



Role of multiplex PCR in the early diagnosis of infective endocarditis

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Abstract

Infective endocarditis (IE) is a serious infection of the endocardium and heart valves that necessitate early diagnosis. The conventional blood culture has lots of false-negative results besides being time consuming. Polymerase chain reaction (PCR) is a rapid diagnostic tool that helps in saving the patients' life. This study aimed to investigate the feasibility of multiplex PCR in early diagnosis of IE compared to the conventional blood culture, and to evaluate its impact on IE diagnosis in cases of negative blood cultures. The current study was conducted on 30 patients admitted to the Cardiology Department, Faculty of Medicine, Ain Shams Hospitals, Cairo, Egypt, which were diagnosed clinically as infective endocarditis according to the modified Duke's criteria. After processing of the patient's blood samples, the blood cultures recorded positivity in 5 cases (16.7 %). The most common recovered bacteria were, *Staphylococcus aureus* 2(6.7 %), *Staphylococcus epidermidis* 1(3.3 %), *Enterococcus faecalis* 1(3.3 %), and *Escherichia coli* 1(3.3 %); however, no other pathogens were isolated. On the other hand, results of multiplex PCR showed positivity in 13 cases (43.3 %), mainly; *Staphylococcus aureus* 5(16.7 %), *E. faecalis* 3(10 %), *Staphylococcus epidermidis* 2(6.7 %), *Pseudomonas aeruginosa* 2(6.7 %), and *E. coli* 1(3.3 %). No other bacterial or fungal pathogens were detected by multiplex PCR. Finally, the multiplex PCR assay exhibited remarkable sensitivity and feasibility in IE diagnosis over blood culture, besides being a rapid and accurate diagnostic assay that enhances proper treatment.

Keywords: Infective endocarditis, Multiplex PCR, Blood culture, Bacterial pathogens

1. Introduction

Infective endocarditis (IE) is a microbial infection of the endothelial lining of the heart, it continues to be

a non-widespread condition; however, it showed elevated levels of associated morbidity and mortality



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([Rajani and Klein, 2020](#)). Infective endocarditis (IE) encompasses native, prosthetic valves or any intracardiac devices within the heart. [Shmueli *et al.*, \(2020\)](#) reported that IE is caused through sowing of any of these cardiac structures by bacterial, or less frequently by fungal pathogens.

The clinical presentation of IE is multiform, this accounts for the difficulty in diagnosis and the risk of delayed treatment. A study conducted by [Lung and Duval, \(2019\)](#) highlighted that in spite of the advances in diagnostic methods; the advent of antibiotic therapy and the performance of valvular surgery during acute infection; however, the prognosis for IE remain poor, accordingly this supports the attempts to improve early diagnosis, therapeutic management and prevention of microbial infection. Notably, the incidence of IE averages 1.7-6.2 cases in every 100 000 individuals per year. [Rizk *et al.*, \(2019\)](#) revealed that neither the incidence nor the mortality of IE have declined in the previous 20 year.

Blood-culture is commonly known as the gold standard for the detection of microbial pathogens in the bloodstream. This methodology possesses some intrinsic restrictions, as it is technically difficult and can only identify microbes growing under optimal cultural conditions. On the other hand, PCR is sensitive to small amounts of pathogen's DNA and can directly detect it in blood samples within 3-6 h, thus supporting subsequent rapid treatment ([Trung *et al.*, \(2019\)](#)).

Nonetheless most microorganisms linked with IE are diagnosed by blood cultures and occasionally with serology; however, diagnosis in the forthcoming decade will expected to rely on molecular biology owing to its noticeable competence in etiological diagnosis with accuracy; efficiency and anticipated wide accessibility. This is significantly crucial and feasible in cases of blood culture-negative endocarditis (BCNE); caused by preceding antibiotic intake and/or incapability of fastidious microorganisms to be cultured, as reported by [Al-Rachidi *et al.*, \(2017\)](#). BCNE may be attributed to the non-capacity of

fastidious microorganisms to be cultured by the routine techniques, in addition to growth inhibition of pathogens due to preceding antibiotic administration, which may be self-administrated, or physician prescribed ([Habib *et al.*, 2009](#)).

Multiplex PCR is a novel methodology that permits simultaneous detection of several microorganisms through introduction of different primers to amplify DNA region coding for genes of each targeted bacterial strain ([Rajapaksha *et al.*, 2019](#)). This assay is sensitive; specific, rapid and economic. Multiplex PCR could be used in clinical laboratories for rapid identification and induction of effective treatment, thus reducing patient's mortality and morbidity rates. Additionally, it may help decreasing abuse of antimicrobials that are toxic and more expensive. There are several multiplex assays for the fast recognition of microorganisms in clinical samples within 8 h ([Ali *et al.*, 2018](#)). The objective of this research was to investigate the feasibility of multiplex PCR in the early diagnosis of IE, compared to the conventional blood culture.

2. Patients and methods

2.1. Patients demographic data

This study was an observational cross-sectional study conducted on 30 patients diagnosed clinically with IE, admitted to the Cardiology Department, Ain shams University hospitals during the period from June, 2019 - June, 2020. Modified Duke's criteria were used for case definition. They were 25 males and 5 females, and their ages ranged from 18- 62 years. Demographic data were collected from all patients including; underlying heart disease and predisposing conditions. Informed consent was obtained from each patient before enrolment.

2.2. Samples collection and processing

Blood samples were withdrawn from all patients promptly after admission and before any antibiotic

use. Approximately 12 ml of venous blood were taken from each participant under complete aseptic conditions; 10 ml were dispensed into a blood culture bottle, 2 ml were inoculated into EDTA treated tubes and then stored at -80°C for PCR analysis.

2.3. Conventional blood culture

Blood culture bottles were incubated at 37°C and examined daily for visible signs of bacterial growth such as; turbidity above the red cell layer, colonies growing on the surface of the red cells, hemolysis, gas bubbles, and clots. Subcultures were carried out on blood agar, nutrient agar, MacConkey's agar and Sabouraud dextrose agar. Moreover, subcultures of the isolates were carried out on other culture media such as Bile esculin agar for identification of Enterococci. Isolated bacteria were identified conventionally according to their Gram stain reaction, followed by several biochemical assays including: Catalase production, Coagulase production, Oxidase production, Sugar fermentation, H₂S production assay (effect on Triple sugar iron agar), Indole production, Urease production and Citrate utilization assays, which were used to identify the Gram-negative bacilli, according to [Cheesbrough, \(2006\)](#).

2.4. Molecular detection of pathogenic bacteria

2.4.1. DNA extraction

DNA extraction was performed using QIAamp DNA blood Mini Kit, as per the manufacturer's protocol. Extracted DNA was eluted in 60 µl elution buffer and then stored at -80°C until PCR was carried out.

2.4.2. Microbial DNA amplification by multiplex PCR

Simultaneous amplification of *Staphylococcus aureus*, *E. coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* was carried out in a single tube (first multiplex PCR reaction), whereas simultaneous amplification of *Streptococcus viridans*, *Staphylococcus epidermidis*, *Enterococcus faecalis*

and *Candida albicans* was carried out in another tube (second multiplex PCR reaction).

-1st multiplex PCR reaction

QIAGEN® Multiplex PCR kit was used. Four pairs of primers specific for *Staphylococcus aureus*, *E. coli*, *P. aeruginosa* and *K. pneumoniae* were used. Primers' sequences and amplicons sizes are demonstrated in Table (1). The reaction mix was assembled into 50 µl volume in a thin walled 0.2 ml PCR tube. DNA amplification was carried out using Labnet MultiGene™ Gradient PCR Thermal Cycler under the following thermal conditions: initial denaturation at 95°C for 5 min., followed by amplification 30 cycles at 96°C for 1 min., 52°C for 30 sec, and 72°C for 1min., and a final extension at 72°C for 10 min., in reference to [Thong *et al.*, \(2011\)](#). Electrophoresis was carried out on agarose gel and visualized using ethidium bromide. Thermo Scientific GeneRuler 100 bp DNA Ladder was used as DNA size marker. An UV transilluminator using UV LUT-300D trans-illuminator (LABNICS, UK) was used to visualize the positive bands in the agarose gel.

-2nd multiplex PCR reaction

Simultaneous amplification of *Streptococcus viridans*, *Staphylococcus epidermidis*, *E. faecalis* and *C. albicans* was carried out in another tube using QIAGEN® Multiplex PCR kit. Four pairs of primers specific for these microorganisms were used; their sequences and amplicons sizes are demonstrated in Table (2). The reaction mix was assembled into 50 µl volume in a thin walled 0.2 ml PCR tube. DNA amplification was carried out using Labnet MultiGene™ Gradient PCR Thermal Cycler following the universal multiplex cycling protocol described by the manufacturer of QIAGEN® Multiplex PCR kit. First, initial activation step was carried out at 95 for 15 min., followed by amplification 35 cycles at 94°C for 30 sec, 57°C for 90 sec, 72°C for 90 sec, and a final extension at 72°C for 10 min. Electrophoresis was performed on an agarose gel and visualized using ethidium bromide. Thermo Scientific GeneRuler

100bp DNA Ladder was used as DNA size marker. An UV transilluminator using UV LUT-300D trans-

illuminator (LABNICS, UK) was used to visualize the positive bands in the agarose gel.

Table 1: List of primers sequences used for 1st multiplex PCR reactions and their amplicons sizes

Microorganism	Target gene	Primer	Amplicon size (bp)	References
<i>Staphylococcus aureus</i> (MRSA)	FemA	FemA-F AAAAAAGCACATAACAAGCG FemA-R GATAAAGAAGAAACCAGCAG	132	(Mehrotra et al., 2000)
	MecA	MecA-F GTAGAAATGACTGAACGTCCGATAA MecA-R CCAATTCCACATTGTTTCGGTCTAA	310	(Geha et al., 1994)
<i>E. coli</i>	PhoA	PhoA-F GTGACAAAAGCCCGGACACCATAAATGC CT PhoA-R TACTGTGCATTACGTTGCGGATTTGGC GT	903	(Kong et al., 1999)
<i>K. pneumonia</i>	Mdh	Mdh-F GCGTGGCGGTAGATCTAAGTCATA Mdh-R TTCAGCTCCGCCACAAAGGTA	364	(Sun et al., 2008)
<i>P. aeruginosa</i>	OprL	OprL-F ATGGAAATGCTGAAATTCGGC OprL-R CTTCTTCAGCTCGACGCGACG	504	(De Vos et al., 1997)

Table 2: List of primers sequences used for 2nd multiplex PCR reactions and their amplicons sizes

Microorganism	Target gene	Primer	Amplicon size (bp)	Reference
<i>Staphylococcus epidermidis</i>	Se705	Se705-1 ATCAAAAAGTTGGCGAACCTTTTCA Se705-2 CAAAAGAGCGTGGAGAAAAGTATCA	124	(Morot-Bizot et al., 2004)
<i>Streptococcus viridans</i>	GtfB	GtfB-F ACTACACTTTCGGGTGGCTTGG gtfB-R CAGTATAAGCGCCAGTTTCATC	517	(Al-Ahmad et al., 2006)
<i>E. faecalis</i>	rrs (16S rRNA)	C1: GGATTAGATACCCTGGTAGTCC C2: TCGTTGCGGGACTTAACCCAAC	320	(Honarm et al., 2012)
<i>C. albicans</i>	IGS 1	Falb AGATTATTGCCATGCCCTGAG Ralb CCATGTCGAACGTAGCGTAT	606	(Arastehfar et al., 2019)

2.5. Statistical analysis

All data were statistically analyzed using Statistical package for Social Science SPSS-20. Numerical data were expressed as mean, standard deviation and percentages, while non-numerical data were expressed as frequency and percentage. Comparison between groups regarding qualitative data was performed through Chi-square test and/or Fisher exact test when the expected count in any cell found was less than 5. Receiver operating characteristic curve (ROC) was used in the qualitative form to detect sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy of PCR on the blood culture as a gold standard. Weighted Kappa agreement was used to assess the agreement percentage between the two methods. The confidence interval was set to 95 %, whereas the margin of error accepted was set to 5 %. So, the *p*-value was considered significant as the following: *p*-value > 0.05: Non-significant (NS); *p*-value < 0.05: Significant (S); *p*-value < 0.01: Highly significant (HS).

3. Results

3.1. Isolation of the microbial pathogens on blood agar

Blood culture was positive in 5 patients that represented 16.7 % of total patients. However, blood culture-negative infective endocarditis (BCNE) represented 83.3 % of all patients (25 cases). Notably, blood culture positivity occurs within 1-6 d of incubation.

3.2. Identification of the pathogens

Results showed that 4 pathogens were isolated. The first was *Staph. aureus* that was demonstrated in 2 cases (6.7 %), and represented the highest detectable microorganism in the blood culture. Then, the other three microorganisms were *Staph. epidermidis*, *E. faecalis* and *E. coli*, which were presented in one case (3.3 %) for each. No *P. aeruginosa*, *S. viridans* or fungal isolates were recovered from any patient by blood culture, as presented in Table (3).

Table 3: Frequency of occurrence of bacterial isolates on blood culture

Blood culture	No.	%
Negative cases	25	83.3%
Positive cases	5	16.7%
<i>Staph. epidermidis</i>	1	3.3%
<i>Staph. aureus</i>	2	6.7%
<i>E. faecalis</i>	1	3.3%
<i>E. coli</i>	1	3.3%

3.3. Multiplex PCR

Multiplex PCR expressed positive results in 13(43.3 %) of IE cases, while the rest of the 17 (56.7 %) patients were negative. Furthermore, 5 pathogens were detected by PCR. The most common isolate was *Staph. aureus* that was detected by positive bands at 310 bp and 132 bp on agarose gel of 1st multiplex PCR reaction in 5 patients (16.7 %). *E. faecalis* was detected through positive bands at 310 bp on agarose gel of 2nd multiplex PCR reaction in 3 cases (10.0 %). *Staph. epidermidis* was diagnosed by positive bands at 124 bp in 2nd multiplex PCR reaction in 2 cases (6.7%), *P. aeruginosa* was detected 2 cases (6.7 %) by positive bands at 504 bp in 1st multiplex reaction. Only one patient (3.3 %) showed *E. coli* bacterium by a positive band at 903 in 2nd multiplex reaction. No *S. viridans* or *C. albicans* were detected in any cases of the multiplex PCR (no positive bands on agarose gel of 2nd reaction at 517 bp or 606 bp, respectively), as demonstrated in Fig. (1 and 2). This study displayed a significant rise in the occurrence of positive cases in results of PCR (43.3 %), compared to the blood culture results (16.7 %), with p -value= 0.024. With regard to the type of microorganism, blood culture detected only

2 cases (6.7 %) of *Saph. aureus*, but PCR detected 5 cases (16.7 %) with p -value = 0.22. Blood culture recorded a single case (3.3%) of *E. faecalis*, however PCR could detects about 3 cases (10 %) with p -value = 0.301. One case of *Staph. epidermidis* (3.3 %) was detected by blood culture, while PCR recorded 2 cases (6.7 %) with p -value =0.554. Through results of blood culture no any case of *P. aeruginosa* was diagnosed, but PCR detects 2 cases (6.7 %) with p -value = 0.150, as shown in Table (4). In this study, the multiplex PCR evaluation compared to blood culture was regarded as the gold standard for diagnosis of IE, which showed a sensitivity of 100 %, specificity of 68.0 %, as demonstrated in Table (5) and Fig. (3), with recorded positive predictive value of 38.5 %, and negative predictive value of 100 %. Five cases were true positive (positive by both blood culture and multiplex PCR), 17 cases were true negative (negative by both blood culture and multiplex PCR), while 8 cases were false positive (positive by multiplex PCR, but negative by blood culture). There were no any observed false negative cases (negative by multiplex PCR and positive by blood culture). Agreement between results of blood culture and multiplex PCR represented by weighted kappa was of 41.5 % (Table 5).

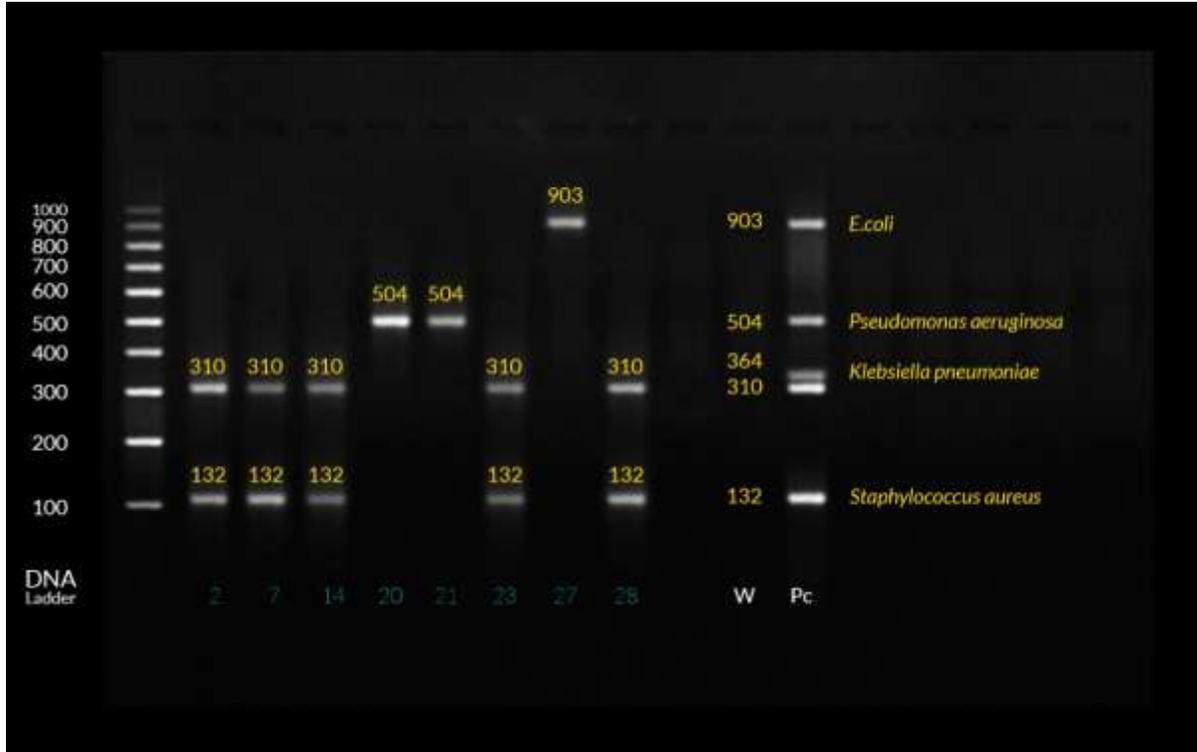


Fig. 1: Agarose gel electrophoresis of PCR amplicons of 1st multiplex PCR reaction

Where; Lane 1: DNA ladder (Thermo Scientific Gene Ruler 100bp DNA Ladder) used as a DNA size marker; Positive bands were detected at: 310 bp and 132 bp for *Staph. aureus* (5 cases; samples no. 2, 7, 14, 23 and 28); 504 bp for *P. aeruginosa* (2 cases; samples no. 20 and 21); 903 bp for *E. coli* (1 case; no. 27); No positive bands for *K. pneumoniae* at 364 bp were detected; *Pc (1st lane on right): Positive control; *W: water as negative control



Fig. 2: Agarose gel electrophoresis of PCR amplicons of 2nd multiplex PCR reaction

Where; Lane 1: DNA ladder (Thermo Scientific Gene Ruler 100bp DNA Ladder): used as a DNA size marker; Positive bands were detected at: 124 for *Staph. epidermidis* (2 cases: no. 1, 15); 320 bp for *E. faecalis* (3 cases: no. 12, 19 and 25). No positive bands were detected for *S. viridans* at 517 or *C. albicans* at 606. *Pc (1st Lane on right): Positive control; *W: water as negative control

Table 4: Comparison between results of blood culture and multiplex PCR

	Blood culture		Multiplex PCR		Test value	p-value
	No.	%	No.	%		
Negative cases	25	83.3 %	17	56.7 %	5.079	0.024
Positive cases	5	16.7 %	13	43.3 %		
<i>Staph. epidermidis</i>	1	3.3 %	2	6.7 %	0.351	0.554
<i>Staph. aureus</i>	2	6.7 %	5	16.7 %	0.351	0.228
<i>E. faecalis</i>	1	3.3 %	3	10.0 %	1.071	0.301
<i>E. coli</i>	1	3.3 %	1	3.3 %	0.000	1.000
<i>P. aeruginosa</i>	0	0.0 %	2	6.7 %	2.069	0.150

Where; p-value > 0.05: Non significant (NS); p-value < 0.05: Significant (S); p-value < 0.01: highly significant (HS)

Table 5: Evaluation of multiplex PCR against blood culture as a gold standard for IE diagnosis

Parameter	True positive	True negative	False Positive	False Negative	Sensitivity	specificity	PPV	NPV	Weighted Kappa
Multiplex PCR	5	17	8	0	100	68	38.5	100	41.5

Where; *PPV: positive predictive value; **NPV: negative predictive value. Multiplex PCR sensitivity: 100%; specificity: 68%; PPV: 38.5%; NPV: 100%. Kappa agreement (which represents agreement between blood culture regarded as a gold standard and multiplex PCR): 41.5%

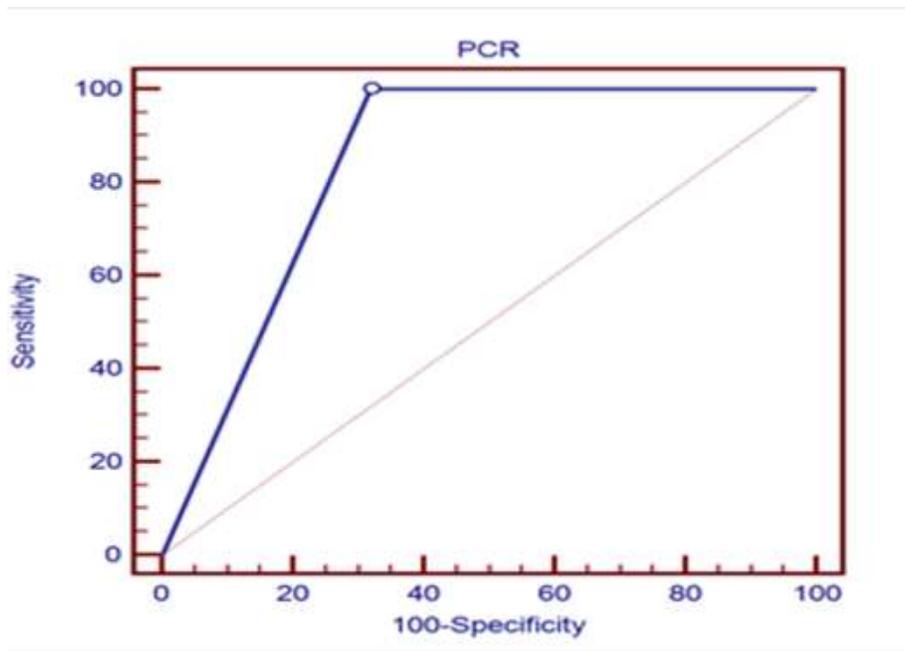


Fig. 3: ROC (receiver operating characteristic) curve: Graphical plotting of sensitivity of multiplex PCR against its specificity. Where; the area under the ROC curve was a measure of the usefulness of multiplex PCR assay

4. Discussion

The current study sheds light on the values of multiplex PCR as a feasible, rapid and accurate diagnostic method of IE, compared to the conventional blood culture. This is verified by the significant increase of positive IE cases diagnosed by multiplex PCR over blood culture. Additionally, multiplex PCR reveals the results rapidly within 6-8 h; thereby reduce sole dependence on blood culture, which requires a long time of 24-48 h, thus multiplex PCR saves patients' life.

Currently, the recorded multiplex PCR sensitivity is 100 % and specificity is 68 %, similar to the previous results reported by [Al-Rachidi *et al.*, \(2017\)](#), which recorded multiplex PCR sensitivity and specificity of 85.7 % and 60.6 %, respectively. In a previous study conducted by [El-Kholy *et al.*, \(2015\)](#), PCR showed a comparable sensitivity of 88.3 % and specificity of 92 %. Moreover, other study performed by [Casalta *et al.*, \(2009\)](#) on 63 patients with infective PCR had a corresponding sensitivity and specificity of 86.7 % and 86.9 %, respectively. In accordance with this study, a systematic review recently conducted by [Faraji *et al.*, \(2018\)](#) to investigate the molecular methods such as multiplex PCR in detecting causative microorganisms of infective endocarditis, the observed highest sensitivity and specificity, were 96 % and 100 %, respectively. With regards to the conventional blood culture and pathogens recorded in this study, results were comparable to those of the study conducted by [Al-Rachidi *et al.*, \(2017\)](#). The study was conducted on 37 patients in which blood culture was positive in 7 (18.9 %) patients of definite IE, while the rest of 30 patients were blood culture negative (BCNE). *Staph. aureus* (6 cases) and *Pseudomonas* spp. (1 case) were the predominant microorganisms isolated from the culture-positive cases. The difference in percentages of pathogens detected in most cases of IE may be attributed to the difference in antibiotic administration therapy.

This study did not detect any streptococcal or fungal cases either by the blood culture or the multiplex PCR; however, in the study of [Rizk *et al.*, \(2019\)](#) that comprised 398 patients; streptococci were detected in 36 cases (9 %) while fungi were detected in 32 cases (8 %) by blood culture. This may be related to the small number of patients included in our study.

According to the recent study of [Talha *et al.*, \(2020\)](#), there had been a considerable shift in the microbiology of IE with the advent of Staphylococci as the most common microorganism; rather than *S. viridans* as reported in the previous years. [Toyoda *et al.*, \(2017\)](#) reported that IE caused by *Staph. aureus* has an increasing standardized incidence from 2.1- 2.7 cases per 100 000 persons annually.

In this study, *Staph. aureus* was recorded as the most common pathogen detected in IE cases; 16.7 % by multiplex PCR, and 6.7 % by blood culture, similar to the previous study conducted by [Wang *et al.*, \(2018\)](#) in which *Staph. aureus* accounted for 40 % of IE cases. Moreover, [Cresti *et al.*, \(2017\)](#) conducted a prospective case-series population and concluded that the most frequent microorganism was also *Staph. aureus* (25 % of cases). In accordance, a retrospective study conducted in tertiary care hospital in New York by [Fatima *et al.*, \(2017\)](#), reported that *Staph. aureus* was detected in 21 cases (38.3 %) out of total 54 patients included in this study. However, another recent population-based study conducted in the United States by [DeSimone *et al.*, \(2021\)](#) observed that *Staph. aureus* and *S. viridans* were the most frequently recorded causative pathogens in the IE patients.

On the contrary, a study carried out by [Patel and Ahmed, \(2020\)](#) that included 355 IE patients observed that Streptococci species were the most common pathogens that accounted for (21.4 %) of IE cases. In addition, another study conducted by [Wu *et al.*, \(2020\)](#) in the tertiary hospital in China, concluded that Streptococci were the main pathogens of IE (24.6 %) of cases.

Currently, *P. aeruginosa* was recorded in 2 cases using multiplex PCR, which were not detected by blood culture, and this reveals the high sensitivity of PCR. Similarly, several previous studies of [Lisby *et al.*, \(2002\)](#); [Casalta *et al.*, \(2009\)](#); [Tsalik *et al.*, \(2010\)](#) also reported that multiplex PCR detected microorganisms in IE cases that were blood culture negative.

Herein we report a considerable difference between positivity by blood culture (16.7 %) and positivity by PCR (43.3 %), in favor of PCR. Similarly, in a previous study of [Millar *et al.*, \(2001\)](#), blood culture was positive in 36.16 % while PCR was positive in 57.44 %. Moreover, in the recent study conducted by [Trung *et al.*, \(2019\)](#) that included 144 patients, blood culture was positive in 49 cases (34 %) while PCR was positive in 83 (57.64 %) cases. On contrary to the current results, a study carried out by [Mencacci *et al.*, \(2012\)](#) that included 23 patients; compared the performance of multiplex PCR with blood culture in diagnosis of IE cases, and recorded that blood culture' and PCR' positivity were the same (65 % of total cases). Furthermore, a study conducted by [Bosshard *et al.*, \(2003\)](#) recorded that blood culture was positive in 44.89 % and PCR was positive in 46.93 % of cases, with no significant difference.

[Veve *et al.*, \(2020\)](#) stated that in the recent years infections due to Gram-negative bacilli (GNB) have raised growing concerns, because of their increasing spread, high mortality, high health care costs, in addition to their tendency to develop and spread antibiotic resistance. In this work, GNB was detected in 3 cases; 2 cases of *P. aeruginosa* (detected by PCR only) and a single case of *E. coli* (detected by both PCR and blood culture), which agree with the recent studies conducted by [Falcone *et al.*, \(2018\)](#); [Merican *et al.*, \(2019\)](#) who reported that the most common GNS detected in IE patients were *E. coli* and *P. aeruginosa*.

Conclusion

In the present study, we concluded that although PCR-based techniques cannot replace the conventional

blood culture as there are microorganisms that are out of the multiplex panel; however, multiplex PCR offers good aid in rapidly detecting infectious causes in many cases of culture-negative IE, and saves time for critically ill patients who cannot wait until results of the blood culture are obtained. Multiplex PCR requires only 6 h to provide results of microorganisms identification, thus directs for rapid diagnosis and treatment programs.

Conflicts of interest

There is no conflict of interests among authors of this study.

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Ethical approval

This study was approved by the Ethical and Moral Committee of Faculty of Medicine Ain Shams University (No. FMASU M S103/2019). The patient's consents and statement of protection of the patient's privacy are provided.

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