99mTc-Ciprofloxacin Imaging: Still an Unsettled Issue?

Mariano G. Portillo[1], Andrea Mendoza Bertelli[2], Fiorella C. Tesán[1], Mariángeles Noto Llana[2], Marcela B. Zubillaga[1], Daniel O. Sordelli[2], Marisa I. Gómez[2] and María J. Salgueiro[1,*]

[1]*Laboratorio de Radioisótopos, Cátedra de Física, Facultad de Farmacia y Bioquímica; Universidad de Buenos Aires, Junín 956, piso bajo, Capital Federal, (C1113AAD), Buenos Aires, Argentina*

[2]*Instituto de Investigaciones en Microbiología y Parasitología Médica (IMPaM), Universidad de Buenos Aires and Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET); Universidad de Buenos Aires Paraguay 2155, piso 12, Capital Federal, (C1121ABG), Buenos Aires, Argentina*

Abstract: The objective of this work consisted in the assessment of 99mTc-ciprofloxacin imaging performance as a diagnostic tool in an experimental rat model of osteomyelitis. Bone (tibia) infection was induced in adult rats by inoculation of a suspension containing *S. aureus* suspended in fibrin glue. *In vivo* studies by means of small animal imaging were conducted using a gamma camera. The study shows the correlation between 99mTc-ciprofloxacin positive images with bacterial bone count but also with histopathological findings in an osteomyelitis animal model, highlighting its potential as a tool in preclinical research and the accomplishment of 3Rs concept regarding welfare of laboratory animals. 99mTc-MDP scintigraphy, failed to show these correlations and therefore it may be proposed as a complementary method to diagnose and follow up the bone physiopathology in this animal model. Future perspectives of small animal imaging in order to potentiate osteomyelitis basic research will derive from numerous research works, and 99mTc-ciprofloxacin may still be a candidate for infectious diagnose and follow-up as demonstrated in this study.

Keywords: Infection, Inflammation, Osteomyelitis, *Staphylococcus aureus*, Small animal imaging.

INTRODUCTION

For decades now, imaging techniques have been installed in clinical practice as a valuable tool for the diagnosis and therapeutic monitoring of different diseases. In the pre-clinical field, specifically in experimental animal models, these modalities are of more recent application [1, 2]. Osteomyelitis is an inflammation of bone caused by an invasive infection, occurring through direct inoculation of different kinds of pathogens such as viruses, parasites, fungi and bacteria, being the last one, the most common one. Osteomyelitis can occur after a trauma, by contiguous spreading of microorganisms from soft tissue infections, or via hematogenous seeding of microbes into bone tissue. *Staphylococcus aureus (S. aureus)* is the most common etiologic agent of osteomyelitis in both children and adults. This bacteria causes over 90% of pediatric hematogenous osteomyelitis [3, 4].

In this sense, small animal imaging techniques have been proposed for monitoring bone remodeling during osteomyelitis caused by *S. aureus* [5]. Thus, the use of sensitive imaging techniques in laboratory animals would contribute to the development of experimental models for the study of virulence factors, host immune responses and the evaluation of experimental treatments with increased statistical power and full compliance of the 3Rs [6]. The concept of the 3Rs is a humane way to implement work with laboratory animals discussed under the three broad headings of Replacement, Reduction, and Refinement. Replacement means the substitution of conscious living animals with senseless material. Reduction means using the lowest number of animals possible. Refinement means the absence of pain or stress and the use of less invasive techniques [5, 7].

In the diagnosis of osteoarticular infections, 99mTc-ciprofloxacin has been proposed as a useful radiopharmaceutical however, it is unknown if preclinical research may benefit from its use as a probe for small animal imaging techniques [6, 8, 9]. Therefore, the objective of this work consisted in the assessment of 99mTc-ciprofloxacin imaging performance as a diagnostic tool in an experimental rat model of osteomyelitis. Additionally, the 99mTc-metilendiphosphonate (99mTc-MDP) three phase imaging protocol was used for comparative purposes.

MATERIAL AND METHODS

Bacterial Isolate and Animal Model

An isolate of *S. aureus* corresponding to the USA300 genotype was used. Bacteria were grown in

*Address correspondence to this author at the Laboratorio de Radioisótopos, Cátedra de Física, Facultad de Farmacia y Bioquímica; Universidad de Buenos Aires. Junín 956, piso bajo, Capital Federal, (C1113AAD), Buenos Aires, Argentina; Tel: 54 11 4964 8202 ext. 33; FAX: 54 11 4964 8202 ext.31;
E-mail: jsalguei@yahoo.com.

E-ISSN: 2408-9788/16

tripticase soy broth at 37°C with agitation over night. Bacteria were washed twice with phosphate-buffered saline (PBS) and the optical density at 600nm was adjusted to 1.5 in PBS.

We used a rat model of osteomyelitis since bone infection was induced in Sprague Dawley rats (280-320 g) as previously described [10] where each tibia was injected with a 5 µl suspension containing 1-2 x 10^6 CFU (colony forming units) of *S. aureus* FRP3757 corresponding to the USA300 genotype suspended in fibrin glue (Tissucol® kit 1 ml; Baxter Argentina-AG, Vienna, Austria). *S aureus* USA300 FPR3757 was kindly provided by Alice Prince (Columbia University, NY, USA).

Radiolabeling Procedure of Imaging Agents and Small Imaging Protocol

The imaging agents 99mTc-ciprofloxacin and 99mTc-MDP were obtained by means of the radiolabelling of purchased cold kits (Laboratorios Bacon SAIC, Argentina) with Na99mTcO4 eluted from a 99Mo-99mTc generator (Laboratorios Bacon SAIC, Argentina) according to the methodology described by the manufacturer. Briefly, 3mL of the Na99mTcO4 solution were added aseptically to the freeze-dried commercial kits in order to reach a final concentration of: 308MBq/mL for 99mTc-ciprofloxacin and 962MBq/mL for 99mTc-MDP. After 10 minutes and mild agitation radiopharmaceuticals, radiochemical purity was assesed. Labeling efficiency exceeded 95% in all the assays performed.

99mTc-ciprofloxacin and 99mTc-MDP were administered intravenously by the tail vein. Small animal imaging was performed in ventral position on the high resolution collimator of a small field of view gamma camera Ohio Nuclear. 99mTc-ciprofloxacin scintigraphy (37-74 MBq) was acquired as static imaging after 60 minutes of biodistribution (1.75 zoom) at 4 hours, 96 hours, 4 weeks, 10 weeks and 15 weeks after inoculation of bacteria. 99mTc-MDP (37-74 MBq) scintigraphic studies were performed at 96 hours and 15 weeks after inoculation of bacteria in a three phase protocol. When positive scans were detected, a pinhole was further used for zoom images.

Ex Vivo Determinations

At the different time points evaluated some of the rats were euthanized using CO_2. Left and right tibias were excised and evaluated morphometrically, and the osteomyelitic index (OI) was determined as described previously [10]. Briefly, the following measurements were made using calipers: (i) the distance between the inoculation point and the distal end of the left tibia (DT); (ii) the left tibia section diameter at the inoculation site (Di) and the perpendicular diameter at the same site (Dp); (iii) Di and Dp were also measured in the uninfected right tibia of the same rat at the DT determined in the diseased left tibia (control). The osteomyelitic index (OI) was determined as follows: OI = (Dp +Di) infected - (Dp +Di) control. The OI was determined by subtraction of values from the healthy right tibia (control). One-centimeter segments of bone involving the inoculation point were crushed, homogenized in sterile mortars and quantitatively cultured on Trypticase Soy Agar (TSA) plates to determine the bacterial load.

Semiquantitative Analysis and Statistics

Images were analyzed qualitatively and also quantitatively by means of drawing regions of interest (ROI) over the infected tibia and its counterpart as well as over the knees of both legs. Radioactivity uptake determined by means of counts in a ROI was expressed with a 95% confidence interval according to Poisson distribution. Ratios of radioactivity uptake between tibias (TR) in the same animal were calculated for each group and are showed as mean±standard deviation. The repeated-measures ANOVA model was used to evaluate differences among the images from different time points. The level of significance was set at $P < 0.05$. Correlation was analyzed with $\alpha = 0.05$ by means of the parametric test (Pearson) for TR vs OI and the non parametric test (Spearman) for TR vs CFU.

RESULTS

The ratio of 99mTc-ciprofloxacin uptake between tibias in the same animal was not significantly different among the time points evaluated (data not shown).

A significant positive correlation between the number of CFU in the infected tibias and the radiopharmaceutical incorporation was observed at 15 weeks after inoculation with *S. aureus* ($r= 0.6565$; $p < 0.05$) (Figure **1**, panel **a**). Similarly, a significant correlation ($r = 0.7470$; $p < 0.05$) was also found between 99mTc-ciprofloxacin uptake and the OI at 15 weeks post bacterial challenge (Figure **1**, panel **b**).

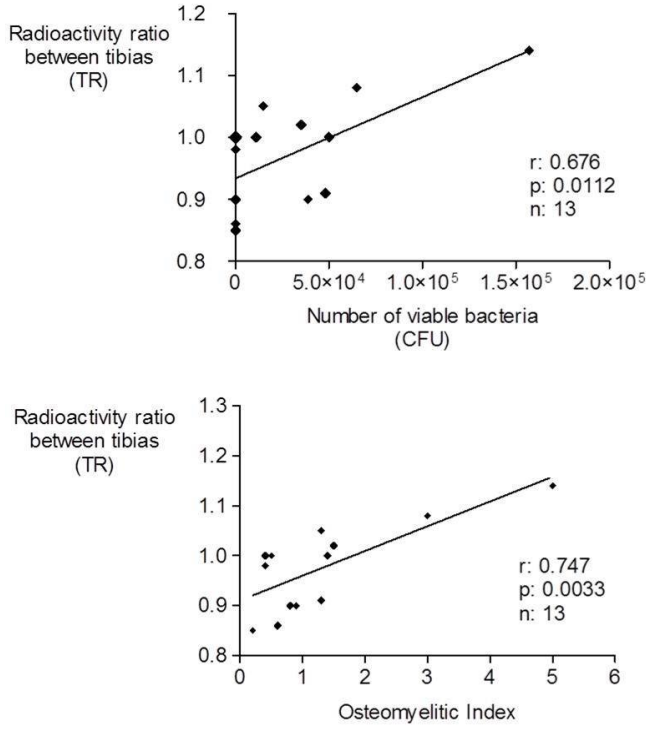

Figure 1: Panel A. Correlation between TR (radioactivity ratio between tibias) and number of viable bacteria at 15 weeks. Correlation parameters: correlation coefficient (r, Spearman); level of significance (p); number of XY pairs (n). Panel B. Correlation between TR (radioactivity ratio between tibias) and OI at 15 weeks (Osteomyelitic Index). Correlation parameters: correlation coefficient (r, Pearson); level of significance (p); number of XY pairs (n).

Scintigraphy studies also showed focal uptake of 99mTc-Ciprofloxacin in the infected tibias at random when OI values were under 1.5 while deliberate positive in cases where OI was higher than 1.5. These imaging findings were independent of the time of evolution of the animal model since focal uptake was detected in individuals of all time points evaluated as shown in the examples of Figure **2**.

99mTc-MDP three phase protocol showed positive scans at time point 96 hours compatible with bone damage and inflammatory reaction of the surgical procedure used to set up the experimental model (Figure **3**). In this sense, images of the blood pool phase showed an increased and diffuse uptake of 99mTc-MDP in the third means of the hind limb which correlates with a focal hot spot at the same level of the tibia in the delayed images.

Images of the three phase scans at 15 weeks (Figure **3**) showed an uptake pattern compatible with a bone remodeling profile after fractures, as compared with clinical setting in humans [11]. At this time point we observed a group of individuals with normal biodistribution of the radiopharmaceutical both at the blood pool images as well as in the delayed images while another group of animals showed a focal increased uptake in the blood pool images that remained, although less hot, in the delayed images. The uptake pattern showed the cortical diaphysis of the tibia as it can be verified in the images acquired with the pinhole collimator. Nevertheless, TR of 99mTc-MDP scans did not correlate with either bacterial quantitative culture or the OI at 15 weeks post-bacterial challenge.

DISCUSSION

Representative animal models of osteomyelitis are valuable tools since they allow diagnostic and therapeutic research to keep moving forward [11].

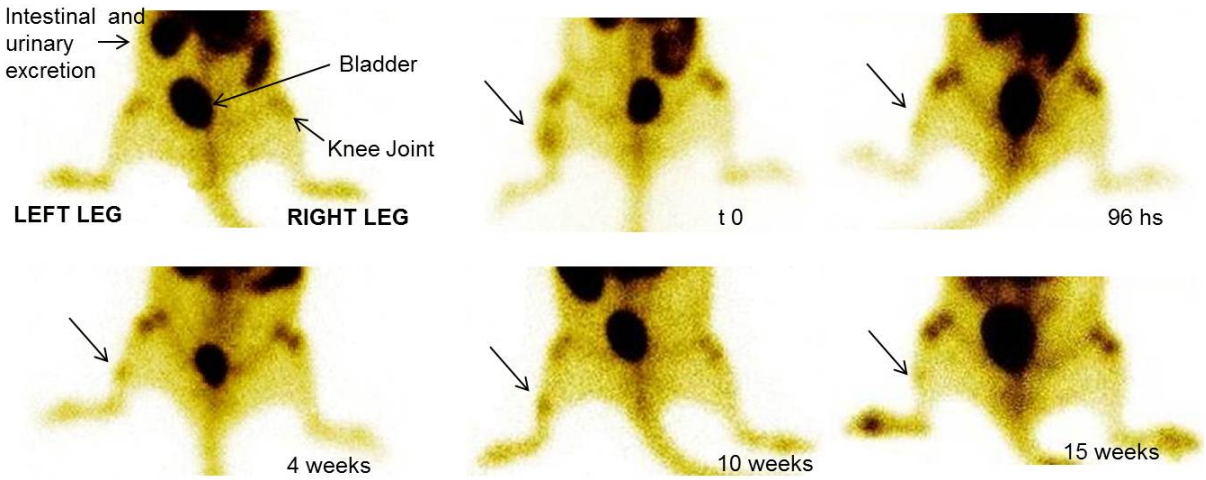

Figure 2: Images from 99mTc-ciprofloxacin scintigraphy. Upper panel from left to right: reference image, 4hs and 96hs. Bottom panel: 4, 10 and 15 weeks. Focal 99mTc-ciprofloxacin uptake is shown with arrows.

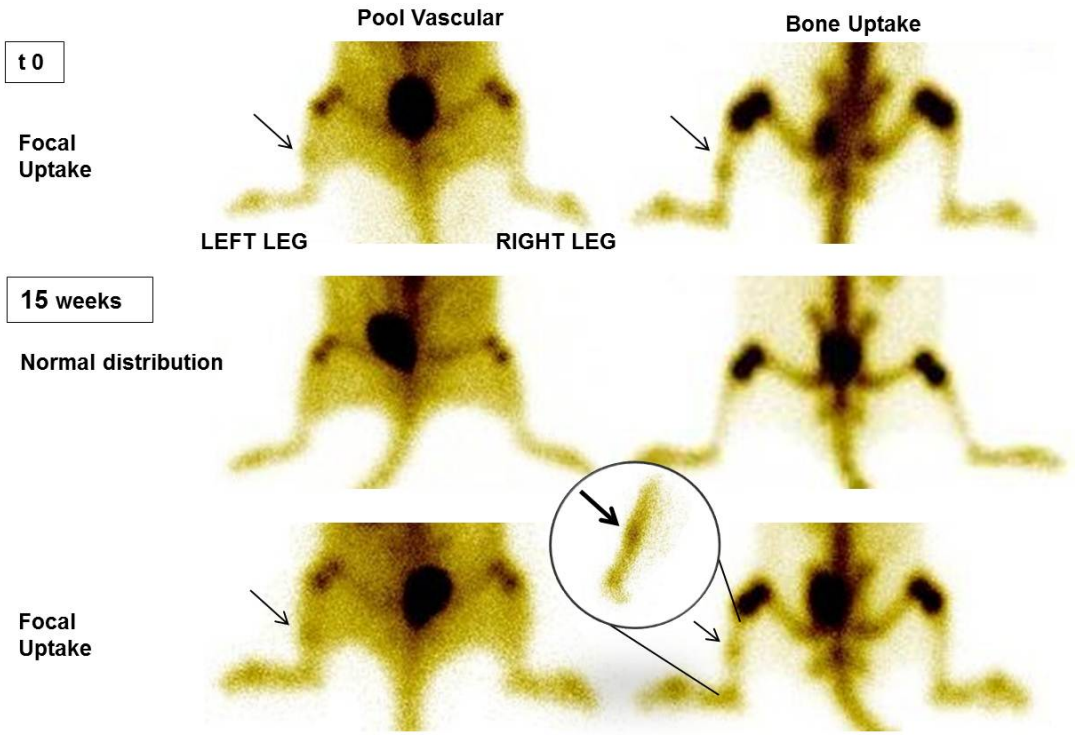

Figure 3: Images from Two Phases 99mTc-MDP scintigraphy (blood pool and delayed bone images). 99mT-MDP images at 4hs (t0) and 96hs 15 weeks. Left panels are blood pool images and right panels are delayed images. Focal uptake is shown with arrows and pinhole zoom with a circle.

Experimental handling of such animal models of infection diseases usually involves microbiological evaluation of the infected organ and searching for macro or micro changes in it such as bacterial colonization as well as morphological and histopathological changes [12-15]. The evaluation of these parameters is related, in a non negotiable way, to animal euthanasia which avoids the follow up of the same animal during the development of the disease or even worse, the follow up of a treatment using the same animal. Small animal imaging techniques contribute with the 3Rs concept in laboratory animal research [5] as well as with the improvement of the statistics of experimental designs [16]. In this work, we evaluated by means of small animal imaging techniques, the performance of a clinically available radiopharmaceutical as a diagnostic agent in an animal model of osteomyelitis.

Our results show a correlation between 99mTc-ciprofloxacin positive images evaluated as infected tibia / non infected tibia uptake ratio not only with bacterial bone count but also with the osteomyelitic index, highlighting the potential of 99mTc-ciprofloxacin scintigraphy as a tool in preclinical research. In this study, the ratio of 99mTc-ciprofloxacin uptake between tibias did not change significantly along the time of evolution of the animal model. However, the remarkable finding was a significant positive correlation between the number of CFU in the infected tibias and the radiopharmaceutical incorporation at 15 weeks after inoculation with *S. aureus* as well as for the Osteomyelitic index. Scintigraphy studies also showed focal uptake, a main goal of imaging diagnosis, using 99mTc-Ciprofloxacin in the infected tibias. These findings were at random when OI values were under 1.5 while deliberately positive in cases where OI was higher than 1.5. This finding is in accordance with results reported by others [17]. Therefore, our results showed up that 99mTc-ciprofloxacin scintigraphy is a worthwhile tool in preclinical research to follow up those parameters without sacrificing the animal when using this experimental model.

On the other hand, it is important to note that 99mTc-MDP scintigraphy could not be correlated to ex vivo assessments. Although 99mTc-MDP is described as a high sensitivity tool for bone infectious diagnoses it lacks specificity because it also accumulates in bone areas of increased turnover [18-20]. In this way, 99mTc-MDP three phase protocol showed the bone damage and the inflammatory reaction of the surgical

procedure used to set up the experimental model. However, images of the three phase scans at 15 weeks showed an uptake pattern compatible with a bone remodeling profile after fractures, as compared with clinical setting in humans, instead of the focal uptake compatible with infection of 99mTc-Ciprofloxacin [18]. Moreover, TR of 99mTc-MDP scans did not correlate with either bacterial quantitative culture or the OI at 15 weeks post-bacterial challenge and therefore it may be proposed as a complementary method to diagnose and follow up the bone pathophysiology in this animal model.

It is noteworthy that TR calculated on 99mTc-ciprofloxacin and 99mTc-MDP scans at time points of 96 hours and 15 weeks were not significantly different. However, our results showed that 99mTc-MDP scan was not specific for infection diagnosis on the basis of our experimental design as was 99mTc-ciprofloxacin. Moreover, correlation with classical index of osteomyelitis diagnosis in this animal model was demonstrated. These findings could give notion on the ability of 99mTc-ciprofloxacin to differentiate inflammation, produced by the surgical trauma of bacteria inoculation for setting the experimental model, from installed infection in this particular animal model. This is a feature of 99mTc-ciprofloxacin that has been discussed in clinical researches [21-23] but not at the preclinical setting as far as the authors are concerned.

CONCLUSION

99mTc-ciprofloxacin imaging demonstrated to be a valuable diagnostic tool in our experimental rat model of osteomyelitis. 99mTc-ciprofloxacin provided functional information by using small animal imaging which correlated with the microbiological and histopathological features of our model and enhanced the accomplishment of 3Rs concept regarding welfare of laboratory animals. Future perspectives of small animal imaging in order to potentiate osteomyelitis basic research will derive from numerous research works, and 99mTc-ciprofloxacin may still be an interesting candidate for infectious diagnose and follow-up as demonstrated in this study.

ACKNOWLEDGMENTS

This work was supported in part by grants from the Agencia Nacional de Promoción Científica y Tecnológica, Argentina (ANPCYT PICT 2013-0941 to D.O.S., PICT 2011-2263 and 2013-1233 to M.I.G.).

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

ETHICAL APPROVAL

All applicable international, national and/or institutional guidelines for the care and use of animals were followed.

All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at wich the studies were conducted.

All the studies were approved either by the “Institutional Committee for the use and care of laboratory animals” (CICUAL) of the School of Medicine and the School of Pharmacy and Biochemistry, University of Buenos Aires, Argentina (EXP-MED N° 2361/2011 and EXP-FYB N° 53406/2013).

REFERENCES

[1] Zanzonico P. Noninvasive imaging for supporting basic research. In: Kiessling F, Pichler B, editors. Small animal imaging. Basics and practical guide. 1st ed. Berlin: Springer 2011; 3-16. http://dx.doi.org/10.1007/978-3-642-12945-2_1

[2] Berry CR, Garg P. Perspectives in molecular imaging through translational research, human medicine, and veterinary medicine. Semin Nuc Med 2014; 44:66-75. http://dx.doi.org/10.1053/j.semnuclmed.2013.10.002

[3] Rosenberg A. El sistema esquelético y los tumores de los tejidos blandos. In: Cotran R, Kumar V, Robbins S, editors. Patología estructural y funcional. 5th ed. Madrid: McGraw-Hill – Interamericana 1996; 1350-1352.

[4] Cassat J, Skaar EP. Recent advances in experimental models of osteomyelitis. Expert Rev. Anti Infect. Ther 2013; 11(12) p. 1263-1265. http://dx.doi.org/10.1586/14787210.2013.858600

[5] Beckmann N, Maier P. Noninvasive small rodent imaging: significance for the 3R principles. In: Kiessling F, Pichler B, editors. Small animal imaging. Basics and practical guide. 1st ed. Berlin: Springer 2011; 47-57. http://dx.doi.org/10.1007/978-3-642-12945-2_4

[6] Cassat JE, Hammer ND, Campbell JP, *et al*. A secreted bacterial protease tailors the Staphylococcus aureus virulence repertoire to modulate bone remodeling during osteomyelitis. Cell Host & Microbe 2013; 13: 759-772. http://dx.doi.org/10.1016/j.chom.2013.05.003

[7] Russell W, Burch RL. The principles of humane experimental technique. 15th ed. London: Methuen; 1959.

[8] Britton KE, Wareham DW, Das SS, *et al*. Imaging bacterial infection with 99mTc-ciprofloxacin (Infecton). J Clin Pathol 2002; 55: 817-823. http://dx.doi.org/10.1136/jcp.55.11.817

[9] Salouti M, Fazli A. Chapter 9: Infectiuos foci imaging with targeting radiopharmaceuticals in nuclear medicine. In: Okechukwu F, editor. Medical imaging in clinical practice. InTech 2013; 193-216. [cited 2016 Feb 15]: Available from:

http://www.intechopen.com/books/medical-imaging-in-clinical-practice/infectious-foci-imaging-with-targeting-radiopharmaceuticals-in-nuclear-medicine. http://dx.doi.org/10.5772/52882

[10] Lattar SM, Noto Llana M, Denoël P, *et al*. Protein antigens increase the protective efficacy of a capsule-based vaccine against Staphylococcus aureus in a rat model of osteomyelitis. Infect Immun 2014; 82: 83-91. http://dx.doi.org/10.1128/IAI.01050-13

[11] Reizner W, Hunter JG, O'Malley NT, *et al*. A systematic review of animal models for staphylococcus aureus osteomyelititis. Eur Cell Mater 2014; 27: 196-212.

[12] Spagnolo N, Greco F, Rossi A, *et al*. Chronic staphylococcal osteomyelitis: a new experimental rat model. Infect. Immun 1993; 61: 5225-5230

[13] Jørgensen NP, Meyer R, Dagnæs-Hansen F, *et al*. A modified chronic infection model for testing treatment of Staphylococcus aureus biofilms on implants. Plos One 2014; 3: 9(10): e103688

[14] Fölsch C, Federmann M, Kuehn KD, *et al*. Coating with a novel gentamicinpalmitate formulation prevents implant-associated osteomyelitis induced by methicillin-susceptible Staphylococcus aureus in a rat model. Int Orthop 2015; 39: 981-988 http://dx.doi.org/10.1007/s00264-014-2582-9

[15] Inzana JA, Schwarz EM, Kates SL, *et al*. A novel murine model of established Staphylococcal bone infection in the presence of a fracture fixation plate to study therapies utilizing antibiotic-laden spacers after revision surgery. Bone 2015; 72: 128-136 http://dx.doi.org/10.1016/j.bone.2014.11.019

[16] Ulbrich HF. Statistical considerations for animal imaging studies. In: Kiessling F, Pichler B, editors. Small animal imaging. Basics and practical guide. 1st ed. Berlin: Springer 2011; p. 69-82 http://dx.doi.org/10.1007/978-3-642-12945-2_6

[17] Pucar D, Janković Z, Dugonjić S, Popović Z. Estimation of 99mTc-ciprofloxacin accumulation in dexes in bone and joint bacterial infections. Vojnosanit Pregl 2009; 66: 395-398. http://dx.doi.org/10.2298/VSP0905395P

[18] Kowalsky R, Falen S. Bone. In: Tarleton Landis N, editor. Radiopharmaceuticals in Nuclear Pharmacy and Nuclear Medicine. 2nd ed. Washington: American Pharmacists Association 2004; 671-693.

[19] Dams E, Nijhof MW, Boerman OC, *et al*. Scintigraphic evaluation of experimental chronic osteomyelitis. J Nucl Med 2000; 41: 896-902.

[20] Gratz S, Doener J, Oestman JW, *et al*. 67Ga-citrate and 99Tcm-MDP for estimating the severity of vertebral osteomyelitis. Nucl Med Commun 2000; 21: 111-120. http://dx.doi.org/10.1097/00006231-200001000-00018

[21] Larikka MJ, Ahonen AK, Niemelä O, *et al*. Comparison of 99mTc ciprofloxacin, 99mTc white blood cell and three-phase bone imaging in the diagnosis of hip prosthesis infections: improved diagnostic accuracy with extended imaging time. Nucl Med Commun 2002; 23: 655-661. http://dx.doi.org/10.1097/00006231-200207000-00010

[22] Britton KE, Das SS, Solanki KK. Ability of (99m)Tc-ciprofloxacin scintigraphy to discriminate between septic and sterile osteoarticular diseases. BrJ Nucl Med 2004; 45: 922-923.

[23] Sarda L, Crémieux AC, Lebellec Y, *et al*. Inability of 99mTc-ciprofloxacin scintigraphy to discriminate between septic and sterile osteoarticular diseases. J Nucl Med 2003; 44: 920-926.

Received on 19-01-2016 Accepted on 19-02-2016 Published on 17-03-2016

http://dx.doi.org/10.15379/2408-9788.2016.03.01.02