ABSTRACT

The doctoral thesis entitled "Studies Regarding the Laboratory Diagnosis, Classical and Non-conventional Therapy of Dermatitis with Bacterial Substrate in Dogs and Cats" consists of 136 pages and, according to standard practices, it is structured in two parts. Information regarding the current stage of knowledge is synthesised in the first part, and the results regarding our own research on the approached subject are presented in the second part. We aimed at approaching the pathology researched from an etiological perspective, a more thorough knowledge of the etiological agents bringing important and relevant information both in the diagnosis and in the therapy of pyodermas.

The first part, comprising of a total number of 28 pages, synthetically presents current information regarding the etiopathogenesis of pyoderma in pet carnivores, and the diagnosis and therapeutic management of pyodermas in dogs and cats.

The second part of the thesis, comprising of a total of 108 pages, presents the results of our own research, and it is structured in 4 chapters. Starting from the purpose and proposed objectives, we presented the results of the epidemiological investigations regarding pyodermas in pets, cats and dogs, then the research is focused on the main etiological agent, *Staphylococcus spp.* In each chapter, the used materials and methods are described, and the results and discussions related to them are presented, as well as the resulting partial conclusions.

In the paper, there are 56 figures and 33 tables, and the thesis was documented with a total of 164 references.

Chapter III – "Purpose and objectives of the research". The arguments regarding the opportunity and importance of the research refer to two aspects: the zoonotic and anthropozoonotic risk of staphylococci and not only, as well as the more and more significant limitation of treatment with antibiotics due to the antibiotic resistance phenomenon. The purpose of the proposed subject was to bring an analytical contribution to the diagnosis and therapy of pyodermas in pets, and to fathom the studies up to molecular level in regard to *Staphylococcus aureus* and *pseudintermedius*, by presenting data useful both in guiding the diagnosis and particularly in initiating etiotropic therapy, as well as by presenting data regarding new therapeutic perspectives with the so-called "non-conventional" substances.

The main objectives of the research were:

- determining the prevalence of pyodermas in dermatitis in dogs and cats;
- determining the prevalence of etiological agents in pyodermas by isolating and phenotypically identifying them;
- molecularly confirming the identification of *S.aureus* and *S.pseudintermedius* species;
- genotypically characterising *S.aureus* and *S.pseudintermedius* in regard to some virulence genes and the resistance to some antibiotics;
- determining the prevalence of MRSA and MRSP strains;
- molecularly confirming the methicillin resistance of strains that are phenotypically classified as MRSA and MRSP;

- *in vitro* assessing the sensitivity of the staphylococcal isolated strains to different classes of antibiotics;
- *in vitro* testing of the antibacterial effect of PAW;
- statistical analysis.

Chapter IV – "Epidemiological investigations regarding pyodermas in dogs and cats in the north-east region of Romania". The casuistry was represented by a number of 163 patients, 98 dogs and 65 cats from the county of Iaşi and localities neighbouring the county of Iaşi. In the first phase, we aimed at classifying dermatitis from a clinical point of view, in superficial dermatitis, deep dermatitis and otitis externa, as well as from an etiological point of view, in mycotic, parasitic and bacterial dermatitis. The association of mycotic and parasitic dermatitis (including otitis) with bacterial dermatitis was noticed in a percentage of approximately 60% in the first case, and approximately 30% in the second case, the higher levels being registered in the case of otitits. After eliminating from the researched batch the cases that no longer had bacterial etiology, the investigations continued in parallel, from a clinical and microbiological point of view. Following thorough anamnesis, in the cases where the suspicion of primary diseases arose, specific investigations were carried out. Four cases of hypothyroidism were confirmed, two suspicions of Cushing being ruled out.

From a microbiological point of view, the phenotypic tests have identified, from the total of 121 pyodermas, 85 cases with staphylococcal etiology, 17 infections with *Pseudomonas spp.*, 15 cases of streptococci, 2 nocardioses, 2 cases of skin infection with *Mycobacterium spp.*, and infections, namely otitis externa, associated with *Proteus spp*. Phenotypic tests were subsequently focused on *Staphylococcus spp.*, the etiological agent with the highest prevalence in bacterial dermatitis. By phenotypic tests, the following were identified: 36 strains of *S. aureus*, 21 strains of *S. pseudintermedius*, and 28 strains were classified in the group "other" staphylococci. Considering the importance of the antibiotic resistance phenomenon, the methicillin resistance was phenotypically tested for the classification in the MRSA.

Of the total of 36 strains of *S. aureus*, 21 were identified as methicillin-resistant (58.33%.). We must highlight that almost half of the owners came for laboratory tests after the patients received one, tow or even more antibiotics, as first-line treatment. It is the method by which antibiotic-resistant bacterial strains are selected, which in the end also affects human health, which is natural considering the cohabitation that is so tight between the human and his pet. Phenotypic tests have included pathogenesis tests such as haemolysis and the presence of coagulase. Pathogenesis tests showed that 14 isolated strains of *Staphylococcus spp.* did not have haemolysis and came from cases of superficial dermatitis, with a faded clinical picture: mild alopecia, erythema, mild desquamation of the tegument. Of the 85 coagulase-positive strains, 19 strains coagulated rabbit plasma within 4 hours, and the rest within 24 hours. This result was correlated with the clinical picture and it has been noticed that the strains came from patients who had purulent material or purulent exudates/ulcerations/fistula.

In chapter V – "Genotypic characterisation of staphylococcal isolated strains and statistical analysis" – the research was fathomed with molecular epidemiology studies on the isolated strains of *S. aureus* şi *S. pseudintermedius*. For the genotypic identification, 83 strains of *Staphylococcus spp.* have been selected. PCR identification of species of staphylococci isolated from pyodermas has confirmed that *S. pseudintermedius* is the main etiological agent in pyodermas in dogs; in cats, *S. aureus* was identified in a percentage of 91.30%. In regard to otitis, genetic identification of *Staphylococcus spp.* was the

following: 10 strains of *S. aureus*, 3 strains of other species, and no strain of *S. pseudintermedius*. For the genetic characterisation of the staphylococcal isolated strains, tests were made on a number of 76 strains of *Staphylococcus spp*. They came from 64 cases of dermatitis and 12 strains of otitis externa.

Genetic characterisation of staphylococcal isolated strains aimed at identifying sets of genes that codify: resistance to antibiotics (*mecA, mecC, mup, fusB, fusC, fusD*), resistance to antiseptics (*smr, qacA/B*), virulence genes (*lukS, lukF, siet, sec$_{canine}$, expA/B, PVL*), and formation of biofilm (*icaA, icaD, bap*).

From a clinical point of view of the aspect of the lesional picture, identification of virulence genes is relevant because: *Luk S* and *Luk F* – present in *S. pseudintermedius* – codify a leukotoxin, similar to PVL produced by *S. aureus* (Futagawa S., et al., 2009), with cytotoxic effect by creating pores in the membrane of the target cells (Guillet V., *et al.*, 2004); *PVL* – extremely virulent exotoxin, characteristic to *S. aureus*, it transforms the host tissues into nutrients necessary for bacterial growth and development (Melles DC., *et al.*, 2006); *Siet*, *exp A* and *exp.B* are genes that codify the production of exfoliative toxins, the presence of *siet* being significantly associated from a statistic point of view with skin infections (Ruzauskas M., *et al.*, 2016); *Sec$_{canine}$* codifies the enterotoxicity of staphylococci, but it has also been identified in strains of *Staphylococcus* isolated from animals from different pathologies, with the ability to cause vomiting and proliferation of T cells. Identification of these genes in strains of *S. pseudintermedius* confirms their enteropathogenic potential (Garbacz K., *et al.*, 2013).

The analysis of the 4 big categories of genes showed that the virulence factors had the biggest impact on the pathogenic mechanism. It has been noticed that in the case of skin infections, in the 44 strains "carrier" of virulence genes, no less than 145 genes have been identified. 16 strains with 4 virulence genes each, 26 strains with 3 virulence genes each and 3 strains with a single gene that codifies virulence factors have been identified. Thus, at the molecular level, the aggressiveness of strains of *Staphylococcus spp.* isolated from pyodermas has been confirmed.

From the point of view of treatment with antiseptics, antibiotics and formation of biofilm (as a form of resistance to antibiotic therapy), we noticed that 83.2% of the strains isolated from otitis had genes from these three categories, specifying that more than half of this percentage comes from the group of genes that codify the formation of biofilm, and in the case of pyodermas the percentage was 37.4. The obtained results explain, at least in part, the therapeutic failures and relapses; also, they argue the need of further research in finding new therapeutic solutions.

Chapter VI – "Initiation of etiotropic therapy in staphylococci in dogs and cats, and perspectives in using new antibacterial compounds" – presents the research focused on the resistance profiles of staphylococcal strains, their classification in MDR, XDR and PDR, and *in vitro* testing of the antibacterial effect of PAW as a therapeutic perspective.

In the first part of these tests, we aimed at establishing the sensitivity/resistance profile to antibiotics, essential in properly choosing the first-line treatment. This is required all the more so since, many a times, due to objective reasons (lack of a microbiology laboratory near the veterinary practice) or subjective reasons (which are related to the owner), the first-line antibiotic therapy is not followed by a bacteriological examination and an antibiogram. It is one of the reasons of developing antibiotic resistance, which hinders the effort of clinicians to treat pyodermas in pets. A therapeutic alternative is the

concept of "rotating" the antibiotics in order to try reducing the pressure of screening resistant strains and extending the duration of action of the compounds. Practically, this involves regularly replacing first-line antibiotics with other classes of compounds (Masterton, 2005).

The tests were carried out on a batch of 85 strains of *Staphylococcus spp*. coming from 61 dogs and 24 cats with pyoderma and otitis externa. Form the anamnesis, it resulted that 55 patients (64.7%) benefited from antibiotic therapy prior to collection of samples for the bacteriological examination and antibiogram; 9 owners (10.5%) stated that they did not give antibiotic, and for 21 cases (24.7%), this information did not exist. Methicillin resistance (MRSA) was confirmed in a percentage of 32.3 strains of *S. aureus*, which increased the resistance to beta-lactam antibiotics in a percentage of 64.7% for AMC, 76.4% for AX, and 64.7% for AML.

Another form of identified resistance was the inducible resistance to clindamycin in 13 tested strains (15.29%). Such strains are reported as resistant to all lincosamides and macrolides, being strains carrier of the *erm.* resistance gene (Andrews *et al.*, 2011; Lewis *et al.*, 2005). The results of testing of strains of *Staphylococcus spp.* in the 11 classes of antibiotics showed that the first three classes of antibiotics in which the tested strains had the highest percentages of resistance were the penicillins, followed by polypeptides and tetracyclines. The results are in correlation with the most used antibiotics that are found in the topical-use products, but also in the first-line antibiotics used in the area from where the cases came. The classes of antibiotics where the tested strains had a high percentage of sensitivity proved to be rifampicins, quinolones and chloramphenicols. We believe that an interesting aspect is the resistance to fusidic acid (11.76%) – phenotypically detected and genotypically confirmed (13.1%), given that from the performed anamnesis it resulted that none of the patients was treated with this antibiotic. This aspect can be a confirmation of the circulation of strains in nature, or it might be about a possible cross-resistance (O'Neill *et al.*, 2002). Also in the first part of chapter VI, due to the studies carried out at the Faculty of Veterinary Medicine Iaşi în 1998 by Prof. Solcan, within his doctoral thesis, we were able to follow the evolution of antibiotic resistance in 13 antibiotics. Thus, we noticed that the highest percentage increases of antibiotic resistance were in the case of Amoxicillin + clavulanic acid (from 0 to 60), followed by Amoxicillin / Polymyxin (from 0 to 57.64), and Neomycin (from 0 to 51.76).

In the second part of chapter VI we presented the results regarding the classification in MDR, XDR and PDR. By initiative of the European Centre for Disease Prevention and Control, an internationally standardised terminology has been created where the resistance profiles acquired by several bacteria, among which there is also *S. aureus*, are described. The lists with the classes of antibiotics proposed for testing the resistance profile have been created and made available by the Clinical and Laboratory Standards Institute (CLSI), the European

According to Committee on Antimicrobial Susceptibility Testing (EUCAST) and the Food and Drug Administration (FDA) from the United States of America, the definitions for MDR, XDR and PDR are: **MDR –** isolated strains resistant to **at least one antimicrobial agent** of **at least three classes** of antibiotics; **XDR** has been defined as the resistance to **at least one agent of all classes**, **except no more than two classes** to which it has total sensitivity; **PDR** are the isolated strains resistant to **all agents, of all classes** of antibiotics.

Following these definitions, we must highlight that: all MRSA isolated strains are considered from the beginning MDR because the resistance to oxacillin/cefoxitin causes the resistance to all existing beta-

lactam antibiotics. Similarly, resistance to clindamycin is reported as resistance to all lincosamides (lincomycin) and macrolides (clindamycin, spiramycin, clarithromycin, azithromycin, roxithromycin), the isolated strains being classified as MDR.

The list of classes and used antimicrobial agents is a rough guide; by representing just a guide, it can be adapted and must be completed as new substances become available for therapy (Magiorakos *et al.*, 2012). In our research, we adapted some antibiotics with the ones used in the geographical area included in the study. Testing the sensitivity/resistance profile in the 11 classes of antibiotics has proved that, of the 85 strains of *Staphylococcus spp.* coming from pyodermas in pets (dogs and cats), 22 strains had sensitivity to all tested classes (with resistance to no more than one antibiotic of no more than two classes), 61 strains can be classified as MDR, having resistance to at least one antimicrobial agent of at least three classes of antibiotics, and 2 strains had resistance to at least one antibiotic of all classes (except for two), classifying as XDR. No PDR strain has been detected.

The last study of our research focused on the *in vitro* antibacterial effect of PAW, its use in dermatology representing a viable solution in regard to mechanisms of resistance to antiseptics and disinfectants. The adaptive mechanisms and the resistance of bacteria to antiseptics and disinfectants are well-known, resistance being a natural (intrinsic) property or an acquired property due to mutation or acquisition of plasmids (self-replicating DNA and extrachromosomal DNA) or transposons (chromosome transposons or integration plasmids) (McDonnel *et al.*, 1999; Grundmann *et al.*, 2006). In the world-wide fight against staphylococcal skin infections, a special case is the methicillin-resistant staphylococcus, which is considered a global threat (Grundmann *et al.*, 2006), the development of alternative topical treatments being beneficial.

Water that is activated with cold plasma acquires an acid pH and contains, among other groups: hydrogen peroxide (H2O2), nitrate (NO3), nitrite (NO2) and anion, responsible for the degradation of the bacterial wall, through an oxidative effect on the proteins, lipids and DNA (Oehmigen *et al.*, 2010; Brisset *et al.*, 2012).

The efficacy of inactivation by using this method depends on abiotic factors (temperature, hardness of water, quantity of organic substances and nutrients for the bacterium) and biotic factors (structure of the cellular wall, presence or absence of capsules, ability to sporulate) (Naitali *et al.*, 2010; Kamgang-Youbi *et al.*, 2008). Previous studies show that the antimicrobial action of PAW increases in the following, in this order: yeast, gram-positive bacteria and gram-negative bacteria (Kamgang Youbi *et al.*, 2009; Ursache *et al.*, 2014).

The tests were carried out on samples that had the initial bacterial load between $4.78x10^8$ and $8.3x10^{10}$. In the 3-minute treatment of strains with PAW, the reduction levels varied from 0 to 7.38 with an average of 5 log, and in the 10-minute treatment the minimum and maximum reduction was 4.99 and 9 respectively, with an average of 6,99 log. Considering that the bacterial loads higher than 10^5 CFU/tissue correspond to infection, and for bacteria such as beta-haemolytic streptococci, *S. aureus* and *P. aeruginosa* much lower loads are sufficient, we believe that the results of the reduction obtained *in vitro* are conclusive for the use of PAW as antimicrobial agent (Heinlin *et al.*, 2011).

In chapter VII – “Final conclusions and recommendations” – 26 conclusions are synthesised, which represent conclusive aspects of the research, and a few recommendations arising from the obtained results are issued.