

Role of procalcitonine, hsCRP and 16SrRNA in diagnosis of culture negative endocarditis

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Abstract

Infective endocarditis infects the heart's inner surface, mostly the valves. It is a lethal disease with a high rate of morbidity and fatality. It mostly caused by bacteria, but fungi can also cause it. Microbiological diagnosis relies on blood culture. Molecular and biochemical indicators add to diagnosis, particularly in culture-negative patients. This study aimed to decide the role of 16s rRNA, procalcitonin, and high sensitivity C reactive protein (hsCRP) in the diagnosis of culture negative infective endocarditis (CNIE) in Assiut university hospitals. This cross-sectional study included 60 patients who were admitted to cardiac hospitals with suspected infective endocarditis according to modified DUKE criteria. A group of 20 apparently healthy subjects served as a control group for investigation of inflammatory biomarkers. We performed blood culture, biochemical markers and molecular investigations. Of the 60 patients, there were 46 (76.7%) culture positives and 14 (23.3%) culture negatives. *Staphylococcus aureus* was the most prevalent pathogen in culture positive patients, followed by *enterococcus* and *klebsiella*. Linezolid and imipenem were the most sensitive antibiotics for Gram-positive and Gram-negative bacteria, respectively. Amplification of bacterial 16S rRNA gene, after DNA extraction from whole blood samples, was positive in 11/14 cases (78.5%) of culture negative patients and in 42/46 cases (91.3%) of culture positive patients. The range of procalcitonin in culture positive patients was (0.32- 89) ng/ml, significantly higher than in culture negative patients (0.02- 8.8) ng/ml, and in the control group (0.01-0.08) ng/ml. hsCRP showed the same pattern. In conclusion, our data suggest that PCR was the most accurate diagnostic tool for diagnosing CNIE, followed by procalcitonin, and hsCRP, respectively.

Keywords: endocarditis; blood culture; culture negative; 16s rRNA.

Date received: 08 July 2021; **accepted:** 27 September 2021.

Introduction

Infective endocarditis (IE) is a potentially lethal infection of the inner surface of the heart,

mainly the valves.¹ Despite the possibility of fungal endocarditis, it is almost always caused by bacteria.² While anyone can get IE, there are specific risk factors that are well known.

Patients with valvar defects, artificial valves, cardiac implantable electric devices (CIEDs), and intravenous drug users are among the most important risk factors.³ The clinical picture might be disparate depending on the host as well as the causative germ. The diagnosis is often difficult, requiring a mix of clinical, microbiological, and echocardiographic evidence.⁴ Over the previous five decades, there have been many changes in the clinical spectrum, etiological organism profile, and diagnostic procedures, as well as major geographic variations in risk factors.⁵ In affluent countries, more than half of those who have IE episodes have normal heart valves, but rheumatic heart disease (RHD) accounts for only 10% of IE cases. However, RHD is still the most common predisposing condition in developing countries.⁶ Blood culture is still the gold standard for diagnosing IE and determining which antibiotics to use. Culture-positive or culture-negative infective endocarditis are two different classifications. Prior antibiotic administration is by far the most common cause of "culture-negative" endocarditis. Some microorganisms might take longer time to grow on culture media, and these organisms are known as fastidious because they have stringent growth needs. Pathogens such as *Aspergillus niger*, *Brucella* species, *Coxiella burnetii*, *Chlamydia* species, and HACEK bacteria (*Haemophilus niger*, *Aggregatibacter actinomycetemcomitans*, *Cardiobacterium hominis*, *Eikenella corrodens*, and *Kingella kingae*) are just a few examples.⁷ Patients may be incorrectly labelled as "culture-negative" endocarditis (CNE) due to a delay in growth and detection of these organisms. NE's next steps are as follows: Incubation for a longer time, special media, serological techniques, molecular diagnostic approaches, inflammatory biomarkers such as procalcitonin (PCT) and high sensitivity C-reactive protein (hsCRP).⁸ The changing demography of the disease in terms of patient profile and microbiology is one of the key hurdles in treating IE. Consequently, the aim of this study was to detect the role of 16s rRNA, procalcitonin, and hsCRP in the diagnosis of culture negative infective endocarditis (CNIE) in Assiut university hospitals.

Patients and Methods

This cross-sectional study was conducted in the Microbiological Unit of the Clinical Pathology Department of Assiut University Hospitals, during September 2019 to September 2020. A total of 60 patients suspected of having infective endocarditis were admitted to cardiac hospitals. Their ages ranged from 1.5 to 64 years old. The study protocol was reviewed and approved by the ethical committee of the Faculty of Medicine, Assiut University (IRB 17100460; 3/2018). The study goals were explained to all the subjects and to guardian of infants and children, and they gave their consent orally. In addition, for school age children (6-18 years of age), the blood collection method and study goals were explained in simple language, and willing to provide blood sample was taken as child assent.

All patients were subjected to the following:

Microbiological testing

A total of 5-10 mL of blood was obtained for adults and 2-3 mL for children for blood culture under aseptic conditions⁹ and put in blood culture bottle of BacT/ALERT (bioMérieux diagnostics, Lyon, France) which is an automated microbial detection system¹⁰. To avoid contamination, we withdrawn two samples from two different veins at a time. At interval of at least one hour, we repeated blood sample collection three times. All bottles were incubated in BacT/ALERT for 21 days for negative samples. The positive blood cultures on BacT/ALERT were subjected to identification of the organism by two methods: 1) a manual method, by culture on one plate of blood agar, chocolate agar, MacConkeys agar, Mannitol agar, EMB agar, and bilesculin agar; and on 2 plates of Sabaroud's agar, one was incubated at 25°C (room temperature) and the other at 37°C, according to the lab's SOP (standard operating procedure of the USA Environmental Protection Agency) for identification of bacterial type based on morphology, and CLSI 2019 (the Clinical Standard Institute) for antibiotic susceptibility. 2) an automated method using the VITEK 2 COMPACT15 system (BioMerieux diagnostics, Lyon, France), according to the

manufacturer's instructions, to confirm the bacterial identification and antibiotic susceptibility of the identified organism.¹¹

Biomarker investigations

A group of 20 apparently healthy persons, age and sex cross matched with patients, formed a control group. Three ml blood were collected from each patient and control subject into plain tube without anticoagulant for assessing procalcitonin, using an immunoassay analyzer (cobas E 411, Roche Diagnostics)¹² and hsCRP using an integrated system (Dimention xpand 2004083091, Siemens Healthineers Diagnostics, USA).¹³ Blood was allowed to clot for 15 minutes at 37 °C and serum was separated by centrifugation at 1560 Xg for 10 minutes then kept at -20 °C until used.

Molecular investigations

All patient's blood samples were subjected to broad range bacterial 16s rRNA PCR assay as the following: a) DNA extraction: Bacterial DNA was extracted from EDTA blood using (QIAamp DNA Blood Mini kit) (QIAGEN, Hilden, Germany) as described by the manufacturer. For testing the yield and quality of the extracted DNA, DNA samples were electrophoresed on 2% agarose gel.¹⁴ For PCR amplification of 16s rRNA genes, five microliters of the extracted DNA were amplified in a 25 µl volume PCR reaction using an oligonucleotide primer set (designed according the USA National Center for Biotechnology Information; NCBI). The forward primer was: CGGTCCAGACTCCTACGGGAGGCA GCA; and reverse primer: GCGTGGACTACCA GGGTATCTAATCC. The PCR reaction consisted of 12.5 µl 2x Tag PCR Master Mix, one µl of each forward and reverse primers, and PCR grade water to a final volume of 25 µl. The PCR amplification consisted of an initial

denaturation step at 95 C for 5 min followed by 35 cycles of denaturation at 95 C for 30 s, annealing at 65 C for 30 s, extension at 72 C for 30 s and final extension at 72 C for 10 min¹⁵. The PCR products were electrophoresed through agarose gel 2%. Molecular sizes of the amplicons were determined by comparison to a 100 bp DNA ladder. A positive result was inferred by detection of 480 bp band¹⁶.

Statistical analysis

Statistical package for social science (SPSS) version 11 was used for data base construction, management and analysis. Quantitative data were summarized in the form of mean \pm SD. Qualitative data were summarized using frequencies and percentages. Student's t-test was done for comparison between two means and one-way ANOVA for more than two means. Probability (p value) less than 0.05 was considered significant. Accuracy of hsCRP, PCT and PCR in diagnosis of culture negative infective endocarditis was assessed by using receiver operator characteristics (ROC) curve.

Results

The study included 60 patients with suspected infective endocarditis based on the modified Duke criteria.

Clinical and demographic data of studied group

The mean age of enrolled patients was 30.5 \pm 16.2 years with a range of (1.5-64 years). Of the 60 studied patients, 33 (55%) patients were males and 27 (45%) females. The most frequent risk factors for infective endocarditis were rheumatic heart disease in 20 patient (33.3%), diabetes mellitus in 19 patients (31.6%), 18 (30%) with prosthetic valve, 11(18.3%) on hemodialysis and 8 (13.3%) with congenital heart defect (Figure 1).

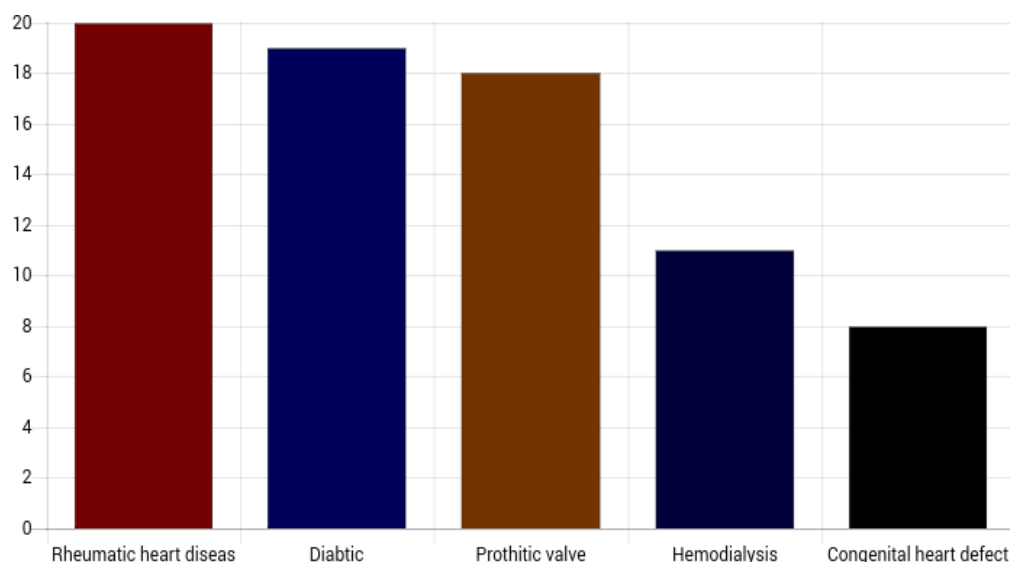


Figure 1. Underlying predisposing factors in study patients

Microbiological results

Positive blood culture samples were identified by conventional culture method according to SOPs of the lab and Vitek2 compact system. The results of organism type obtained by both methods were identical. Of the 60 patients, blood cultures of samples from 46 (76.7%) were positive and negative in 14 (23.3%) samples. Of the 46 culture positive patients, 10 patients had mixed organisms. So, the total number of detected organisms were 59 organisms; 46

(78%) were gram positive organism while only 13 (22%) were gram negative organism.

The result of organism differentiation of culture positive patients

There were 46 (78%) of gram-positive organism and 13 (22%) of gram-negative organism. The most common microorganism causing infective endocarditis was *Staphylococcus aureus* 28 (47.4%) followed by *Enterococcus* 15 (25.4%) then *Klebsiella* 8 (13.5%) and their differentiation is shown in Table 1.

Table 1. Differentiation of organism in culture positive patients

Type of organism (n=59)	Number of patients
<u>Gram positive organism</u>	46 (78%)
- <i>Staphylococcus aureus</i>	28 (47.4%)
- <i>Enterococcus</i>	15 (25.4%)
- <i>Staphylococcus hominis</i>	2 (3.3%)
- <i>Streptococcus</i>	1 (1.6%)
<u>Gram negative organism</u>	13 (22%)
- <i>Klebsiella</i>	8 (13.5%)
- <i>Acinetobacter</i>	5 (8.4%)

Biomarker investigations

The results of biomarkers test of positive culture patients, negative culture patients and the control group are shown in Table 2. It was

observed that the range and mean value of PCT and hsCRP in culture negative patients are less than those in culture positive patients, but more than the control group results.

Table 2. Biomarker data in different study groups.

Biomarker	Culture positive patients (n=46)	Culture negative patients (n=14)	Control group (n= 20)	P value
hsCRP (mg/dl)				
Mean \pm SD	12.8 \pm 4.1	6.9 \pm 5.2	0.3 \pm 0.1	NS
Range	(7.4-20)	(0.3-18)	(0.1-0.7)	
Procalcitonin (ng/ml)				
Mean \pm SD	33.1 \pm 39.6	1.24 \pm 2.3	0.04 \pm 0.02	0.0001*
Range	(0.32-89)	(0.02-8.8)	(0.01-0.08)	

*For comparison between the three groups. $P > 0.05$ is no significant (NS).

Molecular investigations

Broad range PCR for amplification of bacterial 16S rRNA, of DNA extracted from whole blood samples of culture negative patients were positive in 11/14 cases (78.5%), (Figure 3) and 42/46 cases (91.3%) in culture positive patients (Figure 4).

Diagnostic accuracy of hsCRP, PCT and PCR in diagnosis of culture negative infective endocarditis

At cutoff > 10.2 mg/dl, serum hsCRP had 45% sensitivity and 83.33% specificity for diagnosis of culture negative infective endocarditis with overall accuracy of 48.8%. While PCT at cutoff > 1.34 ng/dl had 57% sensitivity and 83.33% specificity for diagnosis of culture negative infective endocarditis with overall accuracy of 59.6%. PCR had the best diagnostic accuracy (95.5%) for diagnosis of culture negative infective endocarditis. It also, had 95% sensitivity and 100% specificity. So, based on the ROC curve analysis, the most accurate method for diagnosis of CNIE was PCR with area under the curve of 0.97 (excellent accuracy) followed by PCT with area under the curve of 0.78 (Good accuracy) and then hsCRP with area under the curve of 0.72 (fair accuracy) Figure (5).



Figure 3. Agarose gel electrophoresis of PCR products of culture negative patients as resulted from universal amplification of 16S rRNA gene. Lane 17:100 bp DNA molecular weight ladder; Lane 16: positive control, Lane 15: negative control, Lanes 14,12,11,8,7,6,5,4,3,2,1: positive PCR result of culture negative patients, Lanes 9,10,13: negative PCR result of culture negative patients.



Figure 4. Agarose gel electrophoresis of PCR products of culture positive patients as resulted from universal amplification of 16S rRNA gene. Lane 18:100 bp DNA molecular weight ladder, Lanes 1,2,3,4,5,6,7,8,9,12,13,14,17: positive PCR result of culture positive patients; Lanes 10,11,15,16: negative PCR result of culture positive patients.

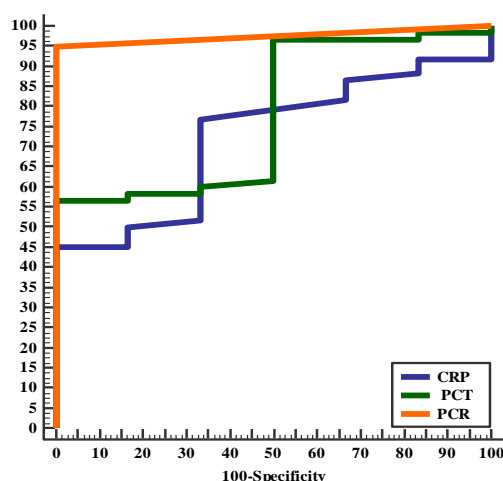


Figure 5. Roc curve analysis for PCT, hsCRP and PCR. Area under the curve: > 0.9 =Excellent accuracy, > 0.8 =Good accuracy, > 0.7= Fair accuracy, ≤ 0.7= Poor accuracy.

Discussion

The present study revealed that the most common risk factors associated with infective endocarditis patients were rheumatic heart disease (30.3%), diabetes mellitus (28.7%), prosthetic valve (27.2%), hemodialysis (18.1%), and CHD (12.1%). These findings agreed with previous studies, reported history of rheumatic fever (30%), prosthetic valve (24%), and CHD (8%) as common risk factors.¹⁷⁻¹⁸ However, another study revealed that chronic hemodialysis was the most common risk factor (26.7%) for IE cases³. Also, another study found that diabetes mellitus was the most common risk factor (18.2%) for IE.¹⁹

In this study the culture positive IE patients were 46 (76.6%) and culture negative IE patients 14 (23.4%). In contrast, a previous study revealed that culture positive IE patients accounted for 30.3% and culture negative for 69.7%.²⁰

Changes in the epidemiology of IE have happened over the past several decades.²¹ In this study the three most common pathogens in culture positive infective endocarditis patients were: *Staphylococcus aureus* followed by *Enterococcus* then *Klebsiella*. In this regard, other studies reported that *Staphylococcus aureus* was the most common organism in culture positive IE patients.^{22,23}

In this study the result of PCR revealed that for culture negative patients 16S rRNA genes

were amplified in 78.5% cases and in 91.3% for culture positive patients. So, in our study, there were 4 samples positive by blood culture but negative for amplification of 16S rRNA genes by PCR, and all were bacteria of gram-positive type. PCR-negative results in culture-positive patients especially with gram-positive pathogens were previously reported.²⁴ The authors indicated that difficulties in breaking the cell wall of gram-positive organisms during sample preparation resulted in failure in the DNA extraction process.^{24,25,26}

In our study PCR showed 95% sensitivity, 100% specificity and 95.5% accuracy while a previous study showed 88.3% sensitivity, 92% specificity and 91% accuracy.²³ In a systematic review, it was concluded that PCT and hsCRP were neither sensitive nor specific for diagnosis of IE²⁷. However, IE should be considered based on clinical information i.e., presence of fever, embolic phenomenon, new or changed heart murmur, risk factors for IE like valvar heart disease, intravenous drug abusers, and bacteremia with incomplete treatment especially with *Staphylococcus*.²⁷

This study revealed that PCT showed a significant difference between IE patients and the control group, but CRP did not reach such significance. A previous study found that PCT had a critical role in the detection of potential IE infection in individuals with a history of cardiac issues who were referred with acute symptoms such as dyspnea. PCT had a predictive value that was linked to clinical outcome and could be utilized as a therapy guide. It also showed a strong link to a positive blood culture, which was an item in the diagnosis of the IE. PCT had a strong correlation with positive blood culture regardless of the pathogen or infection site.²⁸

The PCR approach, as employed in our study, is inexpensive and simple to carry out in a clinical microbiological laboratory. Its cost-effectiveness is measured not only in terms of material costs, but also in terms of the added value of early detection of bacteremia in patients and, as a result, the delivery of proper treatment, which lowers morbidity and death rates. However, DNA amplification approaches do not determine antibacterial susceptibility, which must be tested using traditional methods.

Based on our study findings, we conclude that molecular approaches and biomarkers such as procalcitonin would add value in diagnosis of cases with infective endocarditis, particularly culture negative endocarditis. Nonetheless, PCR-based techniques cannot replace traditional culture procedures. The use of an automated blood culture device, as well as adequate blood culture collection under sterile conditions, would still aid in the identification of infective endocarditis.

Author Contributions

AOA; was responsible for methodology, analysis and interpretation of data, SMA; was responsible for project administration conception and design of the study and overall supervision, AKM; was responsible for review and editing of the study in addition to clinical evaluation of patients, MEA; was responsible for molecular method and experiments in addition to analysis and interpretation of data, SAS; was responsible for writing the original draft in addition to practical work.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) denies receipt of any financial support for the research, authorship, and/or publication of this article.

Ethical approval

The study protocol was reviewed and approved by the ethical committee of the Faculty of Medicine, Assiut University (IRB 17100460; 3/2018).

Informed consent

The study goals were explained to all the subjects and to guardian of infants and children, and they gave their consent orally. In addition, for school age children (6-18 years of age), the blood collection method and study goals were explained in simple language, and willing to provide blood sample was taken as child assent.

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