Evaluation of the pathogenicity of microorganisms involved in infective endocarditis by experimental disease

Evaluarea patogenităţii microorganismelor implicate în endocardita infecţioasă experimentală

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Abstract

Infective endocarditis (IE) is a condition that became a public health problem, due to changes in its etiologic spectrum and to the involvement of resistant bacterial strains or of strains with increased virulence. The experimental animal model allows the assessment of certain pathologic changes that resembles those associated to this infection in humans. The aim of this experimental study is the evaluation of the virulence of the main bacterial species isolated from hospital and community and of the mechanical risk factors in IE pathogenesis. Induction of cardiac lesions in rabbits required surgery, consisting in placing a polyurethane catheter through the carotid artery. The working steps are described in detail. For inoculation we used Staphylococcus aureus strains, Enterococcus spp. strains and viridans group streptococci. Most animals with lesions induced by cardiac catheterization developed endocardic vegetations. S. aureus strains induced the most intense colonization, followed by Enterococcus faecalis strain and Enterococcus faecium strain. The average weight of vegetations was higher for E. faecalis than for S. aureus or E. faecium. Viridans group streptococci did not induced IE. Non-catheterized but inoculated animals did not develop endocarditis lesions. The mechanical irritation of the heart valve is a trigger factor in the pathogenesis of infective endocarditis, the most intense colonization of vegetation being caused by S. aureus, followed by Enterococcus spp.

Keywords: *endocarditis, experimental disease, colonization*

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Rezumat

Endocardita infecţioasă (EI) este o afecţiune care a redevenit o problemă de sănătate publică, datorită modificării spectrului etiologic al acesteia şi datorită implicării etiologice a unor tulpini bacteriene multirezistente sau cu virulenţă crescută. Modelul animal experimental permite aprecierea evoluţiei bolii în mod similar cu boala de la om. Scopul lucrării este evaluarea prin intermediul bolii experimentale a virulenţei principalelor specii bacteriene izolate din mediu spitalicesc şi comunitar, precum şi a factorilor de risc mecanici în patogeneza EI. Inducerea leziunilor cardiace la iepure a necesitat o intervenţie chirurgicală, constând din introducerea prin artera carotidă a unui cateter de poliuretan. Etapele de lucru sunt descrise în detaliu. Pentru inoculări s-au folosit tulpini de Staphylococcus aureus, tulpini de Enterococcus spp. şi streptococ grup viridans. Majoritatea animalelor cu leziuni endocardice induse prin cateterizare au dezvoltat vegetaţii cardiace; S. aureus a indus colonizarea cea mai intensă, urmat de Enterococcus faecalis şi Enterococcus faecium. Greutatea medie a vegetaţiilor a fost mai mare în cazul S. aureus decât în cazul E. faecalis sau E. faecium. Streptococii grup viridans nu au produs EI. Animalele inoculate dar necateterizate nu au dezvoltat leziuni endocardice. Iritaţia mecanică a valvelor cardiace reprezintă un factor trigger în patogeneza endocarditei infecţioase, colonizarea cea mai intensă a vegetaţiilor fiind produsă de S. aureus, urmat de Enterococcus spp.

Cuvinte cheie: *endocardita, boala experimentală, colonizare*

Introduction

Infective endocarditis (IE) is a microbial infection of the endocardium, in which the mortality remains high despite of the improvements in diagnostic methods, medical and surgical therapy.

Two decades ago, the incidence of IE was declining, but nowadays this condition remerges as a public health problem, both due to changes in the etiologic spectrum and due to etiological involvement of resistant bacterial strains or with increased virulence.

The microbiology of IE varies, depending on the site of the disease (native or prosthetic valve), but also on the nosocomial context of the disease. *Staphylococcus aureus*, *Streptococcus* spp. and *Enterococcus* spp. are responsible for more than 80% of IE cases (1,2). Over 75% of nosocomial IE are caused by staphylococci. In these cases, the ratio between the involvement of *S. aureus* and coagulase-negative staphylococci is 3:1 (3). In IE correlated with invasive procedures, the most frequently identified pathogens are *S. aureus* associated with procedures involving the skin and soft tissue and *Enterococcus* spp. following interventions on urogenital and gastrointestinal tract (4).

Both the changes in patient care techniques and the changes in the microbial resistance spectrum due to excessive antibiotic use led to an increased incidence of *S. aureus* bacteraemia in hospitalized patients (5,6,7). The increasing number of infections with Gram-positive cocci might be the result of excessive administration of broad spectrum antibiotics in association with the use of vascular catheters and various prosthetic materials.

Aim of the study

One of the main purposes of the study is to evaluate the involvement of the heart valves irritation (due to mechanical or due to a marked pathogenicity of infectious agents) as a trigger factor in the pathogenesis of infective endocarditis by using the experimental disease.

A second objective followed the pathogenicity of different bacterial species involved in IE (*Staphylococcus* spp., *Enterococcus* spp. and viridans group streptococci); we focused mainly on the idea that the *S. aureus* strains involved in IE (isolated from IE patients) or the *S. aureus* strains with known pathogenicity factors are more virulent than the control strain of *S. aureus* ATCC 29213.

Materials and methods

The working protocol has been approved by the Ethics Committee within the University of Medicine and Pharmacy Tg. Mureş. The experimental animal model chosen for this study was the

New Zealand rabbit, males weighting between 2000-4000 grams. In this study we used two working groups: a first group of rabbits with induced mechanical valve lesions and a second one without valve lesions. In each group, the bacteraemia was simulated by intravenous inoculation of different bacterial strains: strains isolated from patients with infective endocarditis, strains with increased virulence which may cause valve damage without a substrate of mechanical injury, or strains isolated from the community respectively collection strains with known potential to cause endocarditis.

Induction of cardiac lesions in rabbits required surgery, during which a polyurethane 20G arterial catheter (0.6 mm internal diameter, 0.9 mm external diameter, and 80 mm length) was placed through the right carotid artery into the left side of the heart.

Surgical intervention to place catheters

General anaesthesia was achieved by intramuscular injection of Xylazine (0.25 g/kg) and Ketamine (0.35 g/kg). The anesthetized rabbit was tied in dorsal position on the surgical table, with the paws in the extension.

The surgical field was thoroughly prepared, requiring the complete removal of the fur achieved by using depilatory cream, which has an antiseptic and astringent action. The skin was then disinfected with povidone iodine solution. To reduce intra and postoperative trauma, additional anaesthesia was performed by local subcutaneous injection with xyline.

A longitudinal skin incision of 2-4 cm was performed, parallel with the right side of trachea. By crossing through the muscles, the vasculonervous package containing the right carotid artery and vagus nerve was revealed. The artery was properly prepared, requiring the complete removal of the perivascular connective tissue, but without damaging the vagus nerve or the adjacent neural fibres. At the distal (cranial) end of the prepared artery, a ligature with non-resorbable thread was set in order to prevent the blood regurgitation from the brain. Another thread was attached to the proximal end of the artery, without tightening (this thread will fix the catheter to be inserted). The arterial wall was punctured with a needle between the two threads. An ideal puncture of the arterial wall is followed by the exteriorization of a small drop of blood at the time of the needle withdrawal. From this point on, the proximal thread must be kept under tension in order to prevent a massive bleeding. Through the puncture, a polyurethane catheter was introduced in the arterial lumen using a metallic guide. The catheter was carefully advanced to the heart with a forceps, until resistance was met; the characteristic pulsations of the heart were felt when the tip of the catheter was next to the cardiac wall. From this point, the catheter was withdrawn 3-5 mm, until its tip reached the right aortic valve leaflets. The catheter was secured by tightening the proximal wire. The insertion and the fixation of the catheter must be done quickly, in order to minimize blood loss. To ensure the best possible fixation of the catheter and to prevent postoperative blood loss, in some cases we opted for mounting a second proximal thread. During the procedures, a constant moistening with sterile saline water is required to prevent the tissue destruction. After checking all the ligatures, the distal end of the catheter was cut with fine scissors as close as possible to the place of insertion.

The surgical field was checked for the presence of any bleeding and the wound was closed in two planes with absorbable threads, mounted simply or in "U" shape. The catheter was left in place up to a week to produce mechanical damage to the endocardium, followed by sterile vegetation development.

Inoculation of bacterial strains

The bacterial suspensions used for the rabbits' inoculation consisted in six different bacterial strains:

- *S. aureus* - ATTC 29213 strain
- *S. aureus* - isolated from a patient with IE
- *S. aureus* - Community strain of methicillin-resistant SCC*mec* type IV, spa type t044, Panton-Valentine Leukocidin (PVL+) producing, isolated from a fatal case of sepsis associated with necrotizing pneumonia (8)

- *Enterococcus faecalis* - ATCC 29212 strain
- *Enterococcus faecium* - ATCC 19434 strain
- *Enterococcus faecium* - isolated from a patient with IE
- Nutritionally variant viridans group streptococcus (*Granulicatella adiacens*) - strain isolated from a patient with IE.

Bacterial suspension was prepared in saline, adjusted to 0.5 McFarland. After local antisepsis of the external ear with iodine solution, the vasodilatation was obtained by alcohol distempering. Using a fine needle syringe, the animals were inoculated with 0.05 ml / kg bacterial suspension in the ear vein.

Animal euthanasia and bacteriological work-up of vegetations

The animals that did not spontaneously die following the infection were euthanized by inhalation of ethyl ether. The chest was opened by left parasternal incision, revealing the heart. The arteries and veins adjacent to the heart were ligated and then were cut. The heart was opened by incision along the aorta and the presence of vegetations and the catheter position were looked for. If the vegetations were present, they were excised using a sterile forceps and a fine scalpel. The collected vegetations were weighted using an analytical balance and homogenized by vortexing in 1 ml sterile saline in the presence of sterile glass beads (for a more effective homogenization of the vegetation in solution). From the homogenized solution, serial dilutions were created. From each dilution, 200 µl were inoculated on blood-agar plates. After 18-24 hours incubation the colonies were automatically counted using the "Flash & Grow" colony counter. Adjustments for dilution, volume and weight were applied, according to the formula below, thereby achieving the degree of vegetation colonization (CFU / g - the number of colony forming units per gram of cardiac vegetation).

$$CFU/g = \frac{\text{nr. of colonies x dilution factor x 1000 x 5}}{\text{vegetation weight (mg)}}$$

* 1000 = adjustment of weight units (from grams to milligrams)
** 5 = adjustment of the inoculation volume

Results

Catheterization followed by bacterial inoculation was performed in 15 rabbits (group 1). Eight rabbits were inoculated with bacterial strains without prior catheterization (group 2).

In group 1 (*Table I*) most of the animals with endocardial lesions induced by catheterization developed cardiac vegetations (*Figure 1*). Out of seven rabbits inoculated with *S. aureus*, 6 cases presented large vegetations with positive cultures; in one case no vegetation was found. Out of six rabbits inoculated with *En-*

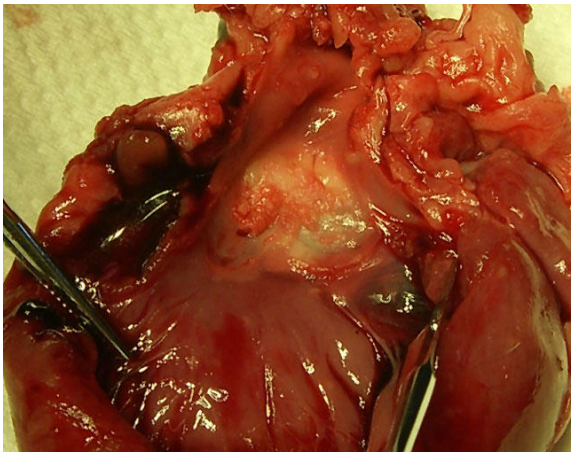

Figure 1. Aortic vegetation

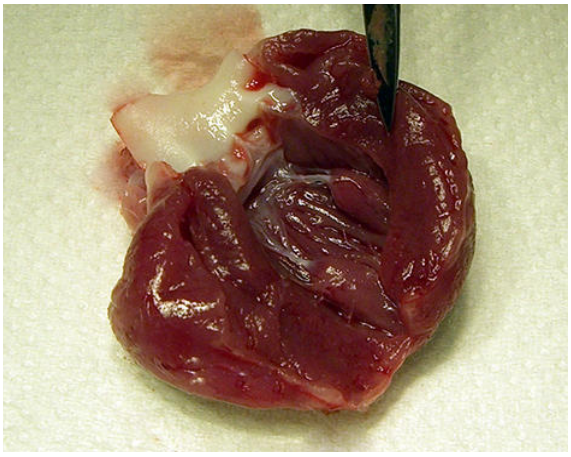

Figure 2. Heart without vegetation

Table I. Catheterized and inoculated rabbits

Inoculated bacterial strain	Days of catheterization	Days of inoculation	Status	Number of vegetations	Vegetation total weight (mg)	CFU/g of vegetation (x 10^4)
S. aureus ATCC	7	3	deceased	3	5.6	714.28
E. faecalis ATCC	7	9	euthanized	3	35	45.71
S. aureus from IE	7	3	deceased	3	27.7	1414
S. aureus PVL+	4	3	deceased	5	39.8	117528.44
E. faecium ATCC*	0	7	euthanized	0	-	-
Granulicatella adiacens	5	7	euthanized	0	-	-
E. faecium from IE	7	6	euthanized	1	0.44	0
S. aureus from IE*	0	14	euthanized	0	-	-
S. aureus ATCC	7	4	deceased	3	6.1	472.13
S. aureus from IE	7	4	deceased	3	35	1536
S. aureus PVL+	7	4	deceased	3	27.2	78607
E. faecalis ATCC	6	8	euthanized	2	21	73.52
E. faecium ATCC	4	7	euthanized	1	12.1	6.77
E. faecium from IE	7	5	euthanized	1	1.32	23.48
Granulicatella adiacens	5	7	euthanized	1	8.5	0

* The catheter was improperly placed and did not reach the endocardial surface.

terococcus spp., five presented vegetations, their cultures being positive in four cases. Viridans group streptococci caused minimal endocardial damage: in one case no vegetation developed, and the second case showed small vegetation, without bacterial growth.

Of the 15 catheterized and inoculated animals, 6 (40%) died spontaneously within 3-4 days after inoculation. In all six cases, the inoculated bacterial agent was *S. aureus*.

Vegetations developed in 12 out of 15 catheterized rabbits. The site of vegetation was different: on the aortic valve (n = 10, 76.92%) and both on aortic valve and mural endocardia (n = 2, 23.08%). In 2 cases no vegetation developed due to an improper placement of the catheter tip.

The average weight of the vegetation was different according to the bacterial species involved (*Table II*). *E. faecalis* produced the heaviest vegetation (average weight 28 mg), followed by *S. aureus* (average weight 23.56 mg) and *E. faecium* (average weight 4.62 mg). Some strains of *S. aureus* with virulence factors such as leukocidin production have formed heavier vegetations. Similarly, the staphylococci isolated from patients with IE formed heavier vegetations than those of the *S. aureus* ATCC 29213.

The colonization degree of the formed vegetation varied by the bacterial species involved (*Table III*). The lowest colonization grade was due to *E. faecium*. *E. faecalis* produced 5.9 times higher colonization degree than *E. faecium*, and *S. aureus* colonization was the most intense, nearly 560 times higher than *E. faecalis*. Among staphylococci, the lowest colonization was produced by the ATCC 25923 strain. The staphylococci isolated from the patient with IE caused a 2.5 times higher colonization than the ATCC strain, and the PVL producing staphylococci produced the most intense colonization (165 times more intense than the ATCC strain).

Table II. The average weight of the vegetations

Inoculated strain	Average weight of vegetations (mg)
S. aureus	23,56
S. aureus ATCC	5,85
S. aureus from IE	31,35
S. aureus PVL+	33,5
E. faecalis	28
E. faecium	8,71

Table III. The average colonization degree of the vegetation

Inoculated strain	CFU/g (average value, x 10^4)
S. aureus	33378.64
S. aureus ATCC	593.20
S. aureus from IE	1475
S. aureus PVL+	98067.72
E. faecalis	59.62
E. faecium	10.08

In non-catheterized group (group 2), the rabbits inoculated with *Enterococcus* spp. and *Granulicatella adiacens* did not developed any symptoms of IE. The autopsy did not reveal any cardiac vegetation (*Figure 2*). Two rabbits inoculated with *S. aureus* (a strain from IE and a PVL+ strain respectively), have died within two days probably due to septic shock, and the autopsy did not reveal any endocardic lesions.

Discussions

Although maintenance and costs associated with the rabbit experimental model represents a certain constrain, we opted for this model at the expense of Wistar rats. Being a larger animal, the cardiac injuries can be easily induced in rabbits (primarily due to the relatively large size of the heart and arteries).

In the IE pathogenesis two important factors are involved: the existence of a substrate for the development of vegetations (usually endothelial lesions of the valves) and a bacteraemia episode (either transient or prolonged) that induces the colonization of the previously formed sterile vegetation. The normal endothelium is resistant to infection, but any mechanical damage generates a local inflammatory process, with deposition of fibrin and platelets, called non-bacterial thrombotic endocarditis, which is the substratum of IE development due to the microbial colonization.

S. aureus is one of the bacterial species with the highest pathogenic potential. In our experiments it caused massive colonization of the endocardial vegetation, inducing septic shock and death in a relatively short time. *S. aureus* has the ability to actively invade the valvular endothelium, inducing apoptosis and emphasizing the local lesions (9).

From the tested staphylococcal strains, those derived from patients with IE and those that harboured virulence factors have produced more intense colonization compared with the ATCC strain. One such case is that of Panton-Valentine leukocidin producing (PVL+) *S. aureus*; the PVL gene is frequently detected in community-acquired methicillin-resistant *S. aureus* (CA-MRSA) strains. These strains are frequently involved in skin and soft tissue infections, but can also cause IE. The possible sources of CA-MRSA are: skin and soft tissue infections (furunculosis, cellulites), osteomyelitis, necrotizing pneumonia and intravenous drug use (10). PVL producing strains have an increased capacity to cause spontaneous and recurrent infections compared to strains that do not produce this

toxin. The nasal carriage of PVL producing *S. aureus* strains (often familial) represents a risk factor for bacteraemia and IE. It seems that PVL producing *S. aureus* is associated with IE in cases where the skin is the portal of entry and not with IE of the intravenous drug users (11).

Huang et al. detected PVL genes in 50% of CA-MRSA isolates (12). The PVL encoding gene can be detected in *S. aureus* strains isolated from hospitals, either as a result of clonal spread of these genes from community, either through horizontal transmission of the prophage (13). The clinical significance of PVL involvement in IE needs further studies. Some authors concluded that CA-MRSA strains carrying *pvl* gene should be considered as potential etiologic agents with high virulence in IE. According to a study that analysed cases of IE caused by CA-MRSA, the suspicion of such IE must be raised in patients with a history of lesions of the skin or soft tissue presenting with tricuspid valve damage (14).

The medical literature describes the ability of *S. aureus* to induce IE on undamaged endocardium (15,16) , but we could not reproduce this by the experimental disease. Neither of our animals without endocardial lesions inoculated with different strains of *S. aureus* has developed vegetations.

It is not known exactly why the staphylococci isolated from patients with IE are able to produce a higher colonization in comparison with the reference strains; this requires further investigations, such as the identification of certain virulence factors or the determination growth rate of these strains. In a recent work the growth rate of the same three species of *S. aureus* that were used within the current study was assessed; the ATCC strain had the slowest growth rate ($16x10^8$ CFU at 4 hours of incubation); the PVL+ *S. aureus* strain had the fastest growth rate ($71x10^8$ CFU at 4 hours), followed by the strain isolated from IE patient ($40x10^8$ CFU at 4 hours) (17).

According to existing data, *E. faecalis* is responsible for the most cases of enterococcal EI. Although *E. faecium* is isolated in many cases too (including patients with nosocomial IE) an association between bacteraemia with this species and its involvement in IE could not be established. This is important because the incidence of *E. faecium* bacteraemic syndromes is increasing and in some centres has become the main isolated species of enterococci (18,19). On the intact endocardium, enterococci are less virulent than other bacteria such as *S. aureus*, the mortality in these patients being much lower (20). In our study, *E. faecalis* produced colonization of the vegetation at much lower level compared with *S. aureus*; also, the IE had a mild evolution, the maximum duration of survival after inoculation until euthanasia being of 9 days. The virulence factors of enterococci include the Esp extracellular proteins and the aggregation substance (Agg), both being involved in colonization of the host (21).

An experimental study on rats conducted in 2005 (22), has also highlighted the fact that the endocardial lesion is one of the most important factors in IE pathogenesis. The injection of human dental plaque suspension induced IE in rats with endocardial lesions induced by catheterization, *Streptococcus oralis* being isolated from the vegetation. The lesions developed on the aortic valve, inflammatory cells being identified in the aortic region, including the adjacent myocardium. The same results were achieved by injecting a pure bacterial suspension of *Streptococcus oralis*.

Another experimental model was described by Imataka and his colleagues (23), in which the endocardial lesion was achieved by electrical stimulation of the vagus nerve. This study also demonstrated the need for pre-existing endocardial lesions prior to the IE development. Injecting a suspension of viridans group streptococci in rabbits simultaneously with the initialization of electro-stimulation led to IE development in 58.8% of cases. In contrast, delayed injection of bacterial suspension, 14 days after electro-stimulation, induced EI in only 18.2% of

cases. The same study examined the susceptibility to infection according to the involved bacterial species; the viridans group streptococci produced vegetations in 58.8%, *Pseudomonas pseudoalkaligenes* in 46.2% of the cases with lesions induced by electrostimulation, while *Staphylococcus epidermidis* produced no vegetation. Viridans group streptococci did not produce vegetations in animals without induced lesions.

The role of bacterial virulence factors in IE evolution has been assessed in other studies. Takahashi and colleagues (24) have studied the role of sialic acid binding adhesins (present in *Streptococcus gordonii* DL1) in IE pathogenesis using rats with endocardial vegetations induced by catheterization. Strains presenting adhesins had greater capacity to induce specific IE lesions compared with the mutant strains, without adhesins. The colonization degree of the vegetations obtained by inoculation of $5x10^6$ bacteria was also higher for strains with adhesins.

The collagen binding adhesins, present in some staphylococci, seems to have importance in IE maintenance, but instead they have a limited role in the adherence of bacteria to the damaged valves. These results were obtained in a Swedish study (25), in which two groups of rats with endocardial lesions induced by catheterization were inoculated with two strains of *S. aureus*: one that presented adhesins and one without it. One hour after inoculation the degree of colonization was not different between the two bacterial strains; in contrast, 24 hours after inoculation, the degree of colonization was significantly higher ($p<0.001$) in the adhesin positive strain.

In addition to the evaluation of the pathogenicity of microorganisms involved in infective endocarditis, during the experiments we managed to establish a working experimental model that can be used successfully in subsequent studies regarding the pathogenicity of different bacterial strains, the pathogenic mechanisms of IE, the IE risk factors and the therapeutic schemes. The effectiveness of antibiotic susceptibility tests in vitro cannot predict their effectiveness in vivo, and for this reason the experimental disease remains one of the most effective methods for evaluating the antibacterial treatment.

Following the suggestive results obtained with *S. aureus* strains and due the fact that at this moment we didn't manage to use *Enterococcus* spp. in a similar way, we would like that in the near future to complete our study with an *E. faecalis* strain isolated from patient with IE and with *Streptococcus gallolyticus* strains.

Conclusions

- The mechanical irritation of the heart valve is a trigger factor in the pathogenesis of infective endocarditis.
- Left heart catheterization induced the vegetation formation, predominately in aortic valve.
- The most pathogenic bacterial species on the induction of infective endocarditis was *S. aureus*.
- The most intense vegetation colonization was achieved due to *Staphylococcus* spp., followed by *Enterococcus* spp.
- *S. aureus*, both the PVL+ strains and the strains isolated from patients with IE, have an increased colonization capacity of the endocardial vegetations, compared with the ATCC 29213 strain.
- The vegetations caused by PVL+ *S. aureus* and those caused by *S. aureus* strains isolated from patients with EI have a greater size and weight compared to those produced by the ATCC 29213 strain.
- *S. aureus* was the only tested bacterial species that induced spontaneous death of the animal.
- The pathogenicity of bacteria involved in IE is different and it is characteristic to the genus, species and strain.

Acknowledgments. This study was partially within the CNMP "Partnerships in priority areas" grant No. 42-119/2008.

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