VERSITA
DOI: 10.2478/rrlm-2013-0005

Original article

Contributions of ENDOBACT multicentric study to the infective endocarditis etiology in Romania

Contribuții ale studiului multicentric ENDOBACT la precizarea etiologiei endocarditei infecțioase în Romania

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Abstract

The multicenter ENDOBACT study aimed at implementing molecular methods for identification of bacterial species encountered in infective endocarditis, and at attempting to reduce the number of cases with unknown etiology. For eighty seven cases was established a diagnosis of definite infective endocarditis. Thirty two of these cases had negative blood cultures. For nine cases out of 32, valve pieces were available and an attempt was made to identify the etiological agent by molecular techniques. Thirty seven available isolates were identified by phenotypical and molecular comparative methods: 16S rRNA (all available isolates), rpoB (staphylococci, streptococci and enterococci), sodA (streptococci and enterococci) genes sequencing. For eight isolates, the comparative results were discrepant. Species identification of one coagulase negative staphylococcal strain was assigned using molecular methods. Molecular identification methods applied here might represent an added value for clinical and conventional microbiological diagnosis of infective endocarditis in Romania.

Keywords: *infective endocarditis, ENDOBACT, DNA sequencing*

Rezumat

Studiul multicentric ENDOBACT a avut ca scop implementarea metodelor moleculare de identificare a speciilor bacteriene implicate in endocardita infecțioasă, precum şi încercarea de a diminua numărul cazurilor cu etiologie neprecizată. Din 87 de cazuri diagnosticate ca endocardita infecțioasă definitivă, 32 de cazuri au

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prezentat hemoculturi negative. Pentru nouă din aceste cazuri, s-au prelevat fragmente de ţesut valvular şi s-a încercat identificarea agentului etiologic din acestea, aplicand tehnici moleculare. Treizeci şi şapte izolate bacteriene au fost identificate simultan, prin metode fenotipice şi moleculare: secvenţierea genelor ARNr 16S (pentru toate izolatele), rpoB (pentru tulpini de stafilococi, streptococi şi enterococi) şi sodA (pentru tulpini de streptococi şi enterococi). Pentru opt izolate bacteriene, rezultatele obţinute prin cele două tipuri de metode nu au coincis. Identificarea unei tulpini de stafilococ coagulază negativ a putut fi completată prin aplicarea metodelor moleculare. Metodele moleculare de identificare aplicate în acest studiu, ar putea reprezenta o valoare adaugată pentru diagnosticul clinic şi microbiologic convenţional al endocarditei infecţioase practicat în România.

Cuvinte cheie: *endocardita infecţioasă, ENDOBACT, secvenţiere ADN*

Received: *19th December 2012;* ***Accepted:*** *26th April 2013;* ***Published:*** *15th June 2013.*

Introduction

Infective endocarditis (IE) is defined as an infection of the endocardial surface of the heart. It is a serious and evolving disease with a persistently high morbidity and mortality, in spite of progress regarding infectious diseases and cardiovascular pathology. The investigation and management of IE in the developed world have changed permanently since the non-invasive imaging, molecular science, diagnostic protocol and curative surgery are all available. However, the incidence remains unchanged, about 1.7 – 6.2 cases per 100 000 patients-year (1-3).

Development of endocarditis is initiated by injury of the endothelium, which disrupts the normal valve structure and exposes underlying tissues. Deposition of host proteins, such as fibrin and platelets leads to the formation of a sterile thrombotic vegetation. Pathogens circulating in the bloodstream as a result of transient bacteremia bind to this coagulum and activate monocytes to produce cytokines, resulting in progressive enlargement of infected vegetation. After valve colonization, the infecting microorganisms become fully enveloped by vegetation. Under this protection, they can survive and avoid host defenses (2).

Infection of the cardiac valves or mural endocardium is caused mainly by bacteria (streptococci, staphylococci, enterococci and less commonly, rickettsiae, chlamydiae, mycoplasmas, etc.), fungi and viruses (4).

The diagnosis of IE requires a multifaceted approach involving clinical examination and laboratory investigation, traditionally microbiological analysis of blood cultures.

As the antimicrobial treatment is essential for therapy, a correct identification of causative bacteria is very important for choosing the appropriate antibiotic.

Conventional phenotypical identification techniques do not always lead to an accurate etiological diagnosis (5). In the genomic era, sequencing of 16 rRNA gene is a widely accepted tool for identifying bacterial isolates. The diagnostic utility of broad range 16S rRNA PCR has been already demonstrated in patients with culture-positive and culture-negative endocarditis (4, 6-10). However, the discriminative power of 16S rRNA gene sequencing, especially for staphylococci, streptococci and enterococci species is limited (11-13). For differentiating such species, alternative targets can be used, namely genes with a higher variability of nucleotide sequences than 16S rRNA gene. Partial sequencing of *rpoB* (encoding-highly conserved β subunit of the bacterial RNA polymerase) and *sodA* (encoding manganese-dependent superoxid dismutase) genes proved to be useful tools for species identification in *Staphylococcus*, *Streptococcus*, *Enterococcus* and other related genera (11, 14, 15).

Blood culture negative endocarditis (BCNE) represents a well-known clinical problem occurring in large range of patients (2.5 - 31%) among IE cases (7, 8, 16, 17). Blood sampling after previous antibiotic treatment, and/or the presence of fastidious microorgan-

isms (e.g. Gram negative bacilli belonging to HACEK group, nutritionally deficient streptococci, intracellular bacteria) are listed among the causes of blood culture negative cases. An important amount of BCNE may be due to Q fever endocarditis, determined by a chronic infection with *Coxiella burnetii* (16).

For surgically treated patients with BCNE, molecular analysis of resected valves proved to be very valuable when fastidious microorganisms are suspected or the patients were previously treated with antibiotics. Bacterial DNA could be extracted directly from anatomic pieces and taxonomic identification assessed by 16S rRNA gene sequencing (7, 9, 17).

In our country, we confront with a scarcity of data regarding the major microorganisms involved in endocarditis etiology. Only few, local studies are available (18-21).

The ENDOBACT project was initiated by a group of researchers working in research-development field, public health system and academic domain. Cardiologists, infectious disease specialists, cardiac surgeons, biochemists and microbiologists created a complex partnership in order to determine the prevalent bacterial causes of IE in Romania, thus diminishing the number of cases without defined etiology.

Material and methods

Patients

ENDOBACT study was carried out from January 1, 2009 until April 15, 2011. Patients were enrolled in four major cardiovascular clinics in Romania. Written informed consent of all patients was obtained and for each patient an information sheet was completed by the physician in charge, with relevant epidemiologic, clinical, echocardiographic and biological data.

Duke's modified criteria were used to classify cases as possible or definite IE (22). When the study ended, all the cases were reanalyzed and only definite IE cases were taken into account for further studies.

Laboratory procedures

Phenotypic conventional methods

Phenotypical methods were carried out at the local laboratories of centers involved in the study.

For each patient at least three pairs of blood cultures (one set including an aerobic and an anaerobic blood culture bottle) were performed in local laboratories. Seven to ten milliliters of blood samples were inoculated in BacT/ALERT standard aerobic and anaerobic blood culture bottles (BioMerieux Inc., Durham, USA). For samples collected from patients on antibiotic treatment, charcoal containing media (FAN aerobic and anaerobic bottles) were used. Bottles were incubated in the BacT/ALERT system for 3 weeks before final negative results were reported. Pathogens from positive bottles were cultured on standard media and identified using API strips (BioMerieux Inc., Durham, USA) or Vitek2 Compact system (BioMerieux Inc., Durham, USA), according to the available infrastructure of the participating laboratories.

Heart valves excised from operated patients were transported from surgery to the local laboratory in sterile dry containers and divided into two parts under laminar flow hood. One piece of abnormal tissue was cultured on enrichment nutrient broth and incubated for a maximum of 7 days with subsequent recultivation. The remaining samples were stored at -70-80°C before DNA extraction for molecular study.

The isolates and valve pieces were transported to the coordinator of this project, "Cantacuzino" NIRDMI for further molecular investigations.

Molecular methods

Available isolates identified by conventional phenotypic methods were subjected also to molecular identification. Additionally, samples of abnormal valves were prepared for bacterial DNA extraction and identification.

DNA extractions were performed using NucleoSpin Tissue commercial kit (Macheray

Nagel, Germany) or PureLink Genomic DNA Mini kit (Invitrogen CA, USA), according to manufacturer's instructions. DNA extracted both from bacterial isolates and directly from valvular fragments was used in PCR reactions.

PCR amplifications. 16S rRNA gene*:* the universal eubacterial forward primer Ad and reverse primer rJ were used for this purpose, to amplify the 1500 bp gene (23). PCR mixtures were subjected to a denaturation step of 94°C for 4 min, 35 cycles of denaturation at 94°C for 1 min, primer annealing at 49°C for 1 min and de novo DNA extension at 72°C for 1 min, final elongation step of 72°C for 7 min.

sodA gene: the degenerate primers d1 and d2 were used to amplify an internal fragment, representing approximately 83% of the *sodA* gene of streptococci species. The above program was slightly modified (annealing at 37°C - 1 min) (15).

rpoB gene: for staphylococci strains primers 2491F and 3554R were used to amplify a 1.081-bp variable fragment in *rpoB* gene (52°C annealing – 45 s) (14). For streptococci: primer pair 2333F and 3073R amplified a 700 bp product (50°C annealing - 30 s) (11).

spa gene: for amplification of the *Staphylococcus aureus* protein A variable repeat region, primers spa-1113f and spa-1514r were used for amplification. Thermal cycling reactions: 1 cycle – 80°C (5 min); 35 cycles – 94°C (45 s), 60°C (45 s), 72°C (1,5 min); 1 cycle – 72°C (10 min) (24).

PCR amplifications were performed in a volume of 50 µl in GeneAmp PCR system 2700 termocycler (Applied Biosystems CA, USA) using High Fidelity PCR Master kit (Roche Diagnostics GmbH, Mannheim, Germany). PCR products were checked by electrophoresis in agarose gel and visualized by ethidium bromide staining. Wizard SV Gel and PCR clean-up system (Promega Corporation, Madison, WI, USA) was used for amplicons purification.

DNA sequencing of purified PCR products.

- 16S rRNA gene: five sequencing primers (3 forward: Ad, E, D, and 2 reverse: rJ, rE) were used.
- *sodA* gene: both strands of the amplified product were sequenced using the primers d1 and d2.
- *rpoB* gene: for staphylococci isolates only 598 bp from amplified 1.081 bp fragment were sequenced using 2643F and 3241R primers. For streptococci isolates, the amplification primers were used also for DNA sequencing.
- *spa* gene: DNA sequences were obtained using spa-1113f and spa-1514r primers.

Primers used for amplifications and sequencing are listed in *Table 1*.

Sequencing reactions were performed using ABI Prism BigDye Terminator V3.1 Ready Reaction Cycle Sequencing Kit (Applied Biosystems CA, USA) as described by the manufacturer, in 20 µl reaction volume. All the sequencing reaction products were run on ABI 3100 Avant Genetic Analyzer (Applied Biosystems CA, USA).

All molecular procedures were carried out in separate rooms to avoid contamination events.

The resulting sequences were edited and assembled using BioEdit Sequence Alignment Editor v7.0.9.

For 16S DNA sequencing, the contiguous sequences obtained were compared with those available from NCBI GenBank (http://blast.ncbi.nlm.nih.gov/), Ribosomal Database Project II (http://rdp.cme.msu.edu/) and BLAST Server for the Identification of Prokaryotes (http://bioinfo.unice.fr/blast/).

spa-type sequences were analyzed by Ridom StaphType software (Ridom GmbH, Germany) and synchronized with Ridom *spa* server (http://spaserver.ridom.de/) (24, 25).

Phylogenetic analysis of *rpoB* and *sodA* loci was used to assign species designation for streptococcal strains. Neighbor-joining trees were constructed using MEGA version 4 software package (26) and DNA sequence databases from http://viridans.eMLSA.net or from www.immi.au.dk/service/download/kilian.

All molecular procedures were performed at the "Cantacuzino" NIRDMI and each partner from the consortium was informed about the results.

Table 1: Primers used for amplification and sequencing

Target gene	*Primer*	*Gene position (GenBank accession no.)*	*5' – 3'direction*	*References*
16S rRNA	Ad (PCR and sequencing)	8 – 28 (X80724)	AGAGTTTGATCMTGGCTCAG	Weisburg *et al*., 1991
	rJ (PCR and sequencing)	1510 – 1492 (X80724)	GGTTACCTTGTTACGACTT	
	D (sequencing)	519 – 536 (X80724)	CAGCAGCCGCGGTAATAC	
	E (sequencing)	787 – 806 (X80724)	ATTAGATACCCTGGTAGTCC	
	rE (sequencing)	787 – 806 (X80724)	GGACTACCAGGGTATCTAAT	
sodA (streptococci)	d1 (PCR and sequencing)	25 - 51 (Z95896.1)	CCITAYICITAYGAYGCIYTIGARCC	Poyart *et al.*, 1998
	d2 (PCR and sequencing)	487- 510 (Z95896.1)	ARRTARTAIGCRTGYTCCCAIACRTC	
rpoB (staphylococci)	2491F (PCR)	2491 – 2511 (X64172)	AACCAATTCCGTATIGGTTT	Drancourt & Raoult, 2002
	3554R (PCR)	3554 – 3573 (X64172)	CCGTCCCAAGTCATGAAAC	
	2643F (sequencing)	2643 – 2660 (X64172)	CAATTCATGGACCAAGC	
	3241R (sequencing)	3221 – 3241 (X64172)	GCIACITGITCCATACCTGT	
rpoB (streptococci)	2333F (PCR and sequencing)	2333 – 2363 (AF535182)	AARYTIGGMCCTGAAGAAAT	Drancourt *et al.*, 2004
	3073R (PCR and sequencing)	3050 – 3073 (AF535182)	TGIARTTTRTCATCAACCATGTG	
spa (staphylococci)	spa -1113f (PCR and sequencing)	1092 - 1113 (J01786)	TAAAGACGATCCTTCGGTGAGC	Harmsen *et al.*, 2003
	spa -1514r (PCR and sequencing)	1534 – 1514 (J01786)	CAGCAGTAGTGCCGTTTGCTT	

Results

Patients

One hundred and two patients were enrolled in this study. Fifteen patients were excluded because they did not meet the diagnostic requirements for definite IE or received other final diagnosis. Only the remaining 87 cases of definite IE were considered for further studies and discussions.

Among the definite cases, 58 were male; mean age of the patients was 55.2 years (range 21 - 79). Fever occurred in 86 cases. Thirty-six patients received antibiotic treatment in the last week before blood samples were taken, 33 patients did not received antibiotics and for 18 patients status was unknown. Transthoracic and/or trans-esophageal echocardiography was/were performed on all patients and showed vegetations in 77 (88.5%) patients. Ve-

Table 2. Strain identification by conventional methods and gene sequencing

Final identification (no. of isolates)	*Identification according to following test methods:*			
	Conventional[*]	*16S rRNA*	*rpoB*	*sodA*
Gram positive bacteria (n = 33)				
S. aureus (4)	*S. aureus*	*S. aureus*	*S. aureus*	-[**]
Staphylococcus epidermidis (5)	*S. epidermidis*	*S. epidermidis*	*S. epidermidis*	-
Staphylococcus hominis (2)	*S. hominis*	*S. hominis*	*S. hominis*	-
Staphylococcus haemolyticus (1)	**CNS**[***]	*S. haemolyticus*	*S. haemolyticus*	-
Kocuria kristinae (1)	***Staphylococcus hominis***	*K. kristinae*	-	-
Streptococcus mitis (1)	*S. mitis/oralis*	*S. mitis /oralis*	*S. mitis*	*S. mitis*
Streptococcus parasanguinis (1)	***Streptococcus anginosus***	*S. parasanguinis*	-	*S. parasanguinis*
S. gallolyticus ssp. *gallolyticus* (5)	*S. gallolyticus* ssp. *gallolyticus*	*S. gallolyticus* ssp. *gallolyticus*	*S. gallolyticus* ssp. *gallolyticus*	*S. gallolyticus* ssp. *gallolyticus*
S. gallolyticus ssp. *pasteurianus* (1)	*Streptococcus pasteurianus*	*S. gallolyticus* ssp. *pasteurianus*	*S. gallolyticus* ssp. *pasteurianus*	*S. gallolyticus* ssp. *pasteurianus*
Streptococcus. equi (1)	***Streptococcus dysgalactiae* ssp. *equisimilis***	*Streptococcus equi*	*S. equi*	*S. equi*
Streptococcus sp. (1)	***S. sanguinis***	*Streptococcus* sp.	*Streptococcus* sp.	*Streptococcus* sp.
Granulicatella adiacens (1)	*G. adiacens*	*G. adiacens*	-	-
E. faecalis (7)	*E. faecalis*	*E. faecalis*	*E. faecalis*	*E. faecalis*
Enterococcus gallinarum (1)	***Enterococcus casseliflavus***	*E. gallinarum/ E. casseliflavus*	*E. gallinarum*	*E. gallinarum*
Brevibacterium casei (1)	***Corynebacterium* sp.**	*B. casei*	-	-
Gram negative bacteria (n = 4)				
E. coli (1)	*E. coli*	*E. coli*	-	-
E. cloacae (1)	*E. cloacae*	*E. cloacae*	-	-
Acinetobacter sp. (1)	***Alcaligenes faecalis***	*Acinetobacter sp.*	-	-
Pseudomonas fluorescens (1)	***P. aeruginosa***	*P. fluorescens*	-	-

[*] Boldface type indicates misidentification or ambiguous (incomplete) identification by conventional methods.
[**] not done
[***] coagulase-negative staphylococci

getations were present on the mitral valve (26 native, 12 prosthetic) in 38 (43.7%) patients, on the aortic valve (20 native, 4 prosthetic) in 24 (27.6%) patients, on the tricuspid native valve in two cases (2.3%) and in 13 cases (14.9%) more than one valve or a valve and another cardiac structure were involved. Surgical treatment of IE was performed in 27 cases (31%). In-hospital mortality occurred in 9 cases (10.3%).

Laboratory procedures

Fifty-five out of 87 patients with definite IE had positive blood cultures. In thirteen

cases out of 55, patients received antibiotic treatment before the blood-cultures were taken, for 17 patients this data were not available. When the blood cultures were negative, in 23 out of 32 cases, patients received antibiotic therapy prior blood samples collection.

Twelve tissue fragments with vegetations on the native valves (6 mitral, 3 aortic, 1 tricuspid) or on the mechanical prosthesis (1 mitral and 1 aortic) recovered from 10 patients were available for culturing and molecular testing. In two cases both mitral (natives) and aortic valves (1 native and 1 prosthetic) were available. Eleven valves were recovered from patients with BCNE and one from a patient with positive blood cultures. For two BCNE cases, both mitral (natives) and aortic valves (1 native and 1 prosthetic) were available. Culturing of available valve tissues did not lead to recovery of etiological agents.

Blood culture positive cases

Bacteria recovered from blood cultures and identified by phenotypic methods were: staphylococci (20 strains), streptococci (16 strains), enterococci (9 strains), other Gram-positive bacteria (3 strains), Gram-negative bacteria (3 strains) and in three cases co-infection occurred. Regarding these last three cases, bacteremia occurred at short time (less than 2 months) after cardiac interventions: two patients received pacemakers and one patient a biological prosthesis, and were considered health-care associated infections.

Only 37 out of 57 strains recovered from definite IE blood cultures were available for molecular identification.

Results obtained by phenotypic methods were in accordance with molecular identification results for 28 bacterial strains (*Table 2*). Similarity with sequences from databases was equal or more than 99%.

Conventional species identification disagreed with gene sequencing results for 8 isolates. All those isolates were phenotypical identified by Vitek 2, except *Corynebacterium* sp/ *Brevibacterium casei*, when specific ANC Vitek 2 card was missing. Molecular identification completed the initial diagnostic for one staphylococcal strain, identified by Vitek 2 only as coagulase-negative staphylococcus (*Table 2*). For three strains another genus was assigned by gene sequencing methods *(Staphylococcus/ Kokuria; Corynebacterium/ Brevibacterium; Alcaligenes/ Acinetobacter).* The five isolates phenotypically identified as *Streptococcus gallolyticus* ssp. *gallolyticus* and one isolate identified as *S. gallolyticus* ssp. *pasteurianus* demonstrated concordance with all three gene targets in molecular identification. All but one streptococcal strain were assigned with species names on the basis of positions on phylogenetic *rpoB* and *sodA* trees. One isolate, phenotypically identified as *Streptococcus sanguinis,* did not fall within known species clusters and had a <99% 16S rRNA sequence similarity with more type strains from mitis group streptococci (*Streptococcus pseudopneumoniae/ Streptococcus mitis/ Streptococcus pneumoniae/ Streptococcus oralis*). A phylogenetic sequence analysis of more housekeeping genes or others methods are necessary in order to assign a species name for this isolate.

In one case, an appropriate piece of valve was available and was analyzed by PCR for bacterial detection. The same *S. aureus* strain recovered from blood was detected in heart valve. Isolates identity was verified by *spa* typing, both isolates sharing the same *spa* type - t284. Additionally, for other three available *S. aureus* strains a *spa* type was assigned: t005, t5891, t5890.

Blood culture negative cases

When conventional techniques did not led to etiological agent identification, the laboratory diagnosis was based on valve tissue analysis for bacterial DNA detection.

Following the DNA extraction from the eleven valvular fragments, four positive PCR amplifications for 16S rRNA bacterial gene were obtained. Using 16S rRNA sequencing, *S. gallolyticus* ssp. *gallolyticus* was identified in both mitral and aortic native valves from one patient. For another two BCNE cases, etiologic-

al agents identified from resected valves were *S. gallolyticus* ssp. *gallolyticus* and, *S. aureus*, respectively. These results, obtained by 16S rRNA sequencing using universal primers, were also confirmed by *rpoB* and *sodA* sequencing for streptococci and *rpoB* for staphylococci.

Discussion

This study aimed at implementing molecular methods for identification of any bacterial species encountered in IE, and to attempt reducing the number of cases with unknown etiology. Therefore, in this study were involved specialists from clinical and laboratory domains, in order to cover all the scientific and logistic requirements.

Positive blood cultures remain the cornerstone of diagnosis and provide live bacteria for susceptibility testing. Phenotypic identification tests for bacteria are inherently subjected to problems: variability of phenotypic expression within a given species, the corresponding databases are not up-to-date, results rely on individual expertise and interpretation and sometimes, alterations in the execution of an assay may give false test results. Due to these limitations, molecular identification could be a helpful alternative in some cases (4, 6-10). However, molecular biology techniques are not fully available to clinical laboratories and are more laborious and expensive than the conventional identification techniques. Also, contamination of samples with foreign DNA can be problematic when the different steps can't be performed in different areas.

In this study, available isolates were identified by phenotypic and molecular comparative methods (16S rRNA, *sodA* and/or *rpoB* genes sequencing).

Molecular identifications were performed to (sub)species level. There are studies that clearly show that species or even subspecies identification may have substantial impact on the management of patients (4). This is the case of *S. gallolyticus* ssp. *gallolyticus* versus other subspecies. The first one is often accompanied by colonic tumours, while ssp. *pasterianus* and ssp. *macedonicus* are less frequently associated with colonic disorders (27-29).

In some studies, up to 25% of the initial microbiological diagnoses were corrected using the mentioned molecular techniques (30, 31).

The requirement for a unique identification method was not established in the study design and can be considered as a weakness of the study; nevertheless, discrepancies between the identification results obtained by molecular and phenotypic techniques were observed. Comparative identification provided here demonstrated that molecular identification was more accurate than classical phenotypic identification in nine cases (eight misidentifications, one incomplete identification) and, when possible or needed, this approach could provide the alternative of an appropriate identification, which can be useful in patients therapy management.

A big concern at the beginning of the study was the high number of BCNE, often delaying etiological diagnosis and the initiation of treatment, with profound impact on clinical outcome. Antibiotic therapy for these cases is based on empirical treatment. The initial choice of empirical treatment depends on several considerations including knowledge of local epidemiology and specific culture-negative pathogens (32).

In the ENDOBACT study, the diagnostic strategy for BCNE included serological testing of fastidious agents, but the methods and results for *C. burnetii*, *Bartonella quintana* and *Bartonella henselae* detection are presented in the article of Cotar *et al*., 2011 (33).

Supplementary, sera from patients with known etiology were tested in order to detect possible co-infections (Cotar, unpublished data). The corresponding sera for patients with positive blood cultures exhibited also IgG phase I antibody for *C. burnetii* in four cases, with titers compatible with endocarditis (Cotar, unpublished data). Co-infections with *C. burnetii* occurred for patients with positive blood cultures for *S. aureus*/*Stenotrophomonas malto-*

Table 3. Causative bacteria in IE – ENDOBACT study (no. of isolates)

Staphylococci (18)	**Streptococci (20)**	**Enterococci (8)**	**Other Gram positive bacteria (3)**	**Gram negative bacteria (10)**	**Co-infections (7)**
S. aureus (8)	*S. gallolyticus* ssp. *gallolyticus* (9)	*E. faecalis* (7)	*G. adiacens* (2)	*C. burnetii* (8)	*C. burnetii* + *S. gallolyticus* ssp. *gallolyticus* (1)
S. epidermidis (5)	*S. gallolyticus* ssp. *pasteurianus* (1)	*E. gallinarum* (1)	*G. sanguinis* (1)	*P. fluorescens* (1)	*C. burnetii* + *S. aureus* (1)
S. hominis (2)	viridans streptococci (6)			*E. coli* (1)	*C. burnetii* + *S. maltophilia* (1)
other CNS (3)	other streptococci (4)				*C. burnetii* + *S. constellatus* ssp. *faryngis* (1)
					E. cloacae + *Acinetobacter* sp.(1)
					S. haemolyticus + *B. casei* (1)
					E. faecalis + *K. kristinae* (1)

philia/ *Streptococcus constellatus* ssp. *faryngis* or *S. gallolyticus ssp. gallolyticus*.

These findings suggest that systematic serological testing for this fastidious bacterium in each case of possible or definite IE could be useful. In common practice, clinicians consider serological testing only when the blood cultures are negative. Raoult *et al.* (34) strongly recommend performing both blood cultures and serological tests at the same time. This approach could shorten the time until initiation of appropriate therapy, the length of hospitalization and, in some cases, could detect poly-microbial endocarditis. A recent study performed in the Netherlands, also revealed the usefulness of systematic serological testing for detecting poly-microbial endocarditis. This approach is recommended especially in areas where Q fever is endemic (35).

Another strategy applied in order to minimize the number of BCNE was molecular analysis of valves with vegetations excised from patients who underwent surgical interventions. Thirty- eight patients had indication for cardiac surgery, but only 27 suffered the intervention.

Only 12 pieces from 10 patients were available for molecular study. Using universal primers for eubacteria a confirmatory identification of *S. aureus* IE was obtained in one case with positive blood cultures. Also, the causative agents in 3 cases with previously unknown etiology were identified: two *S. gallolyticus* ssp. *gallolyticus* and one *S. aureus*. These pathogens are usually easily culturable, but in our cases, all 3 patients received antibiotics prior blood culturing.

The high sensitivity of 16S rRNA PCR technique allows the detection of few or non-viable bacteria in valve tissue. But this issue can be a source of false-positive results, when a small amount of bacterial DNA contaminated culture media, reagents or sample during collection and processing.

As other authors suggested (5, 7, 10), 16S rRNA gene sequencing directly from valve tissue extracted DNA may improve microbiological diagnosis in surgically treated patients with IE, but the results should always be interpreted with cautions.

The causative bacteria of IE detected in ENDOBACT study by phenotypic, serologic and/or molecular testing were listed in *Table 3.*

Several studies indicate that *S. aureus* rate in IE is increasing, especially because of nosocomial acquisitions and drug consumption (38-40). In ENDOBACT study, the *S. aureus* strains were fewer than coagulase negative staphylococci and streptococci. Vegetations (usually with large size) occurred for each patient, in eight cases on native valve, in one case on prosthetic valve. For three strains nosocomial acquisition may be suspected, following gastrointestinal, ophthalmological or dental interventions. No drug consumers were detected in our study group. All available *S. aureus* strains were detected methicillin-sensitive, by PCR targeting *mecA* gene, unlike coagulase-negative staphylococci (CNS) which were all methicillin-resistant (Oprea, unpublished data).

CNS and viridians streptococci endocarditis occurred mostly on the prosthetic devices (70% for staphylococci and 100% for streptococci). Half of the *Enterococcus faecalis* endocarditis involved prosthesis and nosocomial acquisition is suspected in one case when bacteremia with *E. faecalis* appeared at short time after patient received a mechanical valve.

Of note it is the increased number of *S. gallolyticus* ssp. *gallolyticus* isolates. It is estimated that between 18 and 62% of the patients with *S. gallolyticus* ssp. *gallolyticus* endocarditis have underlying colorectal tumors, sometimes with no signs or symptoms referable to gastrointestinal disease. Many studies have been conducted to elucidate this relation (29, 41-43). In a recent study, authors consider that *S. gallolyticus* ssp. *gallolyticus*, based on his pro-inflammatory potential and pro-carcinogenetic properties is most probably responsible for slow progressing carcinogenesis of colorectal mucosal tissues (27). Also, this bacterium is associated with liver diseases or dysfunction (44, 45).

Two from our *S. gallolyticus* ssp. *gallolyticus* patients had already known colonic neoplasia, and one had chronic liver disorders. The physicians in charge to monitor the others were advised to perform a more rigorous control. The early detection of colorectal adenomas or carcinomas via detection of *S. gallolyticus* ssp. *gallolyticus* might be of high value in screening high risk groups for colorectal cancer.

Aerobic Gram negative bacilli are rare causes of IE. We report 3 community acquisitions of *Pseudomonas fluorescens*, *S. maltophilia* and *Escherichia coli*. The poly-microbial Gram negative endocarditis *Enterobacter cloacae* + *Acinetobacter* sp. is probably of nosocomial origin, after a recent pacemaker implantation.

The remaining cases without defined etiology can be attributed to fungal, viral infections, other fastidious bacteria non - tested here and maybe non-infective endocarditis (8). In some cases, administration of antimicrobial agents to IE patients before blood cultures had reduced the chances of bacteria recovery.

It is worth noting that not all of the excised valves in BCNE cases were received for molecular testing. As is a proven fact that is possible to identify the causative bacteria from cardiac tissue with vegetations by PCR targeting 16S rRNA gene, surgeons must better collaborate with laboratory staff in order to improve the findings related to valve pieces.

Overall, the Romanian panel of causative agents of IE is similar with that reported by studies from other countries (36-38), with a high prevalence of staphylococci and streptococci, as presented in *Table 3*.

Conclusion

When phenotypical identification was complemented by molecular and serological testing in IE diagnosis, the percentage of IE cases with unknown etiology decreased from 36.8% to 24.1%. Additionally, in four cases the diagnosis was modified from single causative agent infection to co-infection, after identification of significant levels of IgG phase I to *C. burnetii* along with the bacterial species recovered from blood culture. For nine strains molecular identification changed or completed the initial phenotypic identification.

A better understanding of the spectrum of bacteria causing IE in our country can guide

to the most appropriate empirical treatment in BCNE. Also, a rapid and correct diagnostic will improve the medical act in benefits of patients, resulting in appropriate treatment and shorter stay in the hospital.

We conclude that this study gave a better perspective to investigation and management of IE in Romania.

Acknowledgements

This study was supported by the Romanian Ministry of Education, Research, Youth and Sports, CNMP-UEFISCDI Project PARTENERIATE 42-119/2008, grant awarded to Monica Straut. We kindly thank all the partners in ENDOBACT project and Mrs. Silvia-Mariana Cretoiu which actively participated during the first half of this project.

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