Microbial biofilm correlates with an increased antibiotic tolerance and poor therapeutic outcome in infective endocarditis

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Abstract

Background: Infective endocarditis (IE) is associated with high rates of mortality. Prolonged treatments with high-dose intravenous antibiotics often fail to eradicate the infection, frequently leading to high-risk surgical intervention. By providing a mechanism of antibiotic tolerance, which escapes conventional antibiotic susceptibility profiling, microbial biofilm represents a key diagnostic and therapeutic challenge for clinicians. This study aims at assessing a rapid biofilm identification assay and a targeted antimicrobial susceptibility profile of biofilm-growing bacteria in patients with IE, which were unresponsive to antibiotic therapy.

Results: Staphylococcus aureus was the most common isolate (50%), followed by Enterococcus faecalis (25%) and Streptococcus gallolyticus (25%). All microbial isolates were found to be capable of producing large, structured biofilms in vitro. As expected, antibiotic treatment either administered on the basis of antibiogram or chosen empirically among those considered first-line antibiotics for IE, including ceftriaxone, daptomycin, tigecycline and vancomycin, was not effective at eradicating biofilm-growing bacteria. Conversely, antimicrobial susceptibility profile of biofilm-growing bacteria indicated that teicoplanin, oxacillin and fusidic acid were most effective against S. aureus biofilm, while ampicillin was the most active against S. gallolyticus and E. faecalis biofilm, respectively.

Conclusions: This study indicates that biofilm-producing bacteria, from surgically treated IE, display a high tolerance to antibiotics, which is undetected by conventional antibiograms. The rapid identification and antimicrobial tolerance profiling of biofilm-growing bacteria in IE can provide key information for both antimicrobial therapy and prevention strategies.

Background

Infective endocarditis (IE) is associated with a poor prognosis and a reduced life expectancy [1−4]. The mortality rate for patients with IE is approximately 25%, with more than one-third of patients dying within a year [5−8]. The rapid identification of the specific microbial etiology and targeted antimicrobial therapy are fundamental for optimal patient treatment [9, 10]. Blood culture represents the standard test to determine the microbial etiology of IE, providing identification for almost all cultivable species responsible for endocarditis, including Candida species [11]. However, negative blood cultures are frequent, thus contributing to diagnostic uncertainty [12−15]. In suspected cases, skin examination may provide important indicators to support a diagnostic suspect of IE [16−18]. Staphylococci, streptococci and enterococci are leading causes of IE, accounting for more than 70% of cases [1, 10]. In particular, Staphylococcus aureus is the most...
prevalent microbial isolate, associated with approximately 30% of cases of IE, while coagulase-negative staphylococci account for more than 10% of cases [5, 11, 19]. *Streptococci* account for 30% of IE. Among them, *Streptococcus gollolyticus* appears as the major causative agent, accounting for 20–50% of streptococcal-related IE [5, 11, 19]. *Enterococcus* is the third major cause of both native and prosthetic valve IE, with *Enterococcus faecalis* emerging as the most frequent species (10% of IE) [5, 11, 19, 20]. Conversely, gram-negative bacilli are rare, accounting for 5% of cases of IE, while fungi have been only seldom described [11].

The marked IE pathogenicity relies on the ability of microorganisms to adhere, colonize and chronically persist on native or prosthetic valves, forming septic vegetations which consist of fibrin, platelets, inflammatory cells, and bacteria embedded within a structured biofilm matrix [15, 21, 22]. The mechanisms adopted by bacteria to form biofilms greatly differ among species and environmental conditions. Although the composition and function of the biofilm matrix can vary, some elements remain conserved. For instance, all biofilms contain extracellular polymeric substances (EPS) which mainly consists of polysaccharides, extracellular DNA, proteins and lipids, that holds bacterial cells together [22]. Compelling data from clinical and in vitro studies indicate that, by supporting adhesion and colonization as well as increased tolerance to antibiotics and host immune defenses, microbial biofilms represent a key element in the pathogenesis of endocarditis, being associated with poor treatment outcomes and the frequent need of surgical intervention [8, 23–25].

This study was aimed at measuring biofilm production and antimicrobial susceptibility profile of biofilm-growing bacteria from patients with surgically-treated IE, to support clinical decision-making and more effective therapeutic and preventative strategies.

### Results

The study included eight patients who underwent cardiac surgery for IE caused by Gram-positive bacteria, with a vegetation size on echocardiography ≥1.2 cm. The diagnosis of IE was confirmed by transesophageal echocardiography (TEE) echocardiography following the modified Duke Criteria [26]. The IE was localized on the aortic valve in 50.0% (4/8) of the patients, on the mitral valve in 37.5% (3/8), and on the mitro-aortic valves in 12.5% (1/8). The major predisposing factors were identified in previous surgical interventions (62.5%), followed by cardiac disease (37.5%), pulmonary disease (25.0%), HCV infection (25%) and hypertension (25%). Additional data on the underlying conditions administered treatment and clinical outcome are summarized in Table 1.

Blood cultures gave a positive microbial identification in six cases, while in the remaining two cases, they were repeatedly negative (Table 2). Based on microbial identification and antimicrobial resistance profiles, patients received targeted antibiotic therapy (Table 2). Blood culture-negative patients, but with clinical evidence of IE (N1 and N7), were empirically treated as detailed in Table 2. In particular, antimicrobial regimens containing β-lactams were administered in all patients, Vancomycin was administered in 4 patients, gentamicin administered in 2 patients and linezolid in 1 case. In 89.9% (7/8) of cases, a combination regimen of 2 or more antibiotics was used. The antibiotic therapy was administered for a period of 2 to 6 weeks. Despite antimicrobial therapy, all patients underwent cardiac surgery. Following surgery and the removal of the native valves and the vegetations, samples were sent to the microbiological laboratory for further assessment. The results indicated that the direct culture method, without sonication, allowed microbial identification in 1/8 (12.5%) of samples. Conversely, specimen sonication allowed microbial isolation and identification in all samples, with significant improvement (P = 0.004) as compared to conventional methods. Notably, sonication allowed an etiological diagnosis also in the two patients with repeatedly negative blood cultures (Table 2). *S. aureus* was the most frequently isolated pathogen, present in 4 cases, one of which was identified as methicillin-resistant *S. aureus* (MRSA). *E. faecalis* and *S. gollolyticus* were both isolated in 2 cases (Table 2).

| Table 1 Demographic and clinical data of the 8 patients with IE |  |
|-----------------------------------------------|-----------------|
| Patients with endocarditis (n = 8)            |                 |
| Male/Female                                    | 5/3             |
| Age, years [median (range)]                   | 57 (37–81)      |
| Death due to IE                                | 0               |
| Vegetation cm [median (range)]                | 1.55 (1.2–2.2)  |
| Aortic valve                                  | 4/8             |
| Mitral valve                                  | 3/8             |
| Mitro-aortic valve                            | 1/8             |
| Previous surgical interventions               | 5               |
| Cardiac disease                               | 3               |
| Pulmonary disease                             | 2               |
| HCV                                           | 2               |
| Hypertension                                  | 2               |
| Esophagitis                                   | 1               |
| Rheumatic polymyalgia                         | 1               |
| Rheumatoid arthritis                          | 1               |
| Acute kidney failure                          | 1               |
| Type 2 diabetes mellitus                      | 1               |
| Brain hemorrhage                              | 1               |
| Raynaud syndrome                              | 1               |

| Table 2. In particular, antimicrobial regimens containing β-lactams were administered in all patients, Vancomycin was administered in 4 patients, gentamicin administered in 2 patients and linezolid in 1 case. In 89.9% (7/8) of cases, a combination regimen of 2 or more antibiotics was used. The antibiotic therapy was administered for a period of 2 to 6 weeks. Despite antimicrobial therapy, all patients underwent cardiac surgery. Following surgery and the removal of the native valves and the vegetations, samples were sent to the microbiological laboratory for further assessment. The results indicated that the direct culture method, without sonication, allowed microbial identification in 1/8 (12.5%) of samples. Conversely, specimen sonication allowed microbial isolation and identification in all samples, with significant improvement (P = 0.004) as compared to conventional methods. Notably, sonication allowed an etiological diagnosis also in the two patients with repeatedly negative blood cultures (Table 2). *S. aureus* was the most frequently isolated pathogen, present in 4 cases, one of which was identified as methicillin-resistant *S. aureus* (MRSA). *E. faecalis* and *S. gollolyticus* were both isolated in 2 cases (Table 2).
Assessment of the antimicrobial resistance profiles

The antibiotic susceptibility profiles were determined according to EUCAST clinical breakpoint tables v 9.0. The results show that all the strains appeared susceptible to vancomycin (Table 3). S. aureus was highly susceptible to almost all tested antibiotics, but 100% resistant to benzylpenicillin. E. faecalis were susceptible to ampicillin, ampicillin/sulbactam, linezolid, teicoplanin, and tigecycline but resistant to clindamycin, erythromycin, and trimethoprim/sulfamethoxazole. Finally, S. gallolyticus isolates were susceptible to all antibiotics tested (Table 3).

Quantification of biofilm production

Biofilm production for each bacterial isolate was assessed by the clinical Biofilm Ring Test (cBRT) [27]. The results showed that S. aureus isolates were moderate (2/4 cases, including the MRSA) or high (2/4 cases) biofilm-producers. E. faecalis strains were both high biofilm producers as well as the S. gallolyticus strains, which were found to be

Table 2 Microbiologic Tests for bacterial isolation, antibiotic therapy and biofilm production. The level of biofilm production was measured by the cBRT

| Patients | Blood culture before surgery | Heart valve culture | Heart valve sonication | Antibiotic therapy | Biofilm production | Vegetation (Cm) |
|----------|-----------------------------|--------------------|-----------------------|-------------------|-------------------|-----------------|
| N1       | Negative                    | S. aureus          |                       | Ceftriaxone; Piperacillin/Tazobactam | High              | 1.4             |
| N2       | MRSA                        | MRSA               | MRSA                  | Linezolid; Piperacillin/Tazobactam | Moderate          | 1.2             |
| N3       | S. gallolyticus             | Negative           | S. gallolyticus       | Ceftriaxone; Gentamicin | Moderate          | 1.6             |
| N4       | S. aureus                   | Negative           | S. aureus             | Vancomycin; Meropenem | High              | 1.5             |
| N5       | E. faecalis                 | Negative           | E. faecalis           | Piperacillin-Tazobactam; Ampicillin; Meropenem | High              | 2.1             |
| N6       | S. gallolyticus             | Negative           | S. gallolyticus       | Piperacillin-Tazobactam; Vancomycin | High              | 2.2             |
| N7       | Negative                    | S. aureus          |                       | Ceftriaxone; Vancomycin; Piperacillin/Tazobactam | Moderate          | 1.3             |
| N8       | E. faecalis                 | Negative           | E. faecalis           | Vancomycin         | High              | 1.8             |

Table 3 Antibiotic resistance profile (% of resistance) of S. aureus, E. faecalis and S. gallolyticus (% of resistance) strains, obtained by the Antimicrobial Susceptibility Testing (AST) and the anti-biofilm test (ABT). N represents the number of samples. Ampicillin/ Sulbactam (AMP/SUL), High-level gentamicin (HLG), High-level streptomycin (HLS), Trimethoprim/Sulfamethoxazole (TMP/SMX)
moderate/high biofilm producers (Table 2). Thus, all the bacterial strains were classified in the range of moderate/high biofilm producers.

Confocal microscopy analysis of the biofilms (Fig. 1), examined after 24 h of incubation, was highly consistent with cBRT biofilm assessment. All the strains gave a full coverage throughout the entire extension of the substrate with the development of a thick and structurally complex biofilm of approximately 60 μm in height, with a marked predominance of live (green) cells.

**Antibiotic susceptibility of biofilm-producing isolates**

The susceptibility profiles gathered by conventional antimicrobial susceptibility testing were apparently in contrast to the lack of response to the antimicrobial therapy in vivo. Since all of the strains were substantial biofilm producers, we next evaluated whether the ability to produce biofilm might correlate with the increased resistance to antibiotics. To this end, the antimicrobial susceptibility profiles were assessed in microbial isolates growing in a biofilm. As for the AST, the ABT was performed by the broth dilution-based procedure, to ensure the comparability of the data. The results are summarized in Table 3. The antibiotic susceptibility of the different strains growing in biofilm gave resistance profiles which greatly differed from those gathered by AST. In particular, only 30.0% of the antibiotics tested were found to be in the range of the susceptibility breakpoint. Statistical analyses confirmed a significant difference of the overall antimicrobial susceptibility profiles by comparing the data gathered by conventional susceptibility testing to those obtained in biofilm-growing bacteria (P < 0.001).

All biofilm-growing *E. faecalis* and *S. gallolyticus* were susceptible to ampicillin, while the 3 methicillin-sensitive *S. aureus*, confirmed their susceptibility to oxacillin also when tested by ABT. Teicoplanin was effective against *E. faecalis* and *S. aureus* biofilms in 5 isolates out of 6 (83.3%), while 3 out of 4 isolates of *S. aureus* were susceptible to fusidic acid. Conversely, almost all the isolates were resistant to ceftriaxone, daptomycin, erythromycin, high level gentamicin, levofloxacin, linezolid, tigecycline trimethoprim/sulfamethoxazole and vancomycin. The spectrum of antimicrobial efficacy on different biofilm-growing bacterial isolates revealed that *S. aureus* was susceptible to 23.1–50.0% of the tested antibiotics, while *E. faecalis* and *S. gallolyticus* gave results below the breakpoints in only 33.3–50.0% of the antibiotics (Additional file 2). Notably, *S. aureus* N4, *S. gallolyticus* N6, *S. aureus* N7 and *E. faecalis* N8, which were unsuccessfully cleared by treatment with vancomycin in vivo, were resistant to this antibiotic when grown in biofilm. Likewise, the MRSA N2 and *S. gallolyticus* N3
strains, treated with linezolid and ceftriaxone, respectively, in vivo, were found to be tolerant to these antibiotics when tested in a biofilm. In the absence of positive blood culture, patient N1 was empirically treated with ceftriaxone and piperacillin/tazobactam. Therefore, for the S. aureus strain, subsequently isolated by sonication, comparative analysis between AST and ABT was not possible. E. faecalis N5, which was treated by ampicillin in vivo, was the only bacterial isolate found susceptible by both AST and ABT.

Discussion
IE is a severe multisystem disease with mortality rates of approximately 25% and a generally poor response to antimicrobial therapy [8]. Successful eradication is difficult to achieve and relapse is common even after a prolonged antimicrobial therapy [8]. Possible causes include a limited or compromised host response against biofilms, as well as the relatively high bacterial density within vegetations [8, 23–25]. In the present study, the echocardiographic findings revealed the presence of vegetations (≥1.2 cm) in all patients located on the aortic valve (4 patients, 50%), mitral valve (3 patients, 37.5%) and mitro/aortic valve (1 patient, 12.5%).

The most frequent bacterial etiology was represented by S. aureus, which was isolated from 50% of the samples, followed by E. faecalis and S. galloolyticus, both isolated in 2 cases. This finding is consistent with previous studies showing that staphylococci, streptococci, and enterococci, are responsible for approximately 80% of IE cases [28–35]. Conventional antimicrobial susceptibility assessment showed that all the bacterial isolates were highly susceptible in vitro to most antibiotics, including those considered of choice for the treatment of IE. In particular, all of the clinical isolates appeared susceptible to vancomycin, which is the antibiotic of choice in the treatment of MRSA and a first-line therapy against enterococci and streptococci [8, 10]. S. aureus isolates were all susceptible to daptomycin, fusidic acid, gentamicin, linezolid, rifampicin, teicoplanin, tigecycline and trimethoprim/sulfamethoxazole but were found to be 100% resistant to benzylpenicillin. MRSA was the causative agent of one case of IE (12.5%), and it was found to be resistant to benzylpenicillin, oxacillin and levofloxacin. E. faecalis isolates were susceptible to ampicillin, ampicillin/sulbactam, linezolid, teicoplanin, tigecycline, while S. galloolyticus isolates were found to be susceptible to all the antibiotics tested (Table 3). In all cases, the administered antibiotic therapy failed to achieve complete eradication of bacteria, leading to a clinical relapse and the need for surgical treatment. The high bacterial density in a structured biofilm matrix within the vegetation can pose a physical and metabolic barrier which might, at least in part, explain this failure. The assessment of biofilm production by the cBRT and confocal microscopy analysis showed that all the clinical isolates were able to readily develop a highly adhesive and structurally complex biofilm matrix, being therefore classified as moderate/high biofilm producers. Notably, none of the bacterial isolates was found to be weak biofilm producer. The images collected by confocal microscopy were highly consistent with cBRT measurements, revealing that the moderate/high biofilm production as measured by cBRT gave rise to a compact biofilm matrix of approximately 60 μm in height with all microbial isolates (Fig. 1).

It has been suggested that the antibiotic concentration required to eradicate biofilm-related infections can be a hundred times higher than the MIC for the same microorganism as assessed in planktonic culture by conventional antibiograms [36–38]. In this study, biofilm-growing microbial cultures were found to be highly tolerant to antibiotics as compared to their planktonic counterparts, which is usually explored by the conventional antimicrobial profiling (P < 0.001). S. aureus N4, S. galloolyticus N6, S. aureus N7 and E. faecalis N8, which were challenged with vancomycin in vivo, showed a poor susceptibility to this antibiotic when assessed in a mature biofilm. Likewise, the MRSA N2 and S. galloolyticus N3 strains, unsuccessfully challenged in vivo with linezolid and ceftriaxone, respectively, on the basis of the antibiogram, were found to be tolerant to these antibiotics when tested in biofilm. The S. aureus N1 was empirically, and unsuccessfully treated in vivo with ceftriaxone and piperacillin/tazobactam and only after surgery was subjected to a 4 weeks regimen of gentamicin, to which it was found susceptible by both AST and ABT. E. faecalis N5, which was challenged in vivo with ampicillin, was the only bacterial isolate which was found susceptible to the administered antibiotic by both AST and ABT. Failure of this antibiotic at eradicating E. faecalis N5 can be reasonably attributable to the very short duration of the antibiotic therapy (6 days).

Overall, the most effective antibiotics against biofilm-growing S. aureus were fusidic acid, oxacillin and Teicoplanin, with MBEC values below breakpoints in almost all isolates, while ampicillin and ampicillin/sulbactam were the most active drugs in IE caused by both E. faecalis and S. galloolyticus, respectively. This latter data is in agreement with previous observations, confirming the effectiveness of ampicillin against ampicillin-susceptible E. faecalis, particularly in those patients with aminoglycoside resistance, or at risk of aminoglycoside-nephrotoxicity [7, 8, 39]. Indeed, aminoglycosides are no longer recommended in the treatment of staphylococcal and enterococcal native valve endocarditis due to their limited clinical benefits, the increasing frequency of microbial resistance and renal toxicity [34, 40]. Gentamicin, which was found to be effective against planktonic-growing S. aureus, according to AST, gave minimal biofilm-eradication concentration (MBEC)
values below breakpoints only in 50% of isolates. Besides, gentamicin and streptomycin at high dosage showed a poor efficacy at eradicating biofilm-growing *E. faecalis*, which appeared to be susceptible to both drugs by conventional AST. Short term therapy (2 weeks) with gentamicin in combination with ceftiraxone has been previously suggested for the treatment of uncomplicated *S. gallolyticus* endocarditis [41–43]. Indeed, *S. gallolyticus* strains appeared susceptible to ceftiraxone by AST, but they were found to be resistant when assessed in the biofilm matrix.

Rifampicin is recommended as an adjunctive treatment in prosthetic valve endocarditis caused by staphylococci. However, additional clinical evidence is necessary to support the addition of rifampicin in the treatment of staphylococcal native valve endocarditis and in surgically treated patients [10, 44]. In the present study, rifampicin was found to be effective against 50% of *S. aureus* strains grown in biofilm. Indeed, rifampicin was active only against moderate biofilm-producers but failed against high biofilm-producing *S. aureus*. The poor efficacy of rifampicin against a structured microbial biofilm was also observed in previous studies reporting only a limited bacterial killing against high biofilm-producing *S. aureus* [36–45].

High-dose daptomycin (10 mg/kg/day) alone or in combination with a second antibiotic, is considered a viable alternative to vancomycin for the treatment of staphylococcal endocarditis [10, 46–48]. However, the failure of daptomycin in patients with bacteremia and endocarditis caused by *S. aureus* leading to persistent or relapsing infection was previously reported [49]. In this study, daptomycin had a poor efficacy against biofilm-producing *S. aureus* strains (Table 3). These results are in agreement with previous studies reporting a limited daptomycin efficacy at eradicating biofilm-related infections in vitro and animal models [24, 50–58]. Daptomycin induces a dual action on both the cell membrane and cell wall, creating ion channels that lead to bacterial death [59, 60]. Thus, direct access to the bacterial cell wall is a necessary prerequisite for daptomycin anti-microbial activity [61, 62]. The large, highly structured biomass produced by IE microbial isolates may present a physical barrier to the free drug diffusion, thus limiting daptomycin activity.

Vancomycin is considered a standard treatment for in IE sustained by MRSA, and, more generally, an appropriate empiric choice against Gram-positive bacteria [8, 63], although clinical failures and poor penetration into vegetations have been frequently reported [10, 64]. Indeed, all the strains appeared susceptible to vancomycin by conventional profiling of planktonic growing bacteria. However, drug susceptibility assessment of all the 8 clinical isolates in mature biofilms, gave resistance profiles strikingly different from those gathered in planktonic cultures, showing MBEC values below breakpoints only with the *S. gallolyticus* strain, which was classified as a moderate biofilm producer. The large size and complex structure of the vancomycin molecule might again play a part in the reduced accessibility of bacterial cells within the biofilm both in vivo and in vitro [62, 64]. The result of this study reinforces previous evidence of a poor vancomycin efficacy against biofilm growing Gram-positive bacteria in IE [62, 64]. In contrast, teicoplanin, which is another member of the glycopeptide family of antibiotics, ranked as the most effective drug against biofilm-growing *S. aureus*, showing MBEC values below breakpoints in all isolates (Table 3 and Additional file 2). This is in line with previous studies suggesting teicoplanin as a possible alternative therapeutic option to vancomycin in IE and for patients harboring MRSA and presenting a limited renal function [65, 66].

Early identification of the causative microbial agent and its drug susceptibility profile are crucial in IE, since a delayed antimicrobial treatment negatively affects the clinical outcome [6, 67]. However, microbial isolation and identification remain a challenge in IE, as blood cultures may yield false-negative results in 2.5 to 70% of all cases of endocarditis, particularly among those patients with prior antimicrobial therapy [10, 11, 34, 68, 69]. In this study, blood cultures allowed bacterial isolation in 6 (75%) cases, while in the other two patients (25%), it gave negative results. To improve the chance of a positive bacterial isolation, a sonication procedure was applied to the surgically removed heart valve tissue samples before culture plating. This procedure allowed isolation of pathogenic microorganisms in all samples, thus significantly improving (*P* = 0.004) the assay sensitivity, which by conventional methods allowed microbiological diagnosis in only one case (12.5%). Although gathered in a relatively small group of patients, these results show that by dispersing microbial cells from the tissue/biofilm matrix, sonication can increase significantly the probability to isolate microbial pathogens even in blood culture-negative endocarditis [18, 70, 71]. Even considering that all patients received antibiotic therapy before surgery and tissue collection, the very low sensitivity of the direct tissue culture as compared to the blood culture is intriguing as well as the significant difference observed between the direct tissue culture and sonication. Indeed, the increased sensitivity allowed by the sonication-based procedure may further represent indirect evidence of the presence of biofilm-growing or tightly adherent pathogens, in the valve tissue vegetation [10, 22, 34, 72].

**Conclusions**

IE therapy requires a prolonged administration of antibiotics [8]. The positive therapeutic outcome depends on multiple factors, including the location and size of the vegetation, patient comorbidities and surgical intervention...
The specific cause of most failures of antibiotic therapy is still unclear. Growing evidence suggests that the ability to produce biofilms may play a major pathogenetic role in supporting microbial adhesion and persistence while protecting from antimicrobial drugs [22, 73, 74]. Our results support the notion that IE represents an example of a biofilm-related infection, which, in turn, is associated with an increased antibiotic tolerance. Surgical disruption and removal of microbial vegetation can largely remove the biofilm structure, thus improving patients’ healing. Although this study presents some limitations, due to the small group of highly selected patients and the relatively short period of follow-up, the results strongly point to the need of pursuing novel strategies to allow for early microbiological diagnosis and effective antibiotic therapy against endocarditis sustained by biofilm-growing bacteria. A sonication-based culture method appears as a necessary procedure to allow the detection of a higher proportion of latent microorganisms encased in the biofilm structure. Further, measurement of the biofilm production in blood-culture positive cases may offer a useful biomarker to predict the clinical and therapeutic outcomes. Indeed, the cBRT represents a suitable diagnostic system for most microbiology laboratories. Moreover, the antibiotic susceptibility profile of biofilm-growing bacteria should always be assessed, in addition to standard AST, since it may offer key susceptibility information, particularly in the case of invasive IE, where biofilm represents a recognized pathogenic element.

Methods

Patient’s recruitment and clinical investigation

Eight patients with IE, defined according to the modified Duke criteria [26], were recruited at the Hospital Cardiosurgery Unit during 2016. The epidemiological and clinical data, as well as the therapeutic interventions for each patient, are summarized in Table 1. The presence of IE was confirmed in all patients by transthoracic and transesophageal echocardiography. Blood cultures were performed before antibiotic therapy in all patients enrolled in the study.

Microbiological diagnosis

Blood cultures from each patient were assessed in the Laboratory of Clinical Microbiology, Virology and Bio-emergencies – ASST Fatebenefratelli-Sacco using the BacT ALERT 3D (Biomerieux, Marcy-l’Etoile, France) automated blood culture system. Microbial identification was performed by matrix-assisted laser desorption/ionisation – time of flight mass spectrometry (MALDI-TOF MS system – Bruker Daltonics, Bremen, Germany) and by sequence analysis (ABI PRISM 3130x Genetic Analyzer) of the 16S rRNA gene [75]. Antimicrobial susceptibility testing (AST) was performed by the Vitek 2.0 system (Biomerieux, Marcy-l’Etoile, France) and by the broth microdilution test (Thermo Scientific, Massachusetts, USA) for the definition of the Minimum Inhibitory Concentration (MIC) criteria, according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST clinical breakpoint table v 9.0). The range of antibiotics tested is listed in the additional files section (Additional file 1). Strains were classified as MRSA when presenting both oxacillin resistance (MIC ≥4 mg/ml) and positive agglutination test for Penicillin-Binding Protein (PBP2, Oxoid, Basingstoke, UK) [76].

Specimen collection

In the operating room, the explanted heart valves were removed aseptically placed in sterile tubes and transported to the microbiology laboratory within 1 h. The specimen was then portioned in two parts, one was readily cultured without sonication, whereas the other portion was cultured after sonication as described below. Direct culture: Specimen was placed in Castaneda flasks (DID, Italy), incubated at 37 °C, and subcultured daily in suitable growth medium (Columbia agar + 5% sheep blood, Mac Conkey agar, Sabouraud agar, Mannitol salt agar, and Schaedler agar + 5% sheep blood; Biomerieux, Marcy-l’Etoile, France) [77]. Aerobic and anaerobic agar plates were incubated at 37 °C for 5 days, and the microorganisms were identified using conventional methods.

Sonication

Specimens were placed in separate sterile containers and sterile normal saline was added to cover the sample completely. After vortexing for 30s, the sample was sonicated by use of an ultrasound bath (VWR, Milan, Italy) for 5 min at a frequency of 30 kHz and vortexed for another 30s [78]. The outside of the container was treated with 70% ethanol. The resulting sonication fluid was centrifuged at 4000 rpm for 15 min, and the sediment was used for subsequent microbiological cultures onto chocolate agar, Columbia blood agar, Mannitol salt agar, MacConkey agar, Sabouraud agar and Schaedler agar plates (Biomerieux, Marcy-l’Etoile, France) and inoculated into Brain Heart Infusion and Th yoglycollate broths (TermoFisher, Cornaredo, Italy). Aerobic and aerobic agar plates were incubated at 37 °C for 5 days while broth were incubated for 15 days in the same conditions. Microorganisms were identified using conventional methods.

Biofilm production

Biofilm production was analyzed by the clinical BioFilm Ring Test (cBRT) (Biofilm Control, Saint Beauzire, France) as previously described [27]. S. aureus strains ATCC 25923 and Staphylococcus epidermidis ATCC 12228 were included in each plate as standard reference and internal control. Each strain was analyzed in duplicate and experiments were repeated 3 times.
Biofilm imaging
Bacterial colonies, grown overnight on blood agar plates, were inoculated into 3 ml of 0.45% saline solution (Air Life, Carefusion, CA, USA) to obtain a turbidity of 0.5 ± 0.1 McFarland (McF) corresponding approximately to 1 × 10^6 colony-forming units (CFU)/ml. Samples were diluted 1:1000 and resuspended in 1 ml of brain heart infusion broth (BHI) in a μ-Slide, 8 well glass bottom chamber slides (Ibidi, Germany). The bacterial suspension was incubated at 37°C for 24 h to allow biofilm formation. Afterwards, the medium was removed and biofilms were washed with 0.45% saline solution. The samples were stained using the LIVE/DEAD BacLight kit (Life Technologies, New York, NY, USA), according to supplier specifications. Biofilm samples were analyzed using a Zeiss LSM5 Pascal Laser Scan Microscope (Zeiss, Oberkochen, Germany) as described previously [36].

Antimicrobial susceptibility of bacterial isolates in biofilm
The anti-biofilm test (ABT) was performed by the protocol previously described in Di Domenico et al. [36].

Statistics
Statistical analysis was performed using the chi-square test when appropriate. Observed differences were considered statistically significant, with p-values of 0.05 or less [36].

Supplementary information
Supplementary information accompanies this paper at https://doi.org/10.1186/s12866-019-1596-2.

Additional file 1: List and concentration range (µg/ml) of the antibiotics tested. (PPTX 43 kb)

Additional file 2: Comparison between the Antimicrobial susceptibility test (AST) and the Anti-Biofilm Test (ABT). Susceptibility (S) and Resistance profiles of S. aureus (N1, N2, N4 and N7), E. faecalis (NS and N8) and S. gallolyticus (N3 and N8) clinical isolates to different antimicrobials. Classification was performed according to the European Committee on Antimicrobial Susceptibility Testing clinical breakpoint tables (EUCAST clinical breakpoint table v 5.0). Ampicillin/Sulbactam (AMP/SUL), HLG - High level gentamicin, HLS - High level streptomycin, TXP/SMX - Trimethoprim/Sulfamethoxazole. (TIFF 5897 kb)

Abbreviations
ABT: Anti-biofilm test; AMP/SUL: Ampicillin/Sulbactam; AST: Antimicrobial susceptibility testing; cBRT: Clinical Biofilm Ring Test®; CFU: Colony-forming unit; HG: High-level gentamicin, HLS: high level streptomycin; IE: Infective endocarditis; MBEC: Minimal biofilm-eradication concentration; MIC: Minimum inhibitory concentration; MRSA: Methicillin-resistant Staphylococcus aureus; TMP/SMX: Trimethoprim/Sulfamethoxazole

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Authors’ contributions
Conceived and designed the study: E.D.1, S.R., I.C., and F.E. Performed the confocal microscopy analysis: E.D.1, I.C., D.K., G.C.2. All authors analyzed data. Wrote the paper: E.D.1, I.C., and F.E. Collect the samples and performed in vitro experiments: E.D.1, I.C., S.R., G.D., E.T., G.C.1, A.P., C.P., F.R., E.D.2, M.S., D.S., C.A., G.R., R.D., L.T., M.G.1, M.G.2. All the authors read and approved the final version of the manuscript.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate
The study was approved by the hospital Institutional Review Board (Comitato Etico per la Sperimentazione Clinica, Azienda Ospedaliera-Polo Universitario Luigi Sacco, protocol number 3429/2016). Each participant signed a written, informed consent document. All the procedures and methods have been performed according to the guidelines of the Ethics Committee and in accordance with local laws and regulations.

Consent for publication
Written informed consent was obtained from all subjects.

Competing interests
The authors declare that they have no competing interests.

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Page 8 of 10
Di Domenico et al. BMC Microbiology (2019) 19:228
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