Scientific Works. Series C. Veterinary Medicine. Vol. LXVI (2), 2020
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

COMPARATIVE STUDY OF OSTEOMYELITIS REPRODUCED ON RABBITS USING HUMAN STRAINS OF METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* (MRSA) AND METHICILLIN-RESISTANT *STAPHYLOCOCCUS EPIDERMIDIS* (MRSE)

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Abstract

One of the current concerns regarding orthopedic surgery is represented by the associated infections. Studies show that in 80% of human cases, the primary bacterial agent that causes osteomyelitis is Staphylococcus aureus. On the other hand, the epidemiological data claims that coagulase-negative staphylococcus, especially Staphylococcus epidermidis, have emerged as the predominant pathogens of the associated infection due to their ability to develop biofilm. The goal of the study was to induce osteomyelitis in rabbits using bacterial strains isolated from human patients and to optimize the concentration of the two species of staphylococcus capable of reproducing bone infection. The evaluation of the disease installation and the clinical evolution was completed by hematological and histological examinations. Comparing the results, it can be concluded that the MRSA strain is more pathogenic compared to MRSE. In both cases, the rabbit has been shown to be a good experimental model for the reproduction of osteomyelitis that can be used for the development of new treatments.

Key words*: experimental model, rabbit, osteomyelitis, MRSA, MRSE.*

INTRODUCTION

Osteomyelitis (gr. Osteon = bone, myeloma= bone marrow and itis = inflammation) (Beck-Broichsitter et al., 2015) is the inflammation of the bone marrow, which can be localized or diffuse, which is affecting the surrounding cortex, periosteum, and the surrounding soft tissue (Lew, 2004), even though it can be produced by a wide range of pathogens, the main source of infection is represented by the Gram-positive opportunistic bacteria of the genus *Staphylococcus* (Kavanagh et al., 2018), in particular *Staphylococcus aureus* and *Staphylococcus epidermidis*.

Methicillin-resistant *Staphylococcus aureus* (MRSA) is the leading cause of overlapping infections that produce skin or organ damage (Guardabassi et al., 2019; Thapaliya et al., 2017), with varying degrees of severity (Abd El-Baky et al., 2019). *Staphylococcus epidermidis*, a saprophytic agent of the skin and mucous membranes, has become an opportunistic pathogen, resistant to â-lactam antibiotics and multiple antibiotics, becoming Methicillin - resistant *Staphylococcus epidermidis* (MRSE) (Widerström et al., 2016; Rolo, 2012), which is progressively involved in medical device related infections, through the formation of biofilm (Hofmans et al., 2018) and which increases the costs of care and also the mortality rate among the affected patients (Lakhundi et al., 2018; Wang et al., 2020).

The bacterial biofilm and the exopolysaccharide matrix produced by bacteria during the adhesion of different surfaces (Kývanç, 2018) offers them resistance to antibiotics, phagocytes and bacteriophages. Moreover, the bacterial infections in which the infectious agents have a high capacity for adhesion and biofilm formation are different from infections caused by planktonic bacteria, due to their etiology, sensitivity to various antimicrobial agents and the ability to adhere to non-

animated substrates, such as those used in medicine as prosthetic devices (Raksha et al., 2020).
The ability of the both bacterial strains to form biofilm as well as their resistance to antibiotics was an important criterion in establishing the objectives of this study. Using the rabbit as a model for osteomyelitis, the disease was reproduced by inoculating MRSA and MRSE, bacteria that were isolated from human patients. The bacterial suspensions that were tested at different concentrations, the clinical signs that emerged and also the complementary examinations that were performed have provided important data on the pathogenicity of both MRSA and MRSE, data that can be used to initiate new therapeutic schemes.

MATERIALS AND METHODS

Ethics statement
Animal studies have been approved by the ethics committee of the Cantacuzino Medical-Military Research and Development Institute (CI) and authorized by the competent authority, being carried out in accordance with the provisions of EU Directive 63/2010 respecting the rules of care, the use and protection of animals that are used for scientific purposes. The studies were conducted in Baneasa Station, the Preclinical Testing Unit, a unit authorized as a user by the competent authority. For the induction of the disease, sample collection and clinical supervision were taken all measures to reduce the suffering of animals.

The animals
For each study were used adult rabbits, "White New Zealand" strain, male and female, with an average weight of 3,000 grams. The animals were housed in the experimentation animal facility, in individual cages, with cycles of light and dark of 12 h each.
The rabbits were acclimated for five days under the same conditions of experimentation, at a temperature of 20-24°C and a relative humidity of 45-65%, during this time they received at their discretion food and water. The separation in groups was done by marking the rabbits on the ear using a permanent marker. All the groups have 4 rabbits.

Bacterial strains
MRSA - was obtained from human patients and received at CI from ROMVAC Company, Voluntari, Ilfov, Romania. MRSA was processed in the Microbiology Laboratory of CI by seeding it on Muller Hinton agar plates and by performing repeated passages, incubated at 37°C for 24 h. From the last passage, serial dilutions were made and the concentrations of 5×10^5 CFU/ml and 5×10^6 CFU/ml were the only ones sent for testing.
MRSE - was isolated from the knee prosthesis of a human patient and was received by CI from the Instituto Ortopedico Galleazzo, Milan, Italy. MRSE was processed under the same conditions as MRSA, and the tested concentrations were: 5×10^5 CFU/ml, 5×10^8 CFU/ml and 5×10^{10} CFU/ml.
As the metabolic activity of the bacteria is much more intense when they are organized in biofilms, we have created a favourable environment for its reproduction by using cotton meshes that represented the substrate for adhesion and colonization of the staphylococcal strains.

Surgical procedure
The animals were deeply anesthetized by neuroleptic analgesia using a mixture of Ketamine (50 mg/kg) and Acepromazine (1 mg/kg).
For all the animal groups, the election limb was the left hindlimb, the right hindlimb was taken as a control. The fur in the mid-proximal area of the tibia was trimmed and the skin was disinfected with Iodine 2%. After the incision of the skin layers and the exposure of the cortex, two bone defects were performed under continuous saline jet, using the same technique as in the previous studies (Ancuța et al., 2019). Using a drill with a diameter of 1.5 mm, two cavities were made up to the medullary canal, at a distance of 10 mm between them and approximately 10-15 mm distance from the tibio-femuro-patellar joint.
Each bacterial species, used in the study, were tested on groups of animals inoculated at different concentrations (Table 1).

Table 1. MRSA and MRSE concentrations/animal group

Bacterial species	Group no.	Number of animals/group	The used bacterial concentration
Methicillin-resistant *Staphylococcus aureus*	I	4	5 x 10^5 CFU/ml
	II	4	5 x 10^6 CFU/ml
Methicillin-resistant *Staphylococcus epidermidis*	III	4	5 x 10^5 CFU/ml
	IV	4	5 x 10^8 CFU/ml
	V	4	5 x 10^{10} CFU/ml

The created bone defects were augmented with the cotton meshes previously immersed in the bacterial suspension over which was inoculated an amount of 0.05 ml of bacterial culture/ defect.

The surgery was completed by suturing the wound with non-absorbable thread and applying compressive dressings. After surgery, in the next 3 days the rabbits received analgesic treatment (Ketoprofen - 3 mg/kg SC).

The monitoring period of all animal groups was 30 days, with an intervention point on day 14, when half of the total number of rabbits/group was euthanized by overdose of anesthetic to follow up the osteomyelitis onset and its passage from the acute phase to the chronic one, the rest of the animals were sacrificed at the end of the study. During the surveillance interval, the animals were examined clinically, hematologically, throw the necropsy examination and histologically.

Animal monitoring

Clinical examination: daily, follow - up local and general signs; thermometry, daily for the first 7 days, after that at day 14 and at the end of the study and weekly body weight monitoring.

Hematological examination: performed at CI, the blood samples were collected on day 0 and day 14, and their processing was performed using the ProCyte Dx analyzer (IDEXX Laboratories) for all groups.

The histological examination, performed for all tibias that were inoculated, from all the groups, followed the specific protocol: fixation in 10% formalin solution, 24-72 hours decalcification in a quick decalcification solution, dehydration in ethanol solution and paraffin embedding. The paraffin blocks were cut with the microtome to a thickness of 5 ìm and stained with eosin hematoxylin.

Statistics

Statistical analysis was performed using Microsoft Excel, the current version.

RESULTS AND DISCUSSIONS

Results

Survival rate was100% in groups I, III, IV, V and 75% in group II.

The clinical examination, performed daily to assess the general and local condition, was based on the measurement of body temperature and weight of the animals and care of the surgical wounds, intervening with pain management medication when signs of pain were observed.

Body temperature, measured intrarectally with the digital thermometer: For 2 days in the first 7, were recorded values above the physiological limits in groups II and V, which in the following days it returned to normal parameters (Figure 1).

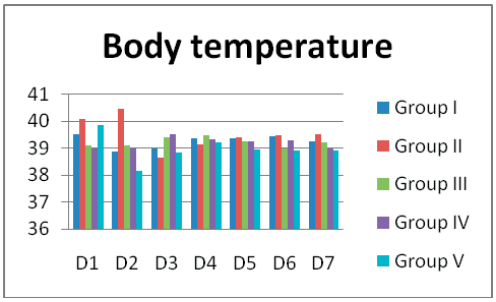

Figure 1. Evolution of average body temperature for each rabbit groups and bacterial concentrations used in the study

The body weight was evaluated at the beginning of the experiment for all subjects, then at day 14 and day 28. In the case of the animals that were inoculated with MRSA, a decrease in weight was observed in the first 14 days, especially in the group where the tested concentration was 5 x 10^6 CFU/ml, which, until the end of the study, these animals recorded increases in body weight. The animals infected with MRSE showed a favourable evolution, in groups III and IV and in the case of group V a slight decrease of the weight was observed in the middle of the monitoring interval, loss that was recovered until day 28 (Figure 2).

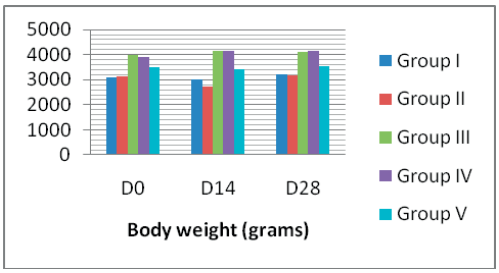

Figure. 2. Evolution of average body weight/groups

Haematological examination: the analysis of the average values for the parameters of interest, provided information about the organism's effort to neutralize the bacteria, without any relevance for the experiments performed (Table 2).

Tabel 2. Hematological average values on day 0 and day 14

The analyzed parameters	WBCx10^9/L		HETEROx10^9/L		LYMx10^9/L	
Reference Values	4.54 - 10.22		0.96 - 3.34		1.49 - 5.21	
	D0	D14	D0	D14	D0	D14
Group I	9.2	10.1	1.62	3.13	6.57	5.24
Group II	9.51	8.82	2.20	2.72	6.15	3.82
Group III	7.2	5.87	1.71	1.70	4.7	3.36
Group IV	5.18	5.91	1.8	2.22	4.36	2.89
Group V	8.57	7.33	2.16	2.72	5.66	3.44

The clinical signs assessment was performed daily, the groups I and II presented specific indicators of *Staphylococcus aureus* infection: fever in the first 2 days in most animals; in group II, the values of body temperature exceeded 40°C, a rabbit passing from the state of hyperthermia into hypothermia followed by death, 3 days after inoculation. Altered general state, lack of appetite, diarrhea and local signs of abscess starting from day 7 were observed. At 2 weeks after disease induction, all animals had open abscesses, thickened and necrotic wound edges. By the end of the study, both local and general signs had improved, in both groups that were inoculated with MRSA, but local lesions characteristic of the infection persisted even after.

The animals inoculated with MRSE have showed a favourable clinical evolution, regardless of the bacterial concentration used, the predominant local signs being inflammation, the indurations and the appearance of the abscesses encapsulated 7 days after the beginning of the experiment. In groups III and IV, abscess resorption has been observed, as well as progression to granuloma in animals from group V at the place of inoculation until the end of the study.

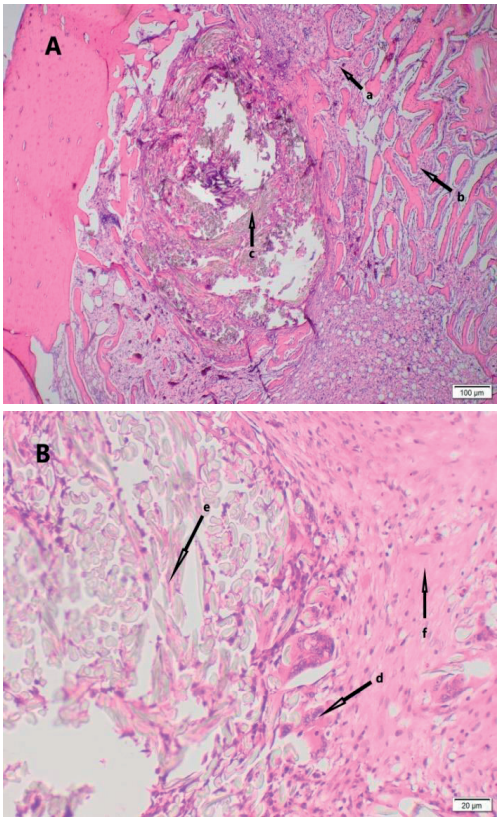

Figure 3. Cross section through tibia inoculated with MRSA 5 x 10^5 CFU/ml: A) Trabecular bone and hematopoietic bone marrow (a, b), textile implant and abundant cell population (c) (Hematoxylin-Eosin stain, 2x); B) Detail of the marginal area of the textile implant (Hematoxylin-Eosin stain, 10x) - presence of giant foreign body cells (d), surrounded by fibrous connective tissue (f), textile implant (e)

Histological examination: the predominant results from the histological analysis, in the groups inoculated with MRSA were (Figure 3):
- Thickened periosteum, with fibrous connective tissue, heterophils and

macrophages. In some rabbits, were observed areas of necrosis, which included even the compact on the implantation area.
- Compact bone with the presence of primary callus and granulation tissue (partial consolidation).
- The medullary area with the presence of abundant cellularity which is composed of giant cells of foreign body type that adhere to the textile implant and bacteria that are found intracytoplasmatically in macrophages.

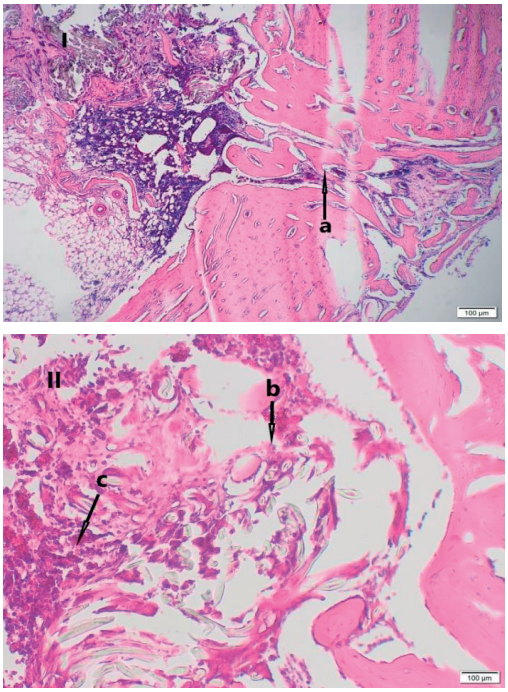

Figure 4. Sections through tibias inoculated with MRSE: I (5 x 10^5 CFU/mL) - Cross section through compact bone: lamellar fragments with a tendency of fusion that consolidates the bone defect (a), fibrous tissue and textile implant fragments in the medullary canal. II (5 x 10^5 CFU/ mL) - medullary area foreign body inflammatory reaction (b), sequestered hematopoietic tissue (c)

In the rabbits where the MRSE strain was tested, the histological changes were different, depending on the concentration used:
Group III (Figure 4):
- thickened periosteum, with fibrous connective tissue.
- Compact bone with regeneration elements - secondary callus.
- Fibrous tissue organized and oriented to the bone defect.
- The medullary with fibrous tissue, foreign body reaction (macrophages and multinucleated giant cells), hematopoietic tissue islands and discrete bacterial colonies (in some cases).

Group IV (Figure 5):
- compact bone with regeneration elements
- secondary callus, fibrous connective tissue
- Foreign body reaction in the marrow, preserved hematopoietic tissue.

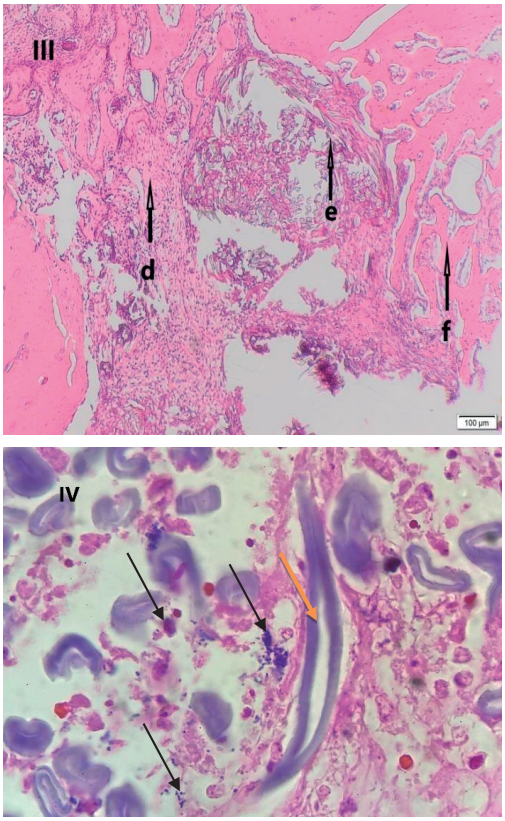

Figure 5. MRSE-inoculated tibia sections: III (5 x 10^8 CFU/mL) - detail of the compact bone with defect, fibrosis (d), textile implant (e) and incompletely fused bone lamellae (f). IV (5 x 10^{10} CFU/mL) - GRAM-positive coccoid bacteria (black arrow), textile implant (yellow arrow), GRAM stain (100x)

Group V (Figure 6):
- thickened periosteum, with the presence of inflammatory cells (macrophages, heterophils).
- Compact bone with regeneration elements - secondary callus, fibrous connective tissue.
- Medullary with debris from the textile implant and foreign body reaction, hematopoietic tissue islands present, heterophils, macrophages,

intracytoplasmatic bacteria and fibrous reaction.

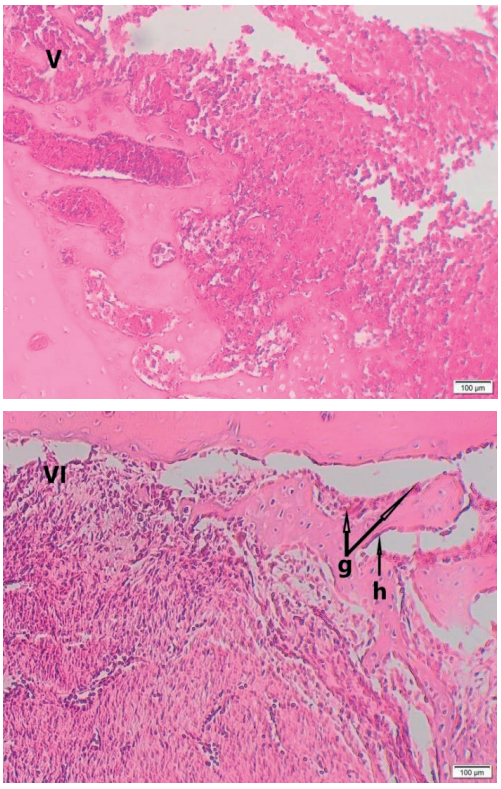

Figure 6. MRSE-inoculated tibiae sections: V (5 x 10^{10} UFC/mL) - Periosteal detail: necrosis area with numerous heterophils, macrophages and osteolysis. VI (5 x 10^{10} UFC/mL) - Detail of periosteal area with abundant fibrosis; compact bone tissue lamellae delimited by osteoclasts (g) and osteoblasts (h)

Discussions

Although, lately, surgical hygiene techniques and preventive antibiotic therapy have been significantly improved, bacterial infections remain a topical issue, especially in the case of orthopaedic surgeries involving the use of medical devices (Tran et al., 2019). Bacteria such as MRSA and MRSE irreversibly adhere to the surface of a device and develop to form a polysaccharide biofilm that protects them from the action of antibiotics or the defence mechanisms of the infected organism (Stewart, 2001).

In the current paper, we have focused on the ability of the two antibiotic-resistant bacteria, of significant importance in orthopaedic infections, MRSA and MRSE, to reproduce osteomyelitis and evaluated their pathogenicity by inoculating different concentrations in a rabbit tibia model.

Based on the premise that osteomyelitis is reproduced in a group of animals if the viability rate is over 75%, it can be said that all concentrations of MRSA and MRSE have been able to reproduce the disease but the correlation with the performed examinations, offers different data.

The longest period of time for the development of bone infection was four weeks (Giavaresi et al., 2008), during which the clinical signs were monitored. The groups inoculated with MRSA expressed a more spectacular symptoms than those in which MRSE was tested. The fact that in group II, a subject died shortly after the beginning of the experiment, presenting at the autopsy examination specific lesions of septicaemia as well as the prolonged febrile condition in the rest of the animals, the more pronounced local signs and the significant decrease in weight in group II in comparison with group I, were indicators of the aggressiveness and the action of the MRSA strain at the bone level, aspects mentioned also in other studies (Nijhof et al., 2000).

The rabbits tested with MRSE showed an attenuated symptoms, only the group that received 5x10^{10} CFU/ml expressed signs of bone infection, an interesting aspect for our study, especially when you take in consideration the rising curve of the body weight and temperature.

The histopathological diagnosis in the case of osteomyelitis has a considerable diagnostic value (Tiemann, 2014), groups I and II presenting specific lesions of active-chronic bone infection, represented by fibrosis, the presence of the multinucleated giant cells of foreign body, heterophils and macrophages (Fig. 4), groups III-IV showed signs of regeneration with the presence of bone spicules in the defect area accompanied by the activity of osteoblasts and the presence of numerous osteoclasts that ensure the remodelling of the newly formed bone (Figure 5, III), and in the last group there were bone remodelling and the activity of osteoblasts, the bone matrix being evidently present in some areas accompanied by different degrees of cartilaginous metaplasia with partial mineralization (Figure 6), probably

due to the negative influence made by the introduction of the inducing agent, that was prior immersed in the bacterial suspension with very high concentration.

If, in the case of the animals inoculated with MRSA, the symptoms and the results of the paraclinical examinations were more than sufficient to establish the diagnosis of osteomyelitis, in the case of the animals inoculated with MRSE with regard to groups III-V, the onset of the disease was insidious, requiring the use of increasing bacterial concentrations, as also mentioned by Park et al. in rats studies (Park, K.H. et al., 2017).

Various studies and models for osteomyelitis in different species have been described in the literature. Reizner et al. have described an extensive set of animal models suitable for the reproduction of osteomyelitis with staphylococcal strains (Reizner et al., 2014). Thus, the chosen animals should have immune and musculoskeletal characteristics similar to humans, and the bacteria should be clinically representative and capable of biofilm formation (Brunotte et al., 2019). In order to reproduce an environment favourable to the adherence and colonization of infectious agents, we used cotton meshes (Bottagisio, 2019), which proved to be effective for the purpose of our study, the reproduction of osteomyelitis being possible in all groups, especially those inoculated with MRSA and MRSE - group V. Comparing groups I and III that have been tested with the same bacterial concentration but different strains it can be concluded that *Staphylococcus aureus* is much more aggressive on the bone area than *Staphylococcus epidermidis*.

One of the our studies weaknesses is the number of animals that has been used, which should be "sufficient but not excessive" for the proposed goal (Penny S. Reynolds, 2019), and in our case, the results could not be quantified as a result of this aspect. Also, the lack of radiological examinations or the analysis of the tibias using the MicroComputer Tomography, is a limitations of our studies.

CONCLUSIONS

Both MRSA and MRSE strains have reproduced osteomyelitis in rabbits, but in relation to body weight and temperature, local signs and histopathological examination results, the optimal concentrations for the induction of the disease are: 5 x 10^5 CFU/ml MRSA and 5 x 10^{10} CFU/ml MRSE and further demonstrates the much higher pathogenicity of MRSA.

Comparing groups I and III that have been tested with the same bacterial concentration but different strains it can be concluded that *Staphylococcus aureus* is much more aggressive in bone than *Staphylococcus epidermidis*.

The work may serve as a basis for further studies because the rabbit is a good experimental model for osteomyelitis reproduction that can be used to optimize therapeutic doses or to develop new treatments.

ACKNOWLEDGEMENTS

This research work was carried out with the support of Ministry of Education and Research from Romania under the Projects PN-III-P1-P1.2 - PCCDI - 2017 - 0728_TERAMED and EuroNanoMed III – ANNAFIB.

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