* (2)	1 (50%)	2 (100%)	1 (50%)	1 (50%)	0
	Coxiella burnetii (2)[a]	0	0	0	0	2 (100%)
	HACEK (2)	0	2 (100%)	2 (100%)	0	0
	Propionibacterium acnes (2)	0	2 (100%)	2 (100%)	0	0
	Bartonella spp. (1)	0	1 (100%)	1 (100%)	0	0
	Kytococcus schoederi (1)	1 (100%)	1 (100%)	0	1 (100%)	0
	Lactobacillus casei (1)	0	1 (100%)	1 (100%)	0	0
	Mycobacterium avium complex (1)	0	1 (100%)	1 (100%)	0	0
	Parvimonas micra (1)	0	1 (100%)	1 (100%)	0	0
	Rothia dentocariosa (1)	0	1 (100%)	1 (100%)	0	0
	Granulicatella adiacens (1)	1 (100%)	1 (100%)	0	1 (100%)	0
Total: 134 cases with etiologic diagnosis, no pathogen identified in 3 cases, 137 cases of IE		75 (55%)	120 (88%)	57	63	14

Abbreviations: HACEK, *Haemophilus, Aggregatibacter, Cardiobaterium, Eikenella, Kingella*; IE infective endocarditis; MA, molecular analysis.

[a]Diagnosed with serology and specific polymerase chain reaction (PCR) (MA was negative).

our diagnostic workflow allowed an etiological diagnosis in 95% of BCNE. Molecular antibiogram showed high concordance with standard AST being a promising method to overcome lack of susceptibility information when the etiological diagnosis is performed with MA.

In our cohort MA showed a high specificity (100%) and sensitivity (88%) in etiological diagnosis of IE, higher than standard culture, with sensitivity 55% and 22% for, respectively, blood and valve culture. The sensitivity of MA in our study is in range with the sensitivity reported from other studies ranging from 81% to 96% [14, 21, 30, 31]. We found 100% agreement between MA and blood culture results when both available, confirming the results of previous similar studies that reported strong agreement of MA results with blood culture [13, 32]. MA resulted negative in 17 IE cases, the reasons may be a low inoculum in the sample, timing of surgery, sampling error, or mutations in the primer target region [33, 34]. In our cohort the proportion of BCNE was 45%, and 92% of them achieved an etiological diagnosis with MA on HV. With addition of *Coxiella burnetii* serology and specific PCR the proportion of etiological diagnosis of BCNE increased to 95%. In a recent case series, Fournier et al identified the etiologic pathogen in 78% of BCNE by implementing a multimodal strategy involving MA, histopathology and serology [2]. Our findings on non-fastidious/fastidious microrganisms ratio in culture negative-MA positive IE confirmed data from previous studies, and all of them show accordance on the main role played by non-fastidious bacteria vs fastidious ones in culture negative IE, being previous antibiotic therapy the main cause for negative BC [14, 16, 30, 35, 36].

In our cohort almost 80% of cases of BCNE diagnosed with MA were caused by a non-fastidious microorganism, confirming data from previous studies in which non-fastidious BCNE diagnosed with MA ranged from 35.7% to 83%, thus demonstrating the reliability of our results [14, 16, 30, 35, 36].

By providing etiological diagnosis, MA had a dramatic therapeutic impact on clinical and therapeutic management: 95% of the BCNE received a targeted antimicrobial therapy thank to MA contribution. In MA-diagnosed IE caused by fastidious or non-culturable micro-organisms and most easy growing pathogens (ie, *Streptococcus* spp.) MA provided the opportunity for appropriate, narrow spectrum and targeted treatment

Table 4. Aetiology of Blood Culture Negative Endocarditis (BCNE) Diagnosed With MA on Valve and Coxiella Serology and Specific PCR

Etiology in BCNE (n = 62)		Number (%)	Impact of MA on treatment
Non-fastidious 47 (76%)	*Streptococcus* spp.	27 (43%)	Narrowing the antimicrobial spectrum
	Enterococcus spp.	3 (4.9%)	Narrowing the antimicrobial spectrum
	Staphylococcus aureus	3 (4.9%)	Narrowing the antimicrobial spectrum
	CoNS	12 (19.6%)	Narrowing the antimicrobial spectrum
	Gram negative bacilli	2 (3.2%)	Change to targeted treatment
Nonculturable/fastidious Slow growing 12 (19%)	*Brucella melitensis*	1 (1.6%)	Change to targeted treatment
	Coxiella burnetii[a]	2 (3.2%)	None
	HACEK	2 (3.2%)	Narrowing the antimicrobial spectrum
	Cutibacterium acnes	2 (3.2%)	Narrowing the antimicrobial spectrum
	Bartonella spp.	1 (1.6%)	Change to targeted treatment
	Lactobacillus casei	1 (1.6%)	Narrowing the antimicrobial spectrum
	MAC	1 (1.6%)	Change to targeted treatment
	Parvimonas micra	1 (1.6%)	Narrowing the antimicrobial spectrum
	Rothia dentocariosa	1 (1.6%)	Narrowing the antimicrobial spectrum
No identification		3 (4.9%)	None

Abbreviations: CoNS, coagulase-negative staphylocci; HACEK, *Haemophilus, Aggregatibacter, Cardiobacterium, Eikenella, Kingella*; MA, molecular analysis; MAC, *Mycobacterium avium complex*; PCR, polymerase chain reaction.

[a]Diagnosed with serology and specific PCR (MA was negative).

and risk factors control (eg, *Streptococcus gallolyticus/bovis* and occult colonic tumors) [37]. In our study the benefit of MA in clinical management is higher from what reported from other studies: a recent study by Fida et al reported that 16S rRNA/Sanger sequenced leading to a change in clinical management in 22% testing of cardiovascular specimens [38]. Peeters et al performed a study that included 127 patients, and in 12 (10%) of them molecular results influenced antimicrobial therapy [21]. Miller et al included 50 patients with definite IE and MA had therapeutic impact in 4 (8%) of them [30]. Marsch et al included 46 cases of culture-negative IE and in 7 (15%) patients antibiotic therapy was adjusted according to 16S rRNA PCR results from HV [39]. The higher therapeutic impact and the better performance of our test in changing clinical management could be attributed to several factors: the prospective nature of our study and the short turnaround time

Table 5. Comparison of Molecular Antibiogram for Detection of Resistance Genes and Standard Antimicrobial Susceptibility Testing

Pathogen/Resistance Mechanism	Antibiotic	Molecular Antibiogram (Detected Genes Encoding for Antibiotic Resistance)	Culture-based Susceptibility Testing (Molecule Tested and MICs[a])
Klebsiella pneumoniae/ KPC	Aminoglycosides	Resistant (aadA1)	Resistant to GEN ≥16, Susceptible to AN ≤2
	Fluoroquinolones	Resistant (AAC(6)-Ib-cr, QnrS)	Resistant to CIP ≥4
	Beta-lactams	Resistant (CTX-M-1 Group, SHV, SHV(156G), SHV(238G240E), KPC)	Resistant to AMC ≥32, AM ≥32, FEP 8, CTX ≥64, CAZ ≥64,TZP ≥128, IPM ≥16 and MEM ≥16
	Others	…	Intermediate to TGC 2, Susceptible to CS ≤0.5, FOS 32 and SXT 40
Proteus mirabilis/ESBL	Aminoglycosides	Resistant (aadA1, aacC1)	Resistant to GEN >4 and TM >4, Susceptible to AN 8
	Fluoroquinolones	Resistant (AAC(6)-Ib-cr)	Resistant to CIP >1 and LVX >2
	Beta-lactams	Resistant (LAT, Class C beta-lactamase)	Resistant to AMC ≥32/2, AM >8, CTX >4, CXM > 8 and CAZ >8 Susceptible to FEP ≤1, TZP ≤4/4, ETP ≥8, MEM ≤0,5, CAZ/AVI 4
	Tetracycline	Resistant (tetA, Tetracycline efflux pump)	Insufficient activity for TGC
	Others	…	Resistant to CS >4, FOS>64 and SXT >4/76
Klebsiella pneumoniae/ KPC	Beta- lactams	Resistant (SHV, SHV(156G), SHV(238G240E), KPC)	Resistant to AMC ≥32, CTX ≥64, CAZ ≥64,TZP ≥128 and MEM ≥16
	Others	…	Resistant to CS ≥16, Susceptible to FOS 32 and SXT ≤20
Enterococcus faecium/ none	…	No resistance genes detected	Susceptible to all antibiotics tested (AM ≤2, SAM ≤2, QD 1, GEN S, IPM 4, LZD 2, STR S, TEC 2, TGC ≤0.12, VA ≤0.5)

Abbreviations: AM, ampicillin; AMC, amoxicillin-clavulanic acid; AN, amikacin; CAZ, ceftazidime; CAZ/AVI, ceftazidime-avibactam; CIP, ciprofloxacin; CS, colistin; CTX, cefotaxime; CXM, cefuroxime; ETP, ertapenem; FEP, cefepime; FOS, fosfomycin; GEN, gentamycin; IPM, imipenem; LVX, levofloxacin; LZD, linezolid; MEM, meropenem; MIC, minimum inhibitory concentration; QD, quinopristin-dalfopristin; SAM, ampicillin-sulbactam; STR, streptomycin; SXT, trimethoprim-sulfametoxazole; TEC, teicoplanin; TGC, tigecycline; TM, tobramycin; TZP, piperacillin-tazobactam; VA, vancomycin.

[a]According to EUCAST breakpoints.

(Figure 1) that allowed us to have the results available in 'real time' leading to prompt de-escalation or adjustment of antimicrobial treatment, high proportion of BCNE in our cohort, and high proportion of susceptible strains detected, while resistant pathogens were covered in the empirical treatment.

We confirm that valve culture has a poor performance. HV culture showed very low sensitivity (22%) and very low negative predictive value (48%), in fact in our cohort majority (78%) of IE cases had negative HV culture in line with previous literature reports [6, 10, 14, 23]. Moreover, valve culture did not allow any additional diagnosis to those performed with blood culture and/or MA.

A common limitation of MA is the lack of information about antimicrobial susceptibility that limit its therapeutic impact. In our cohort we retrospectively performed PCR assay of genes encoding for antimicrobial resistance on culture positive MA-diagnosed IE. The comparison of AST and molecular antibiogram in 13 cases of *S. aureus*, 3 cases of gram-negative bacilli and 1 *E. faecium* showed a full concordance. Interestingly, we found full concordance between the presence of Class C beta-lactamase on molecular antibiogram and cefepime susceptibility in the *Proteus mirabilis* case (Table 5). The identification of mecA gene in 3 strains of *S. aureus* detected exclusively through MA enabled us to confidently de-escalate the therapy. In our laboratory workflow of molecular antibiogram the time-to-response can be as short as 2 hours, and the panel of resistance genes detected is wide. Although it certainly shares the known drawbacks of molecular antibiogram of not providing MICs and having a higher cost, we think that the use of molecular antibiogram may serve as a tool to offer a tailored antimicrobial therapy in short time, especially in cases in which diagnosis is achieved only with MA.

This study has several limitations. First, our study was not large enough to include subgroups of interest, such as right-sided infection, IE due to yeasts or fungi, or prosthetic valve IE.

Second, data on duration of previous antimicrobial therapy and on follow-up are lacking. Third, the technique we used in this study (16S RNA and Sanger Sequencing) was not able to differentiate *Mycobacterium chimerae* from other species of *Mycobacterium Avium complex*. Finally, 16S rRNA and Sanger Sequencing is not useful in polymicrobial infections as it allows identification of a single organism: 16S rRNA gene sequencing cannot be applied to polymicrobial samples, because the presence of multiple microbial DNA results in overlaid Sanger reads that are uninterpretable. This could be overcome by next-generation sequencing of the amplified 16S rRNA gene used in metagenomic studies.

CONCLUSIONS

IE is a severe pathology which requires an early microbiological diagnosis and appropriate treatment. Microbiological diagnosis of IE poses a challenge, particularly in patients with negative blood cultures. MA on HV has substantially improved the diagnosis of IE. To the best of our knowledge, this is the first study to investigate the usefulness of molecular antibiogram in the setting of infectious endocarditis. The molecular antibiogram overcomes the limit of lack of information on antimicrobial susceptibility and it should be used in culture negative-MA positive cases of IE to provide data of susceptibility. We support the implementation of MA and molecular antibiogram in case of BCNE, and samples should be sent to a reference center with expertise and short turnaround time. We advocate for inclusion of MA among diagnostic criteria for IE and for the use of molecular antibiogram when the pathogen is detected only by MA techniques and is a potential carrier of antimicrobial resistance.

Notes

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References

1. Moreillon P, Que YA. Infective endocarditis. Lancet **2004**; 363:139–49.
2. Fournier PE, Gouriet F, Casalta JP, et al. Blood culture-negative endocarditis: improving the diagnostic yield using new diagnostic tools. Medicine **2017**; 96:e8392.
3. Brouqui P, Raoult D. Endocarditis due to rare and fastidious bacteria. Clin Microbiol Rev **2001**; 14:177–207.
4. Fournier PE, Thuny F, Richet H, et al. Comprehensive diagnostic strategy for blood culture-negative endocarditis: a prospective study of 819 new cases. Clin Infect Dis **2010**; 51:131–40.
5. Houpikian P, Raoult D. Blood culture-negative endocarditis in a reference center: etiologic diagnosis of 348 cases. Medicine **2005**; 84:162–73.
6. Munoz P, Bouza E, Marin M, et al. Heart valves should not be routinely cultured. J Clin Microbiol **2008**; 46:2897–901.
7. Shapira N, Merin O, Rosenmann E, et al. Latent infective endocarditis: epidemiology and clinical characteristics of patients with unsuspected endocarditis detected after elective valve replacement. Ann Thorac Surg **2004**; 78:1623–9.
8. Bouza E, Menasalvas A, Muñoz P, et al. Infective endocarditis—a prospective study at the end of the twentieth century: new predisposing conditions, new etiologic agents, and still a high mortality. Medicine **2001**; 80:298–307.
9. Lamas CC, Eykyn SJ. Blood culture negative endocarditis: analysis of 63 cases presenting over 25 years. Heart **2003**; 89:258–62.
10. Giladi M, Szold O, Elami A, Bruckner D, Johnson BL, Jr. Microbiological cultures of heart valves and valve tags are not valuable for patients without infective endocarditis who are undergoing valve replacement. Clin Infect Dis **1997**; 24:884–8.
11. Greub G, Lepidi H, Rovery C, et al. Diagnosis of infectious endocarditis in patients undergoing valve surgery. Am J Med **2005**; 118:230–8.
12. Morris AJ, Drinkovic D, Pottumarthy S, et al. Gram stain, culture, and histopathological examination findings for heart valves removed because of infective endocarditis. Clin Infect Dis **2003**; 36:697–704.
13. Bosshard PP, Kronenberg A, Zbinden R, Ruef C, Bottger EC, Altwegg M. Etiologic diagnosis of infective endocarditis by broad-range polymerase chain reaction: a 3-year experience. Clin Infect Dis **2003**; 37:167–72.
14. Marín M, Munoz P, Sanchez M, et al. Molecular diagnosis of infective endocarditis by real-time broad-range polymerase chain reaction (PCR) and sequencing directly from heart valve tissue. Medicine **2007**; 86:195–202.
15. Goldenberger D, Künzli A, Vogt P, Zbinden R, Altwegg M. Molecular diagnosis of bacterial endocarditis by broad-range PCR amplification and direct sequencing. J Clin Microbiol **1997**; 35:2733–9.
16. Breitkopf C, Hammel D, Scheld HH, Peters G, Becker K. Impact of a molecular approach to improve the microbiological diagnosis of infective heart valve endocarditis. Circulation **2005**; 111:1415–21.

17. Gauduchon V, Chalabreysse L, Etienne J, et al. Molecular diagnosis of infective endocarditis by PCR amplification and direct sequencing of DNA from valve tissue. J Clin Microbiol **2003**; 41:763–6.
18. Lisby G, Gutschik E, Durack DT. Molecular methods for diagnosis of infective endocarditis. Infect Dis Clin North Am **2002**; 16:393–412, x.
19. Millar B, Moore J, Mallon P, et al. Molecular diagnosis of infective endocarditis: a new Duke's criterion. Scand J Infect Dis **2001**; 33:673–80.
20. Lang S, Watkin RW, Lambert PA, Bonser RS, Littler WA, Elliott TS. Evaluation of PCR in the molecular diagnosis of endocarditis. J Infect **2004**; 48:269–75.
21. Peeters B, Herijgers P, Beuselinck K, et al. Added diagnostic value and impact on antimicrobial therapy of 16S rRNA PCR and amplicon sequencing on resected heart valves in infective endocarditis: a prospective cohort study. Clin Microbiol Infect **2017**; 23:888.e1–e5.
22. Ursenbach A, Schramm F, Severac F, et al. Revised version (INFD-D-20-00242): impact of 16S rDNA sequencing on clinical treatment decisions: a single center retrospective study. BMC Infect Dis **2021**; 21:190.
23. Vollmer T, Piper C, Horstkotte D, Korfer R, Kleesiek K, Dreier J. 23S rDNA real-time polymerase chain reaction of heart valves: a decisive tool in the diagnosis of infective endocarditis. Eur Heart J **2010**; 31:1105–13.
24. Shrestha NK, Ledtke CS, Wang H, et al. Heart valve culture and sequencing to identify the infective endocarditis pathogen in surgically treated patients. Ann Thorac Surg **2015**; 99:33–7.
25. Arena F, Giani T, Pollini S, Viaggi B, Pecile P, Rossolini GM. Molecular antibiogram in diagnostic clinical microbiology: advantages and challenges. Future Microbiol **2017**; 12:361–4.
26. Li JS, Sexton DJ, Mick N, et al. Proposed modifications to the Duke criteria for the diagnosis of infective endocarditis. Clin Infect Dis **2000**; 30:633–8.
27. Babowicz F, LaPlante R, Mitchell C, et al. Impact of accelerate pheno and BacT/Alert virtuo on clinical processes and outcomes in patients with sepsis and concurrent gram-negative bacteremia. Antimicrob Agents Chemother **2021**; 65: e02364–20.
28. To H, Kako N, Zhang GQ, et al. Q fever pneumonia in children in Japan. J Clin Microbiol **1996**; 34:647–51.
29. Clopper CJ, Pearson ES. The use of confidence or fiducial limits illustrated in the case of the binomial. Biometrika **1934**; 26:404–13.
30. Miller RJ, Chow B, Pillai D, Church D. Development and evaluation of a novel fast broad-range 16S ribosomal DNA PCR and sequencing assay for diagnosis of bacterial infective endocarditis: multi-year experience in a large Canadian healthcare zone and a literature review. BMC Infect Dis **2016**; 16:146.
31. Vondracek M, Sartipy U, Aufwerber E, Julander I, Lindblom D, Westling K. 16S rDNA sequencing of valve tissue improves microbiological diagnosis in surgically treated patients with infective endocarditis. J Infect **2011**; 62:472–8.
32. Boussier R, Rogez S, Francois B, Denes E, Ploy MC, Garnier F. Two-step bacterial broad-range polymerase chain reaction analysis of heart valve tissue improves bacteriological diagnosis of infective endocarditis. Diagn Microbiol Infect Dis **2013**; 75:240–4.
33. Raoult D. Etiological diagnosis of blood-culture-negative endocarditis. Enferm Infecc Microbiol Clin **2006**; 24:295–6.
34. Raoult D, Casalta JP, Richet H, et al. Contribution of systematic serological testing in diagnosis of infective endocarditis. J Clin Microbiol **2005**; 43:5238–42.
35. Miyazato A, Ohkusu K, Tabata M, et al. Comparative molecular and microbiological diagnosis of 19 infective endocarditis cases in which causative microbes were identified by PCR-based DNA sequencing from the excised heart valves. J Infect Chemother **2012**; 18:318–23.
36. Rampini SK, Bloemberg GV, Keller PM, et al. Broad-range 16S rRNA gene polymerase chain reaction for diagnosis of culture-negative bacterial infections. Clin Infect Dis **2011**; 53:1245–51.
37. Olmos C, Vilacosta I, Sarriá C, et al. *Streptococcus bovis* endocarditis: update from a multicenter registry. Am Heart J **2016**; 171:7–13.
38. Fida M, Khalil S, Abu Saleh O, et al. Diagnostic value of 16S ribosomal RNA gene polymerase chain reaction/sanger sequencing in clinical practice. Clin Infect Dis **2021**; 73:961–8.
39. Marsch G, Orszag P, Mashaqi B, Kuehn C, Haverich A. Antibiotic therapy following polymerase chain reaction diagnosis of infective endocarditis: a single centre experience. Interact Cardiovasc Thorac Surg **2015**; 20:589–93.