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Review

Infective endocarditis: Role of molecular techniques in early diagnosis

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ABSTRACT

Infective endocarditis (IE), a heart valve infection primarily caused by bacteria such as *streptococci* or *staphylococci*, causes significant morbidity and mortality. Despite the long-term use of broad-spectrum antimicrobials, the infection is often difficult to manage. The latest diagnostic modalities for IE are discussed in this study. Blood culture use in pathogen identification can lead to loss of precious time as well as generation of false negative reports. The first steps in diagnosis are blood cultures and echocardiography, but molecular techniques can be extremely useful and may be used for an accurate and early diagnosis.

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1. Introduction

Inflammation on the heart's inner wall is the hallmark of infective endocarditis. Low red blood cell count, fever, small patches of skin bleeding, heart murmur and fatigue are most common symptoms (Holland et al., 2016; Yallowitz and Decker, 2021). Heart risks include backward blood flow, kidney failure, abnormal electrical conduction in the heart and stroke (Njuguna et al., 2017). Bacterial infection is the most common, while fungal infection less likely associated with IE. Rheumatic disease, congenital heart disease, intravascular devices and intravenous narcotics are linked with IE susceptibility (Ambrosioni et al., 2017). Most common bacteria involved are streptococci and staphylococci.

Blood cultures or a heart scan are used to confirm a diagnosis that is suspected based on symptoms. There is also a non-infectious type of endocarditis (Yallowitz and Decker, 2021). Antibiotics' efficacy as a preventative measure during dental procedures is debatable (Cahill et al., 2017a,b). They have been recommended by others for high-risk individuals (Ambrosioni et al., 2017). Antibiotics are typically administered intravenously and are selected based on blood culture findings. Heart surgery is also required because, if left untreated, it is almost always fatal, and those who have been infected have a 25% chance of dying (Ambrosioni et al., 2017). A variety of microorganisms can cause IE. These are usually obtained via blood culture, which entails taking a sample of the patient's blood and recording and recognizing any changes. Since bacteria cause the majority of cases of IE, the term bacterial endocarditis (BE) is often used.

2. IE-related pathogens

2.1. Bacterial infections

Staphylococcus aureus is the most prevalent source of IE in the majority of the world, responsible for nearly 31% of instances (Hubers et al., 2020). *Viridans Streptococci* and *Enterococci* are the second and third most common IE-inducing bacteria, respectively. *Viridans streptococci* infections are prevalent in South America while IE caused by *Streptococcus bovis* is more common in Europe than in North America (Hubers et al., 2020). In North America, bacteria from the HACEK community (*Haemophilus*, *Aggregatibacter*, *Cardiobacterium*, *Eikenella*, and *Kingella species*) may cause IE (Chambers et al., 2013). The viridans genus contains species such as *S. oralis*, *S. mitis*, *S. sanguis*, *S. gordonii*, and *S. parasanguis* (Abranches et al., 2018). The upper respiratory tract and the oral cavity are the main habitats for these bacteria found normally in the mouth and mixing with the blood when dental surgical operations (tooth extractions) or genitourinary manipulation damage oral tissues. Meanwhile, HACEK species are a type of bacteria found on the gums of IV opioid users. Patients can have a history of bad oral health or also have valvular disease currently. When an infection occurs in a population, the most often isolated microorganisms are *Viridans alpha-hemolytic streptococci*, found only in the mouth. In contrast, *Staphylococcus* bloodstream infections are more often obtained in a surgical environment, where they may mix with the blood by treatments that involve a breach in the skin's integrity, such as anesthesia, catheterization, or connection to long- use catheters, or through intravenous injection of narcotics.

Enterococcus may mix with the blood and eventually spreads by gastrointestinal or genitourinary tract anomalies.

Pseudomonas species cause infection through drinking of contaminated water. Endocarditis and septic arthritis may be caused by *P. aeruginosa* infecting a child through a puncture wound on the foot (Gold et al., 2004). The bacteria *S. bovis* and *Clostridium septicum*, found in natural bowel flora, are associated with incidence of colon cancer. Because of concerns about bacteria spreading from the colon into the bloodstream as a consequence of the colon (lumen) and the blood vessels barrier breach by cancer. A colonoscopy is usually done right away when these bacteria are found causing endocarditis (Lamas and Eykyn, 2003). Culture negative endocarditis caused by *Bartonella*, *Chlamydia psittaci*, and *Coxiella* is hard to diagnose using serology, culture, and polymerase chain reaction, however 16S ribosomal RNA sequence can be used to identify these bacteria (Clayton et al., 2006). Rare species have been recorded to cause IE in some cases. *Cutibacterium spp.*, a common skin bacteria, has been linked to IE, which has resulted in death in some prosthetic heart valve patients (Dreier et al., 2004). Endocarditis has been caused by *Tropheryma whipplei* without affecting the gastrointestinal tract (Dzeing-Ella et al., 2009) and *Neisseria bacilliformis* has also been discovered in an individual with a bicuspid aortic valve (Masliah-Planchon et al., 2009).

2.2. Fungal

Fungal endocarditis (FE) caused by *Candida albicans* is a form of IE that is one of the most dangerous. Endocarditis has been linked to IV opioid users, patients with prosthetic valves, and patients who are immunocompromised. *Candida albicans* colonizes and penetrates endothelial cells, forming biofilms around resting structures with thick walls, such as prosthetic heart valves (Tsui et al., 2016). *C. albicans* causes 24–46% of all FE cases, with a mortality rate of 46.6–50% (Yuan, 2016). Other fungi that have been related to endocarditis include *Histoplasma capsulatum* and *Aspergillus* (Clayton et al., 2006). *Aspergillus niger* is responsible for about 25% of FE cases. In a rare incidence, *Tricosporon asahii* was also found causing endocarditis (Izumi et al., 2009).

2.3. *Staphylococcus aureus* (SA)

Native valve IE (NVIE) is primarily caused by *Staphylococcus aureus* bacteremia (SAB), which has a prevalence of 22.9–34% (Abdallah et al., 2016; Miro et al., 2005) while methicillin-susceptible *Staphylococcus aureus* (MSSA) strains are 72–85% prevalent (Miro et al., 2005; Campanile et al., 2012). In 16–27% of MSSA-related IE cases, the aorta is affected, and population acquisition is normal (60–80%) (Miro et al., 2005; Campanile et al., 2012; Hill et al., 2008). The combination of NVIE and community-acquired MSSA (CA-MSSA) is extremely unlikely and can result in fatal evolution. It's linked to severe sepsis and chronic bacteremia, as well as a slew of other problems. A well-known risk factor for IE is persistent SAB, which is described as positive blood cultures for more than three days (Hill et al., 2007). About 75% of MSSA endocarditis patients have no known source of infection (Kaech et al., 2006). Septic metastases manifested as cutaneous lesions along with recurring fever increases the complications in the course of bloodstream infections caused by *Staphylococcus aureus* (Fowler et al., 2003). Janeway lesions, which are extremely specific for IE

but have a lower incidence (5%) than necrotizing vasculitis lesions, as well as later necrotizing vasculitis lesions, suggest embolization in a febrile setting (Murdoch et al., 2009). Three fatal cases of purpura fulminans linked to *Staphylococcus aureus* sepsis were linked to *Staphylococcus aureus* enterotoxin C (SEC) (Kravitz et al., 2005). Rhabdomyolysis is diagnosed by the presence of extreme myalgia and an increase in creatine phosphokinase levels to more than 5 times the normal upper limit. When there were no classic causes of rhabdomyolysis or other contributing factors, muscle injury was caused by a staphylococcal infection in the form of sepsis (Singer et al., 2016). The rapid evolution of infected cases necessitates the surveillance of high-virulence MSSA strains, which have a tendency to mix extracellular toxins and/or virulence factors in a synergic manner, improving pathogeny by increasing adhesion, cellular invasion, and immune response avoidance abilities. CA SAB is a life-threatening illness that necessitates medical treatment right away. Methicillin-susceptible strains can be lethal owing high embolic risk. In the absence of a known cause of infection, extra care is advised. Fever associated with staphylococcal bacteremia is a sign of septic complications warranting a thorough echocardiogram.

2.4. *Streptococcus viridans*

Streptococcus viridans is a genus of alpha-hemolytic streptococci present in the mouth's natural flora that causes dental caries (*Streptococcus mutans*, *Streptococcus sanguinis*), pericoronitis, and subacute IE. *S. viridans* causes 0.3–3% of adult cases of bacterial meningitis and 1% of those cases in pediatric population. *S. viridans* causes pulmonary infections (particularly in cystic fibrosis patients), intestinal abscesses, sepsis in immunocompromised patients, neonatal sepsis (Elting et al., 1992; Bochud et al., 1994), and osteomyelitis (Choudhury et al., 2009), in almost 40–60% of endocarditis cases involving normal valves (Giannakopoulos et al., 2016). Mitral valve prolapse is the most common cause of endocarditis, particularly in men over 45 years old. Aortic valve endocarditis has also been recorded as a result of tongue piercing while colon adenocarcinoma may also cause endocarditis caused by *S. viridans*. Antibiotics with Ceftriaxone and Vancomycin are widely used to rapidly sterilize the endocardium. Cephalosporins alone have been shown to be beneficial in trials, but monotherapy can increase the risk of antibiotic resistance (Francioli et al., 1995). *S. viridans* endocarditis is more often linked to periannular lesions (abscesses, pseudoaneurysms, or fistulas) or heart disease than to renal failure or septic shock with an almost 15% mortality (Lopez et al., 2005). During fixed orthodontics, fixed appliances, and bonding products, an improvement in the volume of subgingival bacterial flora is attributed to the preservation of biofilms. To minimize the chances of oral health problems involved with orthodontic treatment, the orthodontist and dental hygienist should collaborate in a synergistic way. When patients are receiving orthodontic treatment, it is important to educate and encourage them to maintain proper oral hygiene and follow a home oral hygiene routine. During fixed orthodontics, mechanical teeth cleaning by a specialist dental hygienist aids in the maintenance of proper oral hygiene and the reduction of oral health complications (Migliorati et al., 2015). The patients' young age (20–30 years) as compared to the average age of this aetiology, which is 46–64 years, as well as the novel way of repairing the affected mitral valve—mitral valve plasty using video-assisted right minithoracotomy—without prior cardiac surgery—are special aspects of the relationship of endocarditis with oral streptococci attributable to fixed orthodontic device. Even in young or healthy patients, IE caused by oral streptococci and a fixed orthodontic implant appears to be a concern, necessitating caution when using clinical and echographic tests

to diagnose any pre-existing heart complications (such as mitral valve prolapse).

2.5. *Enterococci*

Enterococcus faecalis, the third most infectious bacteria, is the most common cause of endocarditis in patients undergoing transcatheter aortic valve replacement (Chirouze et al., 2013; Amat-Santos et al., 2015). Few case-control and observational reports showed an IE prevalence rates ranging from 5% for mixed enterococcal bacteremia to 13% for monomicrobial *E. faecalis* bacteremia (Pinholt et al., 2014; Dahl et al., 2016). Many studies are limited by low echocardiography rates, with transesophageal echocardiography (TEE) rates as low as 12% (Bouza et al., 2015). The studies with the fewest echocardiographic inspections have the lowest rates of IE (Bouza et al., 2015). However, IE caused by *E. faecalis* can escape because of low echocardiography rates (Fernandez Guerrero et al., 2007). The rise in IE cases caused by *E. faecalis* can be attributed to an ageing population having comorbidities, prosthetic material in the heart, and surgical interventions in the urinary and gastrointestinal tract (Siegmán-Igra et al., 2010; Mohee et al., 2014). Echocardiography is the most common imaging technique used to diagnose IE (Bruun et al., 2014), however poor rates of echocardiography may many endocarditis cases.

2.6. *Coagulase negative staphylococci*

Coagulase negative staphylococci, invariably present in natural skin flora, have a high proclivity for colonizing foreign materials in the human body and causes prosthetic valve endocarditis (Chu et al., 2008; Houkes et al., 2021). Endocarditis caused by this pathogen in a young, otherwise stable person who did not use intravenous drugs and had no other risk factors for IE is uncommon. Internal fixation of the cervical spine with a titanium plate, on the other hand, may be the focal point for the organism to start and then spread to the heart (Al-Tamiami et al., 2011). Cefoxitin susceptibility of *Coagulase negative staphylococci* reduces the fever and clearance of the pathogen from the blood in two days after starting antibiotics.

2.7. *Streptococcus pneumoniae*

Late identification and a high prevalence of complications are behind high mortality rate associated with pneumococcal endocarditis, necessitating early diagnosis and aggressive care. Pneumococcal endocarditis along with contamination of an aortic prosthetic valve, native tricuspid valve, and permanent pacemaker increases significant risk (Lacalzada et al., 2016). Prosthetic heart valves, on the other hand, have recently been identified as a risk factor for Pneumococcal endocarditis (PE) PE, responsible for 22–31% of all cases of valve endocarditis (Fefer et al., 2002). In addition, some cases of penicillin-resistant *Streptococcus pneumoniae* (SP) have been discovered in recent years, and endocarditis caused by these bacteria is associated with a high risk of morbidity and mortality underlining the importance of early diagnosis and treatment (Morita et al., 2011). While the precise rate of PE following penicillin usage is unknown, some reports have recorded rates of about 3% (Straus and Hamburger, 1966). PE has been observed in less than 1% of native heart valves and 22–31% of valvular prostheses, the most frequent of which are prosthetic aortic valves; however, in up to 13% of cases, two or more valves are involved simultaneously (Aronin et al., 1998). SP contamination of a pacemaker lead happens in less than 4% of cases (Morita et al., 2011). Advanced age, alcoholism, malnutrition, immunosuppression, and prior valve disease are the leading causes of PE (Buchbinder and Roberts, 1973; Simeon et al., 2017).

Even though PE cases are uncommon, mortality rates are still high, ranging from 28 to 60% (de Egea et al., 2015). In most cases, the clinical presentation is sudden with intense progression. Congestive heart disease affects 48.6% of patients, while valve perforation, paravalvular abscesses, and embolization affect 33.7 percent and 24.3 percent, respectively (Lacalzada et al., 2016).

2.8. *Escherichia coli* (*E. coli*)

E. coli-induced IE is an unusual condition, present in about 0.51 percent of IE cases (Morpeth et al., 2007). Between 1909 and 2002, the literature revealed 36 cases of *E. coli* native valve IE meeting the Duke criteria. Urinary tract infection was found to be the most frequent cause of *E. coli* endocarditis (Micol et al., 2006). The low prevalence of *E. coli* IE is likely because of *E. coli*'s inability to adhere to the endocardium, and also the presence of antibodies against *E. coli* (Watanakunakorn and Burkert, 1993). However, the number of IE patients above the age of 70 harboring *E. coli* has recently increased, with older women accounting for roughly 70% of those affected (Micol et al., 2006). Furthermore, *E. coli* IE has a higher mortality rate (21%) than IE caused by HACEK-group gram-negative bacteria (4%). *Haemophilus* spp., *Aggregatibacter* spp., *Cardiobacterium hominis*, *Eikenella corrodens*, and *Kingella* spp. are among the bacteria involved (Chambers et al., 2013). *E. coli*-related IE is rare, affecting mainly older people, including those with diabetes. *E. coli*-related IE, on the other hand, is more likely to affect children. An early study found *E. coli* in 8.4% of 20–40-year-old IE patients, with only about 60% of the *E. coli* detected were amikacin and amoxicillin/clavulanate sensitive (Fayyaz et al., 2014). Another study suggest that *E. coli* is the most common gram-negative bacillus causing IE, as much as one-third of non-HACEK, gram-negative bacilli-induced IE (Loubet et al., 2015). These findings suggest that *E. coli*-related IE could be more prevalent than previously thought.

3. Necrotizing heart infection

Life threatening necrotizing infections commonly affect perineum, extremities, and wounds or surgical incisions. Patients with the disease are also immunocompromised, and a connection to diabetes has been established. Computed tomography (CT) can reveal gas that dissects contaminated tissue or fascial planes and can define the degree of disease in the case of a necrotizing infection affecting the myocardium (McGillicuddy et al., 2011). Gas-forming infections in the pericardial space caused by implantable cardiac instruments, such as pacemaker generator reservoir sites in the chest wall, have been connected to superficial soft tissue necrotizing fasciitis (Ivey and Gross, 1993; Ott and Hodge, 2009; Subramanian et al., 2012). Perforated ulcers and other causes of fistulous contacts between the esophagus or stomach and the pericardium have been linked in the past, supporting the pathological hypothesis that the free gas in the myocardium was caused by a perforated gastrointestinal viscus (Pickhardt and Bhalla, 2000). Necrotizing infections with gas-producing species in the pericardial space have been identified and referred to pneumopericardium can be caused by perforated esophageal or gastric tumors, as well as perforated peptic ulcers (Brander et al., 2002). Of such cases, the root cause is not always found. There has been at least one instance of immediate expansion from an infectious ascending aortic aneurysm (Ivey and Gross, 1993). A prior myocardial infarction is the most likely cause of necrotizing inflammation, which causes pneumomyocardium.

4. The effect of manganese on bacterial endocarditis

Diet, especially dietary metals like manganese (Mn) can influence infection risk. Systemic infections caused by *Staphylococcus aureus*, a major cause of bacterial endocarditis, can be predicted by dietary Mn levels. Mn levels and distribution within contaminated tissues were altered in mice fed a high Mn diet, as well as elevated *S. aureus* virulence and heart infection (Juttukonda et al., 2017). While calprotectin, Mn-sequestering protein in mammals, is present around staphylococcal heart abscesses, it does not reach the abscess nidus and thus has little effect on Mn levels in this organ. Excess Mn in the heart is available to *S. aureus* which in turn detoxifies reactive oxygen species and protects neutrophils, all of which are good for cardiac protection (Brophy and Nolan, 2015). Available data suggest that any change in Mn intake can influence the host's antimicrobial defenses, resulting in deadly staphylococcal infection. The host has pathways that block pathogens from accumulating vital metals through infection, which is known as "nutritional immunity" (Hood and Skaar, 2012). Metal chelators and transporters, both small molecules and proteins, that work together to reduce metal availability during the inflammatory response make up nutritional immunity. However, unbalanced dietary metal consumption may deplete nutritional immunity (Maggini et al., 2018).

5. Molecular basis of antimicrobial resistance in infective endocarditis

Methicillin-resistant Staphylococcus aureus, or MRSA, causes serious endovascular infections like bacteremia and IE (Abdallah et al., 2016; Miro et al., 2005). 15 to 30% of MRSA infections are caused by persistent MRSA bacteremia (Campanile et al., 2012; Hill et al., 2008). In vitro, most PB strains are vulnerable of CLSI breakpoints to standard-of-care anti-MRSA antibiotics (e.g., vancomycin [VAN] and daptomycin [DAP]), but they survive in vivo through antibiotic treatment (Ellis et al., 2009; Jarraud et al., 2002). Prophage components have been found to play a part in the pathogenesis of staphylococcal infections (Kaech et al., 2006; Fowler et al., 2003). Prophages, which are genetically linked to immunomodulatory virulence factors (e.g., lukF-PVL) and staphylococcal global regulators, may affect bacterial health and host-microbe interactions in certain instances (like sigB) (Kaech et al., 2006; Fowler et al., 2003). Multiple studies indicate that prophages can play a role in bacterial survival by stimulating biofilm formation and nutritional stress may lead to a metabolic signaling cascade (Hill et al., 2007; Kaech et al., 2006). Bacteria are associated with some kind of biotic or abiotic surface in many natural and clinical settings, allowing them to form biofilms, a multicellular lifestyle with bacteria present in extracellular matrix. The most common causes of biofilm-associated infections on indwelling medical devices, *Staphylococcus aureus* and *Staphylococcus epidermidis*, may move between single free-floating cells and multicellular biofilms. Cells bind to a surface and then multiply to form microcolonies during biofilm formation. They then create the extracellular matrix, which is made up of polysaccharides, proteins, and extracellular DNA and is a hallmark of biofilm formation. The biofilm population goes through a disassembly phase after maturation into three-dimensional structures, which leads to staphylococcal cell propagation. *Staphylococci* have developed a large network of regulatory mechanisms to alter and fine-tune biofilm production in response to changes in environmental conditions, as biofilms are dynamic and complex biological systems. As a result, biofilm formation is used as a survival and persistence mechanism in the human host, as well as a reservoir for spreading to new infection sites. Furthermore, staphylococcal biofilms provide improved resistance to antibiotics and the

immune response, presenting major therapeutic challenges in clinics around the world (Schilcher and Horswill, 2020).

5.1. Formation of biofilms

The elucidation of biofilm role in the pathogenesis and/or outcomes of IE is the target of many recent studies. It is self-evident that IE associated with implantable cardiac devices can result in the formation of *peri-device* biofilms. In these cases, biofilm formation has a significant impact on the evolution of device-associated vegetation proliferation. In native valve IE, on the other hand, the role of biofilm formation is uncertain. Early studies in *S. aureus* IE provide the most compelling evidence for the importance of biofilm formation in native valve IE (Bouchiat et al., 2015). *S. aureus* strains that can form biofilms is also associated with clinically “persistent” methicillin-resistant *S. aureus* (MRSA) bacteremia in humans (Oyama et al., 2016).

5.2. Quorum sensing detection

Since IE vegetations involve high densities of organisms, the importance of quorum-sensing genetic control (regulation of gene expression dependent on bacterial cell density) of virulence factors has increased (Yarwood and Schlievert, 2003). Quorum-sensing regulon *agr* (accessory gene regulator) of *S. aureus* has been studied extensively (Turkey et al., 2018). In both clinical and experimental IE, the propensity of MRSA strains to cause vancomycin-resistant IE is likely dependent on their triggering *agr* early in the growth cycle. However, *agr*’s “early activation” profile is more of a biomarker for recurrent IE strains than a pathogenetically linked result, according to *agr* gene knockout reports (Tan et al., 2018). hVISA (heterogeneous vancomycin-intermediate *Staphylococcus aureus*) infections are not completely understood (Bae et al., 2009). Clinical MRSA isolates from all over the world have the hVISA phenotype described as the presence of MRSA subpopulations with intermediate vancomycin resistance (typically 1 organism per 10⁵–10⁶ bacteria). Fundamental concerns about hVISA remain unanswered, such as its occurrence, global spread, and therapeutic significance. MRSA-related IE is a situation where antibiotic effectiveness is important. Vancomycin remains the first-line therapy for this condition, owing to need rather than preference. Vancomycin, on the other hand, has a slow bactericidal function and poor penetration into valvular vegetations, limiting its effectiveness. In addition, several studies have discovered a connection between the hVISA phenotype and deep-seated endovascular infections such as IE (Bae et al., 2009; Howden et al., 2010).

5.3. Use of fluorescence in situ hybridization (fish) for microbial detection

Blood culture and, if heart valve replacement is needed, valve culture are the gold standards for diagnosing endocarditis. Negative culture outcomes are often caused by previous antimicrobial therapy or slow-growing organisms, rendering adequate therapy difficult (Brouqui and Raoult, 2001; Brouqui and Raoult, 2006). In these complex cases involving fastidious or slowly developing organisms like *Coxiella burnetii*, *Tropheryma whippieii*, or *Bartonella species*, FISH is used to diagnose endocarditis (Geissdorfer et al., 2012; Gescher et al., 2008). In several cases, FISH allowed for a microscopical identification of the causative agent in the absence of positive culture results, demonstrating its diagnostic value (Mallmann et al., 2010; Frickmann et al., 2017; Melenotte et al., 2016). Thorough specificity checking of the respective FISH probes is an essential feature of using FISH in diagnostics. Specific probes for the most frequently occurring clinically important species (*streptococci*, *staphylococci*, and *enterococci*) as well as some

rare species like *T. whippieii* and *Bartonella quintana*, have been reported, but the probes for *C. burnetii* have yet to be evaluated (Geissdorfer et al., 2012; Gescher et al., 2008; Mallmann et al., 2010). Furthermore, formamide concentrations are not available for any of the published *Coxiella* probes (Melenotte et al., 2016; Jensen et al., 2007), despite the fact that this chemical is needed to change the stringency and ensure the specificity of hybridizations in FISH (Wagner et al., 2003). A comparison to the SILVA 16S rRNA database (Quast et al., 2013) revealed that at least one of the reported probes has a perfect match to over 8000 non-target species in the respective region of the 16S rRNA of different bacterial families (Melenotte et al., 2016), resulting in false-positive hybridization signals for all of those species. In the absence of properly tested probes, a previous study used the general bacterial probe EUB (Amann et al., 1990) in conjunction with PCR to diagnose *C. burnetii* in a patient with IE (Kumpf et al., 2016). The high autofluorescence level of the heart valve tissue was a concern in this research and other studies analyzing tissue parts of endocarditis patients (Gescher et al., 2008). This may be attributable to calcification in the parts or non-specific probe binding to sample material, and it makes unambiguous identification of a causative agent in these samples difficult. This problem can be solved by including a non-sense probe in the hybridization mix (e.g. nonEUB338), which allows for the detection of unspecific fluorescence caused by unspecific probe binding. The fluorophore used for this additional nonsense probe, however, cannot be used for a specific FISH-probe in that hybridization, limiting the number of bacteria that can be detected at the same time. FISH may provide valuable information for up to 30% of all cases of endocarditis that remain culture-negative if additional protocols to address the challenges presented by autofluorescence and a range of specific probes for use in endocarditis are established (Brouqui and Raoult, 2006).

FISH has been shown to be effective in diagnosing fastidious bacteria (Geissdorfer et al., 2012; Gescher et al., 2008; Mallmann et al., 2010; Kumpf et al., 2016), however, it can only detect intact and (at the time of fixation) viable bacteria because it takes at least 400 ribosomal target molecules per cell (Hoshino et al., 2008). FISH also indicates the location of the causative agent at the site of infection, as well as spatial details and the likelihood of the presence of additional co-infecting bacteria. Based on these benefits, combining FISH with other diagnostic methods such as blood cultures, PCR, or immunohistochemistry is a viable choice, especially in complex cases where the causative agent is difficult to identify. However, in order for FISH to be a desirable addition to the diagnostic toolbox, protocols must be established to deal with actual, and thus often difficult *ex vivo* samples. False-positive results could be caused by autofluorescent particles that imitate bacteria, while false-negative results could be caused by autofluorescence masking the presence of bacteria in the sample. However and perhaps more importantly, improperly validated probes can result in a false-positive identification of bacteria in a sample. As a result, for each newly developed probe, hybridizations with target- and non-target organisms with the fewest mismatches at the probe binding site at various formamide concentrations should be performed, and the respective non-target species must be used as a separate control in any hybridization experiment in diagnostic applications (Frickmann et al., 2017). The right choice of specific probes, as well as the fact that the same bacterial cell cannot be evaluated with more than three probes due to the limitations of available wavelengths and filter sets in the routine diagnostic laboratory, is a problem in cases where there is insufficient material or few available parts. The use of double-labeled FISH probes for fastidious and slow-growing bacteria with low ribosomal content can also aid detection and identification since the probe signal is doubled (Stoecker et al., 2010).

5.4. Amplification of the 16S rRNA gene

Amplification and sequencing of the gene encoding the 16S rRNA can lead to the crucial early detection and diagnosis of bacterial DNA in heart valve specimens (Nikkari et al., 1992; Kotilainen et al., 1998; Rantakokko-Jalava et al., 2000) which in turn can help in diagnosis of blood culture-negative cases of IE, especially the ones harboring rare or non-cultivable microbes (Greub et al., 2005; Rovero et al., 2005; Houpiqian and Raoult, 2005).

Direct 16S rDNA PCR have been found to be useful in ascertaining the causes of IE in samples of blood or biopsy culture-negative cases, particularly with species including *Brucella* spp., *Coxiella burnetii*, *Bartonella* spp., *Tropheryma whippelii*, *Mycoplasma* spp., and *Legionella* spp (Allen et al., 2020; Miller et al., 2016; Habib, 2016).

An early study found hard to culture or anaerobic organisms in five of eight culture-negative IE cases (Horstkotte et al., 2004). Detection of bacteria that are difficult to culture or non-viable bacteria has been significantly enhanced by broad-spectrum PCR (Habib, 2010). 16S rDNA PCR can detect *K. pneumoniae*, a multidrug-resistant bacteria that could only be extracted in enrichment media (Morris et al., 1995) accurately, however certain artifacts such as the presence of PCR inhibitors in clinical samples and the risk of contamination in clinical samples and PCR reagents can reduce sensitivity.

6. Use of metagenomic sequencing for microbial detection

Staphylococcus aureus is present in almost 30% of IE cases, followed by oral streptococci (20%), other streptococci (10%), enterococci (10%), coagulase-negative staphylococci (10%), HACEK organisms, zoonoses, and fungi (Cahill et al., 2017a,b). The blood culture-based diagnostic algorithm faces difficulties due to the diverse etiology of IE. To increase diagnostic sensitivity, molecular methods have been suggested to be included in Duke criteria (Tak and Shukla, 2004). Heart valve 16S rRNA PCR-Sanger sequencing has been shown to be useful for etiologic diagnosis (Kim et al., 2017; Peeters et al., 2017), but heart valves are not always available and are only available after surgery, which can prolong diagnosis. When required, a broad-range, culture-independent blood test is added to the diagnostic workflow. When traditional tests fail to provide a diagnosis, retrieving the entire microbial genome for analysis of antibiotic resistance-associated features is extremely useful if surgical material is accessible. These provide clues about the etiology as well as the role of resistance genetic determinants, which may further enhance treatment outcomes. There were only a few studies in the literature that looked at broad-range identification of IE causative agents on clinical blood specimens. Nakajima et al. used whole blood 16S rRNA PCR-Sanger sequencing to diagnose a case of *S. aureus*-caused blood culture-negative IE (BCNIE) (Nakajima et al., 2016). The same technique was used by Fournier et al., who established the etiologic agents in 35 of 257 (13.6%) seronegative BCNIE cases (Fournier et al., 2010). To get around this diagnostic bottleneck, it appears that a more sophisticated method is needed. Recent advancements in metagenomic sequencing technologies have allowed the identification of a wide range of pathogens in a short amount of time (Wilson et al., 2014; Grumaz et al., 2020). The massively parallel design of metagenomic sequencing, unlike the Sanger process, facilitates the detection of several bacteria in a one sample. This benefit has sparked a lot of interest in pan-microbial pathogen identification directly from clinical specimens. In general, there are two primary methods for conducting metagenomic studies: 16S and shotgun methods. The former entails using consensus primers to amplify a single taxonomically informative genomic marker (i.e., the bacterial 16S rRNA gene), the most widely used housekeeping genetic marker for bacterial phylogeny

and taxonomy studies (Janda and Abbott, 2007), followed by amplicon sequencing to facilitate bacterial abundance analysis. Shotgun metagenomic sequencing, on the other hand, is non-targeted, and all of a sample's DNA is sheared into fragments and sequenced. This whole-genome, highly multiplexed approach broadens the 'radar' of pathogen detection beyond bacteria, allowing it to identify practically every part of a microbial genome.

The whole genome sequences of *B. quintana* and *Propionibacterium* sp. oral taxon 193 were retrieved directly from heart valves using metagenomic sequencing (Chan et al., 2019), allowing pathogen detection and antibiotic resistance prediction. S83A mutation in the region determining quinolone-resistance of the *gyrA* gene for *B. Quintana*, 1408 A > G mutation in the 16S rRNA gene, and 2576 G > T mutation in the 23S rRNA gene were among the resistance-associated mutation hotspots. There are two prerequisites for molecular antibiotic resistance prediction. The first is target genome coverage, which is affected by microbial load and the degree of nucleic acid contamination in the host (Hasan et al., 2016). Increased sequencing depth or other enrichment methods based on preferential lysis of human cells can boost microbial genome coverage (Thoendel et al., 2016). The cost of achieving high-sequencing depth and the degree of microbial loss are also essential factors to consider when deciding the best procedure. The second issue concerns the degree to which mutation hotspots are related to resistance phenotypes. In *Staphylococcus* and *Enterococcus* species, for example, the connection between linezolid resistance and the 2576 G > T mutation in the 23S rRNA gene has been well studied, but not in *Propionibacterium* species (Besier et al., 2008; Bocanegra-Ibarias et al., 2016). The reliability of molecular resistance prediction is heavily reliant on experimental and clinical evidence supporting the mutation-resistance relationship, which is based on adequate coverage depth. By studying the metagenomes of plasma and resected heart valves, fastidious etiologic agents of IE cases have been identified broadening the range of pathogen detection and allowing the prediction of antibiotic resistance.

6.1. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection and the risk of Infective endocarditis

COVID-19 has been linked to an inflammatory response as well as a hypercoagulable condition, which can lead to a variety of conditions. According to the available research, the most prevalent comorbidity linked with COVID-19 infection is cardiovascular illness. It's crucial to realize that severe Coronavirus Disease 2019 (COVID-19) illness can have major long-term consequences. A recent study showed damage to the mitral valve structure possibly mediated by the cytokine storm combined with systemic inflammation caused as the patient had no risk factors for IE but had previously been hospitalized for severe COVID-19 illness (Kumanayaka et al., 2021).

Interstitial pneumonia and respiratory failure leading to Acute respiratory distress syndrome (ARDS) have been linked to SARS-CoV-2 infection, particularly in individuals with prior cardiovascular illness. Lately a study showed a case of IE induced by MRSA, further complicated with SARS-CoV-2 infection leading to the requirement of mechanical ventilation for interstitial pneumonia (Spinoni et al., 2020).

Organ damage caused by the inflammatory response and hypercoagulable condition consequent to COVID-19 infection can be a potential risk factor for IE, even in the absence of other predisposing factors. More research into the benefits of therapy for COVID-19 infection in terms of post-disease sequelae is needed.

7. Conclusion and future directions

Staphylococcus aureus, *Viridans streptococci*, *coagulase-negative staphylococci*, *Streptococcus gallolyticus*, *HACEK species*, *enterococci* along with fastidious organisms like *Candida*, *pneumococci* and *gram-negative bacilli* are responsible for the majority of IE cases. Because of valvular injury or turbulent blood flow, these pathogens mixes with the blood via the skin, mucosal surfaces, or an early infection, eventually binding to nonbacterial thrombus. These pathogens are able to proliferate, form small colonies, and shed in the bloodstream in the absence of host defenses. IE is emerging as one of the most morbid infections lately especially caused by hard to treat infections like *MRSA*. Current data suggest a multidisciplinary approach to an early diagnosis as the key to a better prognosis. Many studies suggest that molecular techniques holds an edge over echocardiography for the detection of IE along with endocarditis caused by intracardiac device, prosthetic valve endocarditis and peripheral septic emboli. The molecular techniques namely *FISH*, *Quorum sensing detection*, *16S rRNA PCR* and *metagenomic sequencing* can overcome the limitations like diagnostic delay, false negatives/ positives, and presence of non-culturable pathogens. However, few studies have also shown that conventional technique like blood culture and modern molecular technique complement each other and thus should be used in tandem leading to better clinical management and an improved outcome. Most of the current research evidence is primarily based on nonhuman data and thus corroborating latest diagnostic modalities performing comparative efficacy studies in human system is of paramount importance. Further, newer diagnostic technique holds great promise of enhancing the sensitivity and specificity of IE diagnosis.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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