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ORIGINAL ARTICLE

Molecular and serological techniques for the diagnosis of culture negative infective endocarditis in Alexandria Main University Hospital

Salama M. Sadaka [a], Iman F. El-Ghazzawy [a], Mahmoud M. Hassanen [b], Ahmed S. Abu El Kasem [c], Amina M. Nour El Din [a], Marwa A. Meheissen [a,*]

[a] *Microbiology and Immunology Department, Alexandria University, Egypt*
[b] *Cardiology and Angiology Department, Alexandria University, Egypt*
[c] *Cardiothoracic Surgery Department, Alexandria University, Egypt*

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Abstract *Background and aim:* Culture-negative infective endocarditis (CNIE) is a diagnostic dilemma. The study was carried out to estimate the prevalence of CNIE among definite IE cases, to describe the epidemiologic and clinical characteristics of CNIE patients and to diagnose the microbial etiology of CNIE using molecular and serological techniques.

Subjects and methods: Sixty-five definite IE cases were enrolled in a prospective observational study between January and December 2010. CNIE cases were tested by 16SrRNA and seminested PCR for 35 blood samples, serological tests and the study of ten valve tissue specimens.

Results: CNIE constituted 39 (60%) cases. The mean age of CNIE patients was 31 years. Male to female ratio was 2.9:1. Healthcare associated IE accounted for 15.4%, native valve IE for 66.7% and intravenous drug abuse for 20.5% of cases. The mitral valve was the most frequently involved (56.4%). Out of 39 CNIE cases, seminested blood PCR detected 12 cases (ten *Staphylococci*, two *Streptococci*). Five cases were reactive by serology (three *Bartonella*, one *Coxiella*, and one *Brucella*). Six cases were positive by analysis of valve tissue (three *Staphylococci*, three *Streptococci*). The combined results of all diagnostic tools decreased the percentage of non-identified causes of CNIE from 60% to 24.6%.

Conclusions: Our data underlined the role of collecting blood culture before starting antibiotics and the role of seminested PCR in the diagnosis of conventional causes of CNIE. The importance of serology to identify non conventional causes was also highlighted.

* Corresponding author. Tel.: +20 01005667342.
E-mail address: mmeheissen@hotmail.com (M.A. Meheissen).

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1. Introduction

Infective endocarditis (IE) is an uncommon but serious and often life-threatening condition. The overall incidence of IE has remained relatively stable for about 3.6–7.0 cases per 100,000

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patient-years.[1] Despite the introduction of improved, often automated, blood culture systems and the development of improved culture media, studies have indicated that this approach fails to identify an etiologic agent in 2.5–31% of cases. These negative cultures result in the classification of such cases as blood culture-negative infective endocarditis (CNIE).[2] The proportion of CNIE varies from country to country and among different centers in the same country. These variations reflect the local epidemiology of IE, diagnostic criteria used, initiation of antibiotics in patients prior to obtaining blood cultures, and the diagnostic protocol used to establish an etiology.[3] This situation is a major problem for the choice of the optimal antibiotic regimen, which should be as specific and efficient as possible for this life-threatening disease. In view of a changing epidemiology and a growing number of resistant strains, the correct diagnosis of IE-causing pathogens has become even more important. Furthermore inadequate therapy carries the risk of relapse or re-infection after heart valve replacement, with a poor prognosis.[4]

Two main reasons account for CNIE. First, in many critical clinical situations, empirical antibiotic therapy is administered prior to diagnosis or even before the patient is suspected of having IE. Blood cultures drawn under antibiotic treatment have a significantly lower sensitivity. Second, fastidious or yet uncultured microorganisms that are regularly missed by routine culture methods may be involved in CNIE cases.[2]

Considerable efforts have been made to establish the etiological diagnosis of CNIE. PCR amplification of universal rRNA loci and specific gene targets for bacteria has been done in blood, as well as in valve material from patients with CNIE.[5] The main limitation of studies performed in heart valves is that they are profitable only for patients needing surgery. Theoretically, the ideal rapid diagnosis of CNIE by broad range PCR would be performed in circulating blood, including that of patients with IE who do not require valvular surgery.[6] However, to date, the studies performed on blood samples are scarce and their utility has been hampered by low analytical sensitivity and the presence of contaminant DNA in PCR reagents.[7–9]

Serological tests are now included as diagnostic criteria for IE in the modified Duke criteria.[10] Testing for CNIE includes the systematic determination of antibody titers against *Coxiella burnetii*, *Bartonella* spp., *Chlamydia* spp., *Brucella melitensis, Mycoplasma pneumoniae,* and *Legionella pneumophila* which are among the most common pathogens in CNIE. However, in other instances, one must be cautious when relying solely on serologic methods to diagnose endocarditis because of cross-reactions.[2]

This study was conducted to estimate the prevalence of CNIE among definite IE cases in Alexandria Main University Hospital, to describe and analyze clinical data from CNIE patients and to diagnose the microbial etiology of CNIE by using molecular and serological techniques.

2. Patients and methods

Sixty-five patients admitted to the Cardiology as well as to the Cardiothoracic Surgery Departments of Alexandria Main University Hospital (AMUH), with definite IE according to modified Duke criteria were enrolled in a prospective, observational study starting from January 2010 through December 2010. The study protocol was approved by the Ethics Committee of AMUH. Informed written consent was obtained from all study subjects before enrollment in the study.

2.1. Data collection

A structured sheet was used to collect the following clinical, laboratory and echocardiographic data of the patients: name, age, gender, type of work (contact with animals), predisposing and triggering factors, history of previous attacks, clinical manifestations and complications, the affected valve, echocardiography results, patient outcome, and history of previous antibiotic intake.

2.2. Sample collection

Three blood samples were withdrawn simultaneously from all patients under strict aseptic technique; 10 ml blood in Oxoid signal blood culture system for blood culture (Three blood cultures were taken for each patient separated by at least one hour), 3 ml blood in EDTA tubes for molecular testing and 3 ml blood in plain tubes for serology. Bottles and tubes were rapidly delivered to the Microbiology laboratory of AMUH where Oxoid signal blood bottles were incubated at 37 °C, EDTA tubes were stored at 4 °C till DNA extraction (maximum 72 h after specimen collection) and plain tubes were used to separate serum samples which were then stored at −20 °C. Blood culture was carried out according to manufacturer's instructions and bacterial identification was performed according to standard microbiological procedures.[11]

2.3. Molecular tests from whole blood samples for CNIE

2.3.1. Broad range bacterial 16S rRNA PCR assay[12,13]

DNA was extracted from whole blood with EZ-10 spin column genomic DNA minipreps kit, Blood, BS483 (Bio Basic Inc., Canada) according to the manufacturer's instructions.

Five microliter of DNA extract was amplified in a 25 ml volume PCR reaction using the oligonucleotide primer set DG74:5′-AGG AGG TGA TCC AAC CGC3-′ and RW01: 5′-AACTGG AGG AAG GTG GGG AT3-′. These broad range PCR primers are located at the nucleotide positions 1522–1540 and 1170–1189 within the *Escherichia coli* 16S rRNA genes respectively. The PCR reaction consisted of 12.5 μL DreamTaq™ Green PCR Master Mix (2X) (Fermentas), 30 pmol DG74 and RW01 each, and PCR grade water to a final volume 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 60 °C for 30 s, extension at 72 °C for 30 s and final extension at 72 °C for 10 min. (Techne Genius, Cambridge, UK). The PCR products were electrophoresed through a 2% (w/v) molecular biology grade agarose gel (Conda, Pronadisa) in Tris–Borate EDTA buffer, pre-stained with ethidium bromide (0.5 mg/ml) for 45 min. Molecular sizes of the amplicons were determined by comparison to a 100–1000 bp DNA ladder. A positive result was inferred by detection of 371 bp band.

An internal control was added into each 16S rRNA PCR vial. BGF: 5′-CAACTTCATCCACGTTCACC-3′ and BGR: 5′-GAAGAGCCAAGGACAGGTAC-3′ in a concentration

of 30 pmol/μL were used to amplify 268 bp fragment of the human β-globin gene.

Positive controls for Gram positive bacteria (*Staphylococcus aureus*), Gram negative bacteria (*Escherichia coli*), and positive blood sample taken from a patient with *S.* aureus endocarditis, as well as, an extraction negative control (distilled water instead of blood sample) and master mix negative control (distilled water instead of DNA template) were also included into every PCR reaction. These controls enabled monitoring of PCR performance and specificity, as well as checking for crossover and carryover contamination.

The sensitivity of the broad range PCR assay, including the efficiency of DNA extraction, was tested using blood samples spiked with known concentrations of *S. aureus* (*S.* aureus) determined using a modification of the Miles and Misra technique. Tenfold serial dilution was done till reaching a concentration of one CFU of bacteria per 1 mL of blood. After DNA extraction and PCR, agarose gel electrophoresis was used to establish the lowest number CFU/ml of blood that is required to generate a band visible by ethidium bromide staining.[14]

The occurrence of false-negatives due to the presence of inhibitory substances in the whole blood extract was investigated by performing PCR amplification on PCR-negative template samples inoculated with staphylococcal DNA extract. The PCR-negative samples were 'spiked' with 5×10^4 CFU DNA extract per 25 μL PCR reaction. Amplification was performed by the standard PCR protocol. PCR inhibition was indicated by the failure of PCR to generate an amplicon from template spiked with bacterial DNA extract.[12]

2.3.2. Seminested PCR assays[15]

Five microliter of each broad range 16S rRNA PCR product was further identified in separate amplification reactions using the genus or species specific primers of *Streptococcus* spp., *S. aureus* and coagulase negative *Staphylococcus* (CONS) spp. The primers used were initially designed and used as probes by Greisen et al.[16] They anneal to genus or species-specific internal sequences within the 371-bp region of the detected bacteria obtained using universal primers RW01 and DG74. For *Streptococcus* spp., the primer pair 5′-AAC TGA GAC TGG CTT TAA GAG ATT A-3′ and RW01 was used. These primers flank a 132-bp region within the 16SrRNA gene of *Streptococcus* spp. For *S. aureus,* the primer pair 5′-GCC GGT GGA GTA ACC TTT TAG GAG C-3′ and DG74 was used. These primers flank a 105-bp region within the 16SrRNA gene of *S. aureus*. For CONS, the primer pair 5′-CGA CGG CTA GCT CCA AAT GGT TAC T-3′ and RW01 was used. These primers flank a 297-bp region within the 16SrRNA gene of CONS. The reaction conditions were the same as those used in the 16S rRNA PCR.

2.4. Serological tests from serum samples of CNIE cases[3]

Serum samples were subjected to serological assays to detect specific antibodies to *Coxiella burnetii*, *Bartonella henselae* and *Bartonella Quintana* using immunofluorescence assay (IFA) (Vircell, Spain), and to *Brucella* spp. by BRUCELLAC-APT (Vircell, Spain) according to the manufacturer's instructions. The cutoff titer to diagnose IE was 1/800 for phase I *C.* burnetii IgG and for *Bartonella* IgG, while that for *Brucella* was 1/160.

2.5. Laboratory processing of valve tissue samples of CNIE cases[17]

Excised valvular material was aseptically divided for further analysis in the operating theater by the surgeon: (1) A sample for PCR analysis and valve staining in a DNA-free and sterile container without any additive. The sample was frozen within 45 min at −20 °C till DNA extraction. (2) The remaining tissue was aseptically transferred to a bottle containing 50 ml thioglycollate broth (Oxoid, UK) and transferred as soon as possible to the microbiology laboratory for incubation at 37 °C.

2.5.1. Direct smears and valve cultures

A smear of each valve tissue sample, after grinding, was prepared and stained using Gram stain. The section of heart valve tissue that was transferred to a sterile bottle containing 50 ml of thioglycollate broth was incubated for 3 weeks at 37 °C. Subsequently subcultures were done on day 2, 7 and 21 onto a range of microbiology media, including blood agar, chocolate agar (Columbia agar base supplemented with 5% defibrinated blood, Oxoid), and blood agar inoculated in its middle with *S. aureus*. These plates were incubated for 18 h in a 5% enriched CO_2 at 37 °C. Subculture was also done on MacConkey's agar incubated aerobically at 37 °C, fungal media (Sabouraud dextrose agar, Oxoid) incubated aerobically at 30 °C. Subculture was done on one chocolate agar plate and the plate was incubated anaerobically for at least 48 h at 37 °C. All isolates were identified by morphology and standard microbiological methods.[11]

2.5.2. Molecular tests from valve tissues

Native valve tissue samples (a piece of the valve, vegetation, or abscess) were cut into small pieces using a sterile scalpel blade, homogenized in a sterile physiological solution, and a 200 μl aliquot of this suspension, was purified on a spin column (QIAamp blood kit; Qiagen, Hilden, Germany). Prosthetic valves were carefully scraped with a scalpel and then vigorously washed in a sterile physiological solution. The suspension obtained was then subjected to DNA extraction procedure using a spin column (QIAamp blood kit; Qiagen, Hilden, Germany). Broad range bacterial 16SrRNA and seminested PCR assays were performed with blood samples.[12,13]

3. Results

3.1. Clinical and demographic data

Patients were considered to have definite CNIE if the results of standard blood cultures were negative and if clinical and echocardiographic findings met the modified Duke Endocarditis. Among the 65 definite IE cases, 39 (60%) patients had one major and three minor criteria and were classified as definite CNIE.

The mean age of CNIE patients was 31 years. Male to female ratio was 2.9:1. Endocarditis was considered nosocomial healthcare-associated IE (HCA-IE) when developed in a patient hospitalized for more than 48 h prior to the onset of signs and symptoms consistent with endocarditis. Non-nosocomial HCA-IE was defined as endocarditis with the onset of specific symptoms within 48 h of admission in a patient with health

care contact.[18] HCA-IE accounted for six (15.4%) episodes (one nosocomial and five non-nosocomial). Native valve IE (NVE) accounted for 26 (66.7%) cases, while prosthetic valve IE (PVE) constituted 12 (30.8%) cases and intracardiac devices one (2.6%) case. The mitral valve was the most frequently involved, affecting 22 (56.4%) cases, followed by the tricuspid and aortic valves affecting eight (20.5%) and six (15.4%) cases respectively. Combined aortic/mitral valve affection was seen in two (5.1%) cases. One patient (2.6%) was on a pacemaker. The most common underlying heart disease was rheumatic heart disease (RHD), which was responsible for 64.1% (25 cases) of all CNIE, followed by non-rheumatic valvular heart diseases and mitral valve prolapse (MVP) (2 cases each) 5.1% each, and one congenital heart disease (2.6%). Two patients (5.1%) experienced a previous IE attack. Intravenous drug abuse (IVDA) accounted for eight cases (20.5%). There was no predisposing heart disease or previous risk factor in only one patient (2.6%). All CNIE patients (100%) had received a previous antibiotic course before being admitted to our hospital. (Figs. 1 and 2).

The mean time between onset of symptoms and diagnosis of IE was 39 days (total range 3–210 days). All symptoms and signs were nonspecific and occurred in a significant number of patients. Fever was the most common symptom (100%) at initial evaluation. Other major symptoms were dyspnea, fatigue, arthralgia, headache, chills, anorexia, weight loss and low back pain. The most frequent sign found at the initial examination was heart murmur (84.6%). Anemia was present in 95% of patients, and the inflammatory parameters (ESR, CRP) were constantly elevated. Peripheral signs like splinter hemorrhage, clubbing of the fingers, Janeway lesion and Osler's node were not observed. Complications were found in 17 (43.6%) patients. Manifestations of heart failure were found in 11 patients (28.2%). Major embolic events (cerebral, pulmonary and peripheral) were present in 12 patients (30.8%). One patient had periannular abscess and another patient had renal failure. Some patients presented with more than one complication. The endocarditis associated mortality was observed in five (12.8%) CNIE. The major causes of mortality included neurological and cardiovascular complications.

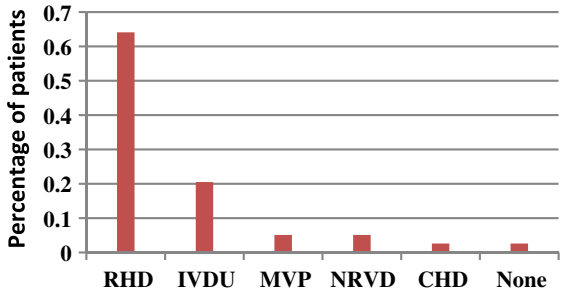

Figure 1 Predisposing factors in CNIE patients. (RHD: rheumatic heart disease, CHD: congenital heart disease, MVP: mitral valve prolapse, NRVD: non-rheumatic valvular disease, IVDU: intravenous drug user).

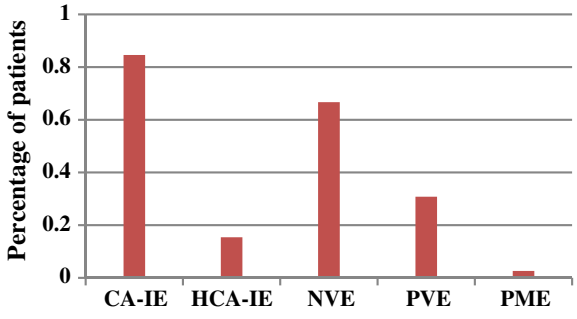

Figure 2 Type of IE in CNIE patients. (CA-IE: community acquired IE, HCA-IE: health care acquired IE, NVE: native valve endocarditis, PVE: prosthetic valve endocarditis, PME: pacemaker endocarditis).

3.2. Microbiological results

Out of 39 CNIE patients, 29 whole blood and serum samples were collected from 29 CNIE patients and 10 valve tissue samples were procured during valve replacement, corresponding to 10 surgically treated patients. Six surgically treated patients had corresponding blood and serum samples and four patients had only valve specimens but confirmed results of negative blood cultures, collected before surgery, were evaluated retrospectively in these four patients. So, out of the 39 CNIE patients, 35 blood and serum samples as well as 10 valvular samples were collected.

3.2.1. Whole blood PCR results

3.2.1.1. Broad range bacterial 16S rRNA PCR results. PCR amplification of 16S rRNA showed negative results with all tested whole blood samples. No inhibition of PCR was detected; β-globin amplicons were obtained in all cases (Fig. 3).

The amplification sensitivity assay using whole blood spiked with S. aureus demonstrated that 2.5×10 bacteria per

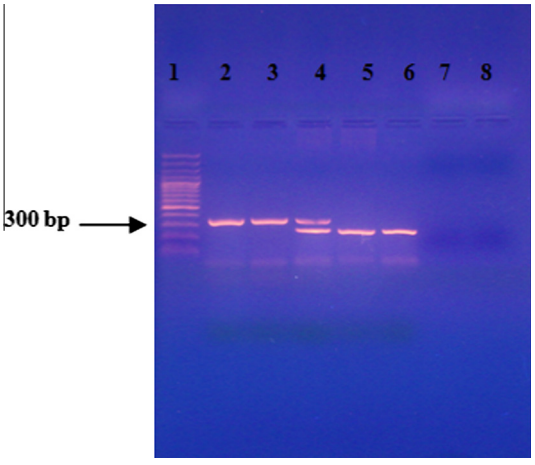

Figure 3 Agarose gel electrophoresis for whole blood broad range 16S rRNA. A band of 371 bp corresponds to 16S rRNA DNA and a band of 268 bp corresponds to internal control β-globin DNA. Lane 1: molecular size DNA marker (100–1000 bp); lane 2: *S. aureus* ATCC 25923 positive control (371 bp); lane 3: *Escherichia coli* ATCC 25922 positive control (371 bp); lane 4: positive blood sample control (268 and 371 bp); lane 5 and 6: negative blood samples results (268 bp); lane 7: extraction negative control; lane 8: master mix negative control.

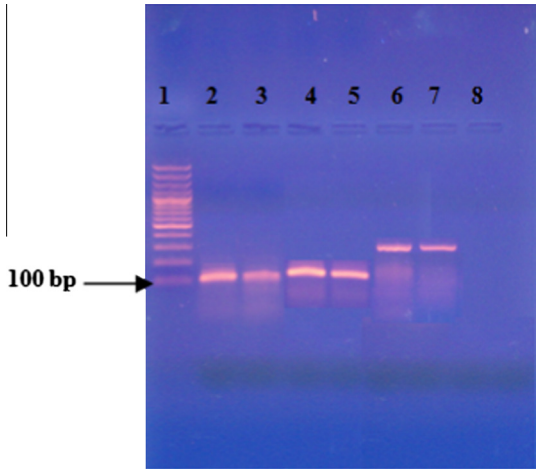

Figure 4 Agarose gel electrophoresis for whole blood seminested PCR. A band of 105, 132, and 297 bp corresponds to *S. aureus*, *Streptococcus* spp. and CONS spp. DNA respectively. Lane 1: molecular size DNA marker (100–1000 bp); lane 2: *S. aureus* ATCC 25923 positive control (105 bp); lane 3: *S. aureus* positive sample (105 bp); lane 4: *S. pneumoniae* ATCC 49619 positive control (132 bp); lane 5: *Streptococcus* spp. positive sample (132 bp); lane 6: *Staphylococcus epidermidis* ATCC 12228 positive control (297 bp); lane 7: CONS positive sample (297 bp).

1 mL of spiked whole blood were required to generate a detectable PCR amplicon. No false negatives were detected due to the presence of inhibitory substances in whole blood extract as proved by positive amplification of all negative PCR samples spiked with staphylococcal DNA.

3.2.1.2. Seminested PCR results. The semi-nested PCR assay detected DNA from 12 out of 35 blood specimens (34.3%). Identified bacteria were as follows: Eight (66.6%) *S. aureus,* two (16.7%) CONS and two (16.7%) *Streptococcus* spp. (Fig. 4) The combined results of PCR assays applied on CNIE blood specimens succeeded to identify the causative agent of CNIE in 12 out of 35 patients (34.3%). Thus, the percentage of CNIE patients with no identified etiology dropped from 60% to 41.5% by considering the PCR results.

3.2.2. Serology results

A positive reactive *Bartonella* IFA test result was observed for three patients. One patient had *Bartonella henselae* IgG titer $\geqslant$ 1:3200 and *Bartonella quintana* IgG titer equals to 1600, another patient had *B.* henselae IgG titer $\geqslant$1:3200 and *B.* quintana IgG titer equals to 400, a third patient had *B.* henselae IgG titer $\geqslant$1:3200 while *B.* quintana IgG titer was negative. We considered the three patients as possible *B.* henselae endocarditis. Another patient had a typical profile of Q fever endocarditis (Phase I: IgG $\geqslant$ 3200; IgM 48 and IgA $\geqslant$ 192 /Phase II: IgG $\geqslant$ 3200; IgM negative and IgA $\geqslant$ 192) as proved by *C.* burnetii IFA test. A positive *Brucella* serologic result with titers of $\geqslant$5120 was observed for one patient using BRUCELLACAPT agglutination test. Therefore, the combined results of all serological tests applied on CNIE serum specimens succeeded to implicate a causative agent of CNIE in (5/35) 14.3% of patients, decreasing the percentage of CNIE patients with no identified etiology to 52.3%.

3.2.3. Valve tissue testing results

When excluding the two contaminated valves, the sensitivity of different tests used for valve analysis was as follows: valve Gram staining (2/8; 25%), valve culture (5/8; 62.5%), valve 16S rRNA PCR (6/8; 75%). This decreases the percentage of CNIE patients with no identified etiology from 60% to 50.8% (Table 1).

In 59% (23/ 39) of CNIE patients, we were able to identify the bacteria to the species and/or genus level using all diagnostic tools. This was possible by PCR analyses of whole blood samples, serological testing, culturing materials obtained from cardiac valves and PCR analyses of valves in 12/35, 5/35, 5/8 and 6/8 cases, respectively. An etiologic agent could not be determined for 16 (41%) of CNIE cases (Table 2). Thus, the combined results of all diagnostic tools used in our study, decreased the percentage of non-identified causes of CNIE from 60% to 24.6%.

4. Discussion

In our study, the mean age of CNIE patients was 31 years which reflects the effect of rheumatic fever and its sequelae on the heart. The same young mean age was also observed in other CNIE studies.[19,20] In contrast with CNIE reports from Europe in which CNIE cases occurred in patients over the age of 50 years.[5,21] Male: female ratio was 2.9:1. Male sex predilection has been observed in previous CNIE studies.[19–21]

In the present study, 15.4% of cases were HCA-IE. HCA-NVE currently accounts for more than one third of all cases of NVE in non-injection drug users throughout much of the world.[22] Our CNIE group included a large proportion of PVE (30.8%) in agreement with the study of Rizk et al.[20] which reported a similar proportion (37.7%). The commonest valve affected was the mitral valve (56.4%) which is likely due to the predilection of mitral valve by rheumatic heart disease which represented the majority of our patients. Right-sided IE affecting the tricuspid valve accounted for 20.5% of our patients, it has been suggested that a significant increase in the proportion of right-sided IE in our study is related to IVDA. According to our study, RHD remains the most common underlying heart disease as indicated by a positive past history in 64.1% unlike western countries where the incidence of rheumatic fever has decreased[23,24] MVP has been reported as a common underlying heart lesion in western studies[23,25] but constituted only a small fraction in our study (5.1%). The proportion of CNIE patients without previously known cardiac disease or risk factor was 2.6% only. This is in contrast to other studies[26,27] where half of their cases belonged to this category. Antibiotic treatment preceded blood culture in 100% of our CNIE episodes. It differs from other studies that report on the previous antibiotic treatment in 45–50% of episodes.[21,28]

The mean duration elapsed between the beginning of the clinical picture and the definite diagnosis of IE was 39 days which is longer than earlier reports studying CNIE.[21] Among the presenting symptoms, fever was observed in all patients (100%), in contrast to previous CNIE studies where fever was not present in all cases.[5,19] When comparing our CNIE group with other CNIE series[5], our rate of heart failure was less (28.2% vs 50%); while the rates of major embolic events (cerebral, pulmonary and peripheral) were almost similar (30.8% vs 29%).

Table 1 Results of the ten operated CNIE patients.

Case no.	Type of IE	Valve gram stained smear	Valve culture	Valve broad range 16SrRNA PCR	Valve seminested PCR	Blood broad range 16SrRNA PCR	Blood seminested PCR	Serology
1	PVE (MV)	Negative	CONS	Positive	CONS	Negative	Negative	Negative
2	NVE (MV)	Gram + ve cocci	*Viridians streptococci*	Positive	*Streptococcus* spp	No blood specimen	No blood specimen	Negative
3	PVE (MV)	Negative	Negative	Negative	Negative	Negative	Negative	Negative
4	PVE (MV)	Negative	*Viridians streptococci*	Positive	*Streptococcus* spp	No blood specimen	No blood specimen	Negative
5	NVE (AV)	Negative	Contaminated	Not done	Not done	Negative	Negative	Negative
6	NVE (MV)	Negative	Negative	Positive	*Streptococcus* spp	Negative	Negative	Negative
7	PVE (MV)	Negative	Negative	Negative	Negative	No blood specimen	No blood specimen	Negative
8	PVE (AV)	Negative	CONS	Positive	CONS	Negative	Negative	Negative
9	NVE (MV)	Negative	Contaminated	Not done	Not done	Negative	Negative	Negative
10	NVE (TV)	Gram + ve cocci	MRSA	Positive	S. aureus	No blood specimen	No blood specimen	Negative

NVE: native valve endocarditis, PVE: prosthetic valve endocarditis, MV: mitral valve, AV: aortic valve, TV: tricuspid valve.

Blood culture was negative in 5–90% of IE patients in previous studies.[29–33] We reported a high rate of CNIE (60%) in our study as reported from developing countries. We realize that the main reason of negative blood cultures in our study was prior administration of antibiotics, so that the introduction of an automated blood culture system in our hospital is mandatory for proper diagnosis of IE.

In the present study, the 16S rRNA PCR assay was negative with all blood samples. The negative result obtained could be due to the presence of a lower number of microorganisms in the sample or, probably, due to failure to detect bacteria present in a sample at a level below the sensitivity of the conventional PCR method. We tried to determine whether false-negative results could have been avoided by analyzing multiple samples, larger blood volumes, larger DNA extract or even increasing the number of PCR cycles, but this was not successful. On the contrary this resulted in false positive results (amplification of environmental contaminants; *Bacillus* spp.) confirmed by sequencing. Specimen storage might be an alternative explanation. Some of our sample extracts were stored for six months till PCR was performed. Fenollar et al.[34] reported that the sensitivity of their PCR assay was strongly affected by serum storage at −20 °C, most likely through the progressive degradation of frozen DNA. The inhibition of the PCR reaction was previously reported by Goldenberger,[35] as a potential cause of negative PCR result. However, in the present study, we applied all measures (internal β-globin gene, positive controls) to exclude inhibition of PCR reaction, also, no false negatives, due to the presence of inhibitory substances in blood, were detected as proved by positive amplification of all negative PCR samples spiked with staphylococcal DNA.

Fournier PE et al.[36] reported that the PCR output varied depending on specimens. When applied to blood, 16S rRNA amplification exhibited a poor sensitivity, with 35 positive specimens of 257 tested (13.6%). Valvular biopsies, when available, proved to be of great diagnostic value, the 16S rRNA PCR assays of valves allowed the detection of a microorganism in 150 out of 227 patients (66.1%). Similarly, in the study of Casalta et al.,[37] among the 20 CNIE patients previously treated with antibiotics, the SeptiFast test has detected only three bacteria. Based on the results of the previous studies and taking into consideration that we tested a conventional PCR and not a real time assay, the negative results of our 16S rRNA PCR assay are not surprising.

The sensitivity of the different PCR assays was different among studies. In the study of Ley et al.,[14] the PCR methods detected 4.3×10^3 colony-forming units (CFU) of *E.* coli per mL of spiked whole blood. Casalta et al.[37] reported that the analytic sensitivity of the SeptiFast assay was tested using bacterial dilutions of 100, 30, and 3 CFU/ml in EDTA blood. A minimum sensitivity of 30 CFU/ml was obtained for most the species. Our 16S rRNA PCR detected 2.5×10 CFU of *S. aureus* per mL of spiked whole blood. Although successful amplification of the PCR product after spiking bacteria in the blood demonstrates the sensitivity of the assay, this does not guarantee reproducible amplification of the pathogen DNA in true IE bacteremia, in which the number of organisms tends to be low with the previous antibiotic intake.

We included specific primers for *S. aureus,* CONS and *Streptococcus* spp., the most encountered bacterial etiological agents of CNIE due to previous antibiotic treatment, in a seminested PCR assay using the same 16S rRNA primers in

Table 2 Identified pathogens obtained with different diagnostic tests in 39 CNIE patients.

	Whole blood broad range PCR	Whole blood seminested PCR	Serology	Valve culture	Valve broad range PCR	Valve seminested PCR
S. aureus ($n = 9$)	–	8	ND	1	1	1
CONS ($n = 4$)	–	2	ND	2	2	2
Streptococcus spp ($n = 5$)	–	2	ND	2	3	3
Coxiella burnetii ($n = 1$)	–	ND	1	ND	–	ND
Bartonella henselae/*B. quintana* ($n = 3$)	–	ND	3	ND	–	ND
Brucella spp. ($n = 1$)	–	ND	1	–	–	ND

–: negative, ND: not done.

order to increase the sensitivity of the assay and to detect the species of the causative organism in one step. The same assay was previously tested on cerebrospinal fluid samples for the diagnosis of bacterial meningitis.[15,16] The seminested PCR assay allowed the diagnosis of 12 out of 35 CNIE (34.3%). To the best of our knowledge this is the first time to apply a seminested PCR assay on whole blood samples for the diagnosis of CNIE.

In the present study, etiologic agents identified by serology constituted 12.8% of identified causes of CNIE. *Bartonella* was implicated in 7.7% of CNIE cases, while *C*. burnetii and *Brucella* represented 2.6% of CNIE cases each. Our results were consistent with the results of Cairo University[20] which reported 53 CNIE cases with 9.4% of cases caused by *Bartonella* and *C. burnetii*. Moreover, 10% of cases of IE in Tunisia were caused by *C*. burnetii and *Bartonella*.[38] Our findings are not in agreement with the results the French National Reference Center for Rickettsial Diseases study[39] which reported 348 cases of CNIE with 48% of cases caused by *C. burnetii* and 28% by *Bartonella*. Also, in Algeria, *C. burnetii*, *B. melitensis*, and *B.* quintana constituted one quarter of the performed diagnoses.[40] In our study, the low prevalence of *Coxiella* and *Bartonella* could be attributed to differences in living conditions, or animal exposure, or availability of louse borne infections in our country.

Comparative results from recent studies using molecular approach indicate the clear superiority of valve material PCR over conventional valve culture and microscopy.[41] This is in agreement with our results where the sensitivity of valve PCR (75%) exceeded that of valve culture (62.5%) and valve microscopy (25%). An explanation for the negative PCR results in the two CNIE valve cases might be that only parts of the infected heart valve tissue was subjected to DNA extraction. It is therefore possible, that the actual infected site was not analyzed as the two patients had prosthetic valves. On the other hand, we reported one CNIE case positive by valve PCR for *Streptococcus* spp. while negative by valve culture; this could be attributed to previous antibiotic therapy which inhibited the growth of fastidious streptococci. Similarly, Bosshard et al.[42] found that the results of PCR for three patients were positive, even though the results of microscopic examination, and culture of endocardial specimens were negative.

An unexpected finding of previous CNIE studies was the identification of patients with non-infective endocarditis associated with cancers and autoimmune diseases.[36,43,44] In our study we did not evaluate the presence of non-infective endocarditis and it is possible that some of the 16 CNIE cases with unidentified etiology belong to this category. Further, previous authors reported that truly negative IE is far less frequent than initially expected.[5,39] We could not estimate the percentage of truly CNIE cases in our study, as we did not search for all possible etiological agents of CNIE identified either by serology (for example Legionella, Mycoplasma, Chlamydia), or PCR analysis (enterococci, HACEK, Gram negative bacilli, fungi).

5. Conclusions

CNIE may be a diagnostic dilemma but in many cases the causative pathogen can be determined. Proper collection of blood culture before starting antibiotics, as well as, the use of an automated blood culture system will improve etiological diagnosis of IE. PCR offers a very good opportunity to diagnose CNIE from blood samples especially in case of previous antibiotic therapy. The results obtained by seminested PCR should be acceptable as a starting point for optimization of the method for diagnosis of CNIE. Serum samples should always be tested for antibodies to fastidious organisms, and excised valves or vegetations should undergo microscopy, culture, and relevant PCR analysis.

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