Fundació La Marató de TV3
21st SYMPOSIUM
Heart diseases

EXPERIMENTAL AND CLINICAL STUDY OF THE RELATIONSHIP BETWEEN VANCOMYCIN MIC IN METHICILLIN SUSCEPTIBLE *STAPHYLOCOCCUS AUREUS* (MSSA) AND THE PROGNOSIS OF LEFT-SIDED MSSA INFECTIVE ENDOCARDITIS

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1. **Summary study**

In a previous study (Cervera C et al. Clin Infect Dis 2014), from a case series of 93 methicillin-sensitive *Staphylococcus aureus* (MSSA) left-sided endocarditis, results showed that the presence of a vancomycin minimum inhibitory concentration (MIC) equal to 1.5-2 mg/L was associated with a higher mortality even if the patients had been treated with cloxacillin.

1.1 Objectives

The objectives of the study were the following:

1. To validate our findings with 114 cases of MSSA IE from the International Collaboration on Endocarditis (ICE-PCS: 2000-2006).
2. To determine whether a high vancomycin MIC in MSSA strains is associated with specific genetic patterns by analyzing clonal complexes, the agr gene, adhesins and toxins and biofilm formation in MSSA isolates from both cohorts.
3. To describe the natural history of left-sided MSSA IE with high and low vancomycin MICs in the experimental endocarditis model (EE).
4. To evaluate the efficacy of cloxacillin, ceftaroline or daptomycin monotherapies and the combined therapies of daptomycin plus cloxacillin or ceftaroline in the treatment of MSSA experimental endocarditis caused by a strain with a low (<1.5 mg/L) vancomycin MIC and a strain with a high (1.5-2 mg/L) vancomycin MIC.

1.2 Design of the study and methods

1.2.1. Clinical study

Subjects of the study: About 114 cases of left-sided IE caused by MSSA that were treated with nafcillin or cloxacillin and collected prospectively from the International Cohort of Endocarditis (ICE-PCS: 2000-2006) with a 1-year follow up.

Analyzed variables: Demographic and baseline clinical characteristics: age, sex, geographical area, year of diagnosis, Charlson comorbidity index. General features of IE: duration of symptoms, type of IE (native or prosthetic), focus, type of acquisition (community, nosocomial or nosohusial), and presence of complications. Microbiological characteristics: vancomycin MIC, number of positive blood cultures, other cultures. Echocardiographic findings: affected valve, presence of vegetations, size of

vegetations, presence of periannular complications, valvular dysfunction. Treatment: antibiotic and surgical treatment.

Outcome of the episode: Hospital mortality, mortality at 6 months, relapses. Data handling was anonymous with no variables that could identify patients either in our database or in international cohort of endocarditis (ICE). No new patients were included. In both databases we identified all the patients with endocarditis caused by methicillin-susceptible *S. aureus* (MSSA) and with their respective strains stored in our collection and the ICE collection.

Statistical analysis: Categorical variables were expressed as percentages and were compared using the chi-square test or Fisher's exact test. Continuous variables were expressed in means or medians and compared by using Student's t-test or the Mann-Whitney respectively. Survival was analyzed using the Kaplan-Meier method. The graphs were compared using the log-rank test. The correlation rate was calculated using Spearman's rho. To identify the independent variables associated with mortality a multivariable logistic regression was used, including variables with P <.30 in the invariable analysis. A value of bilateral P <.05 was considered statistically significant.

1.2.2. Experimental study

1.2.2.1 Molecular in vitro study: The strains used in the study were 93 MSSA isolates from patients diagnosed with aortic left-sided endocarditis from the Barcelona Hospital Clinic from 1995 to 2014. All the isolates are susceptible to vancomycin (EUCAST MIC ≤ 2 mg/L). Clonality of the strains was studied by the molecular technique Spa and multilocus sequence typing (MLST). Adhesins, toxins, and the agr group was identified by gene screening using the technique of multiple polymerase chain reaction (PCR) and delta-hemolysin for agr operon functionality phenotypic test. To measure the biofilm formation, tests of detection and production of biofilm were carried out.

1.2.2.2. Study of the susceptibility pattern: Determination of the MIC (minimum inhibitory concentration)/MBC (minimum bactericidal concentrations) for vancomycin, daptomycin, cloxacillin, and ceftaroline were carried out by the broth microdilution method following the recommendations of the CLSI (Clinical and Laboratory Standards Institute) and by E-test following the manufacturer's recommendations.

1.2.2.3. In vitro synergy study: Prior to the in vivo study, we selected six MSSA strains with low vancomycin MIC and six MSSA strains with high vancomycin MIC to assess the in vitro activity (synergy and bactericidal activity) of cloxacillin, ceftaroline and daptomycin (alone or in combination with cloxacillin) by time-kill curve methodology. Antibiotics were tested at concentrations equal to ½xMIC and 1xMIC. To perform the test, the recommendations of the American Society for Microbiology were followed. (Knapp C, Moody JA Tests to assess bactericidal activity in HD Isenberg ed. Clinical Microbiology Procedures Handbook Washington DC: American Society for Microbiology, 1992; 5.16:1-33.) From the results obtained in the in vitro study two MSSA strains were selected for the in vivo study: MSSA-673 a high biofilm producer with a MIC vancomycin equal to 0.75 mg/L, clonal complex 30 and agr III; MSSA-236 a low biofilm producer with a MIC vancomycin equal to 1.5 mg/L, clonal complex 45 and agr I.

1.2.2.4 I*n vivo* study: New Zealand white rabbits, 2 kg each, were obtained from San Bernardo Farm, (Tulebras, Navarra).

MSSA Experimental endocarditis natural history: The experimental aortic endocarditis model (see below) was used. The aim of the study was to observe and characterize the course of the infection until the fifth day after the inoculum and to see what differences occur, depending on the infecting strain. For the in vivo monitoring of the disease, positron emission tomography (PET) studies were carried out in the animals. This metabolic imaging technique is based on the intravenous administration of a glucose analogue labeled with a positron emitter (18F-FDG) and in clinical practice has demonstrated its ability to detect endocarditis and foci of infection. In this animal model, this technique can be used to monitor noninvasively the establishment of infection at heart level and to locate septic emboli prior to necropsy. In addition to the qualitative analysis, the technique allows a semiquantitative analysis that quantifies the differences between groups. Four PET scans were made on each animal at the following stages: baseline (day 1, before MSSA inoculation); then 24 h, 48 h and 72 h after the inoculum. To carry out these studies we collaborated with the Research Unit Micro-PET CIMA-CUN from Pamplona. After the death of the animals the necropsy was performed and samples were taken.

- Necropsy and sample taking: After administering a lethal i.v. injection of sodium pentobarbital to the animals, we obtained the vegetations attached to the aortic valve, and samples from the brain, kidney and spleen. The samples obtained were weighed and homogenized in a tissue homogenizer (Stomacher 80; Seward Limited, London, UK). Homogenates were quantitatively cultured onto plates containing Columbia agar with 5% sheep blood. The results were expressed as $\log_{10}$ CFU per gram of tissue.

Evaluation of the efficacy of new antibiotics in MSSA experimental endocarditis (EE) against two strains with different virulence and vancomycin MIC profile:

Pharmacokinetic study: Studies of humanized pharmacokinetics and therapeutic efficacy were carried out in the EE model, applying the human-like model to ceftaroline. For cloxacillin and daptomycin, these studies had already been done. The human-like pharmacokinetics model is based on the administration of decreasing i.v. doses of antibiotics to the rabbits using infusion pumps controlled by a computer. Thus we can simulate in the animal, the human profile of the antibiotic serum levels for a known antibiotic dosage.

Experimental aortic endocarditis model: Experimental aortic valve infective endocarditis will be induced in the rabbits according to the technique described by Garrison and Freedman (Garrison P & Freedman L. Yale J Biol Med.1970;42: 394-410). Briefly, a polyethylene catheter was inserted through the right carotid artery into the left ventricle and kept in place throughout the experiment. One or two catheters were inserted into the inferior cava vein through the jugular vein to administer the test antimicrobials. 24 h after the placement of the intracardiac catheter animals were infected via the marginal ear vein with 1 ml of saline solution containing an ID_{90} (90% infecting dosage) of the selected MSSA strain. A 1 ml sample of blood was obtained 18 h after infection and immediately before the initiation of antimicrobial therapy to confirm the presence of endocarditis. Infected rabbits were randomly assigned to one of the treatment groups. Antimicrobial therapy, administered using the computer-controlled infusion pump system, was initiated 18 h after inoculation and maintained for 48 h. Following 48 h of treatment and 6 half-lives of the antibiotic after ending antimicrobial therapy the rabbits in the treatment groups were euthanized with a lethal i.v. injection of sodium pentobarbital. In the control group animals were euthanized 16 h after infection. The chest cavity was opened, the heart excised and opened, and the

aortic valves were removed aseptically. Aortic valve vegetations were weighed and homogenized. Homogenates will be quantitatively and qualitatively cultured. The bacteria recovered will be retested to confirm their antibiotic MICs.

Antibiotic treatment groups: Two different MSSA strains selected from the previous in vitro study were studied. A strain with vancomycin MIC <1.5 mg/L and a strain with vancomycin MIC >1.5 mg/L.

MSSA strain showing a low vancomycin MIC (<1.5 mg/L): Control group (non-treated); cloxacillin (2 g/4h); daptomycin (10mg/kg/24h); daptomycin (10 mg/kg/24h) + cloxacillin (2 g/4h) or ceftaroline (600 mg/8h).MSSA strain showing a high vancomycin MIC (1.5-2 mg/L): Control group (non-treated); cloxacillin (2 g/4h); daptomycin (10 mg/kg/24h); daptomycin (10 mg/kg/24h) + cloxacillin (2 g/4h) or ceftaroline (600 mg/8h).

Monitoring the resistance to antibiotics: Bacteria recovered were retested to confirm their antibiotic MICs (vancomycin, daptomycin and ceftaroline) by E-test. Population analysis profile was done to detect the presence of resistant subpopulations.

2. Results obtained (ordered according to the objectives)

1. The results of the clinical study are collected in the article published in Clinical Microbiology and Infection (Pericas JM, 2017). The study concludes that in this international cohort of patients with left endocarditis caused by MSSA and treated with beta-lactam antibiotics, the vancomycin MIC phenotype was not associated with a worse clinical prognosis (higher mortality). There were also no differences in genetic virulence patterns, clonal complex, or biofilm formation among strains with a high or low phenotype to vancomycin.

2. The results and discussion of the second objective proposed in this study are collected in the article published in Antimicrobial Agents and Chemotherapy (Pericas JM, 2020). The study shows that in the strains of the study no differences were found among the virulence genes, clonal complex or differences in the biofilm formation related to the vancomycin MIC. So the obtained results did not explain the worse

prognosis of MSSA endocarditis that was presented in the previous study (in the patients who had endocarditis caused by strains of MSSA with a high MIC to vancomycin Cervera et al. Clin Infect Dis 2014).

3. The results of the third objective of the study were presented at the 2018 SEIMC scientific meeting, obtaining one of the best oral communication awards (six prizes were awarded in this category). See conference presentations in bibliography section 4 below. The corresponding manuscript is currently being prepared.

This study concluded that although mortality was significantly higher in the group of animals infected with the MSSA strain with the high phenotype MIC of vancomycin, the phenotype of vancomycin (high or low) did not influence the concentration of bacteria in valve vegetations or extracardiac spread of infection in the experimental MSSA endocarditis model in the rabbit.

4. Regarding the fourth objective: evaluation of the efficacy of cloxacillin, ceftaroline and daptomycin in monotherapy or combinations of daptomycin with cloxacillin or ceftaroline in the experimental endocarditis model against MSSA with high and low vancomycin MIC phenotype. The results of this study have been presented at different national (SEICAV 2018 [2]) and international (ECCMID 2019 [3], ISCVID 2019 [4]) congresses in poster form and then as two oral communications respectively; see presentations to congresses in bibliography section 4 below. The corresponding manuscript is currently being prepared.

The results of this study showed that there were no differences in the efficacy of the different antibiotic treatments between the two selected strains (representative of the high and low vancomycin MIC phenotypes), so the groups were evaluated together. Our study concluded that in the experimental MSSA endocarditis model, ceftaroline at both doses studied showed superior activity to cloxacillin, although the differences were not statistically significant. Results also showed that when daptomycin was administered as monotherapy at doses of 6 or 10 mg/Kg/day, 4/20 (20%) and 1/19 (5%) of the recovered isolates had developed resistance to daptomycin both in valve vegetations and in kidney and splenic abscesses. Daptomycin monotherapy was less active than beta-lactams in extracardiac metastases (spleen and kidney). The addition of cloxacillin or ceftaroline significantly improved daptomycin activity. The

combinations of daptomycin plus cloxacillin or ceftaroline showed a synergistic and bactericidal activity, presenting a better activity than cloxacillin or ceftaroline in monotherapy ($p < 0.05$ in both cases) in the sterilization rate of the vegetations and preventing in all cases the appearance of daptomycin resistant strains. Thus, these results justify the use of the combination of daptomycin with cloxacillin or ceftaroline in the treatment of MSSA endocarditis regardless of the vancomycin phenotype.

3. Relevance and possible future implications

This study has shown that:

1. The MIC of vancomycin is not a prognostic factor in infectious endocarditis due to MSSA.

2. A relationship between vancomycin MIC and some virulence or pathogenicity factor has not been demonstrated.

3. A relationship between elevated vancomycin MIC and increased mortality has been shown to exist in the experimental natural history model of MSSA infective endocarditis.

4. The combinations of daptomycin and cloxacillin or ceftaroline were superior to the respective monotherapies and avoided the appearance of resistance to daptomycin. Daptomycin would be the treatment of choice and should always be administered in high doses and combined with beta-lactam antibiotics to avoid appearance of resistance.

For all these reasons, the results of this Marató project will help to improve antibiotic treatment of methicillin-sensitive *Staphylococcus aureus* (MSSA) endocarditis and will have an impact on the design of clinical trials and clinical practice guidelines.

4. Scientific bibliography generated

Publications

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Scientific meetings presentations

Title: La CMI a la vancomicina no influye en la historia natural de la endocarditis experimental (EE) por *Staphylococcus aureus* sensible a meticilina (SASM). Authors: C. García de la Mària, M. Collantes, D. Fuster, J.M. Pericàs, J. García-González, M. Almela, M. Hernández-Meneses, J. Ambrosioni, E. Sandoval, B. Vidal, J.M. Tolosana, E. Quintana, C. Falces, J. Llopis, F. Marco, A.S. Bayer, V. Fowler, A. Moreno, I. Peñuelas, J.M. Miró. Conference: XXII Congreso de la Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica, Bilbao, 24-26 May, 2018. Presentation: Oral communication PO#0161). This oral communication won an award for best communication.

Title: Eficacia de daptomicina y cloxacilina en el tratamiento de la endocarditis experimental por *Staphylococcus aureus* sensible a la meticilina. Authors: C. García-de-la-Mària, J. García-González, M. Villamonte, M. Almela, J. Ambrosioni, E. Quintana, M. Hernandez-Meneses, C. Falces, A. Téllez, J.M. Pericas, B. Vidal, J. Llopis, A. Moreno, J.M. Miró y el Grupo de Estudio de la Endocarditis del Hospital Clínic. Conference: Sociedad Española de Infecciones Cardiovasculares (SEICAV). Seville, 16-17 November, 2018. Presentation: Poster P-32.

Title: Efficacy of daptomycin plus cloxacillin, cloxacillin and ceftaroline in the treatment of methicillin-susceptible *Staphylococcus aureus* (MSSA) Experimental Endocarditis. Authors: C. García-de-la-Mària, J. García-González, M. Villamonte, M. Almela, J. Ambrosioni, E. Quintana, M. Hernandez-Meneses, C. Falces, A. Tellez, J.M. Pericas, B. Vidal, J. Llopis, A. Moreno, J.M. Miró and the Hospital Clinic Endocarditis Study Group. Conference: European Congress of Clinical Microbiology and Infectious Diseases (ECCMID).
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