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SUMMARY OF PhD thesis

Reciprocal relationship of *Parapoxvirus* virus with secondary infection microbiome and the immune system in sheep with orf

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REZUMAT

Sheep farming is a traditional activity, closely related to the history of the Romanian people. Shepherding, an important side of Romanian agriculture, ensured the need for meat and the continuity of civilization in Romania's pastoral lands. Meat, dairy products and leather obtained from sheep farming ensure the subsistence of households, trade and the stability of the labor force in rural areas. Sheep meat is one of the products of animal origin with a high export rate in Romania, sheep breeding sector showing a significant development, by ensuring the need for domestic consumption and creating availability for export. The variety of products obtained from sheep breeding has been and is a particularly important economic advantage (Gulyaz et al., 2020).

Contagious ecthyma is an acute infectious disease of sheep and goats, caused by an epitheliotropic virus, clinically characterized by vesiculopustular eruption, with oral, feet, genital, mammary and ocular involvement (Moga et al., 2005, Haig and Mercer , 1998). The disease is also known as contagious pustular dermatitis, infectious labial dermatitis, scabby mouth disease or orf (Haig, 2006). Contagious ecthyma has been known since the eighteenth century in Germany, its viral etiology being first mentioned by Aynar in 1923 ("chancre du mouton"). In Romania, the contagious ecthyma was first documented by Riegler in 1935 near Bucharest, but sheep breeders knew it beforehand as " curd in the mouth". The first to study the contagious ecthyma in our country were Grigore, 1955, Verdeş et al. (1960), Ivana Filea (1968).

Currently contagious ecthyma is endemic in all continents, but with a higher incidence in Australia, New Zealand, Asia. In Europe, the existence of contagious ecthyma has been reported in France, Norway and Greece, in other countries, including Romania, only isolated outbreaks being recorded (Moga, 2005). According to the World Organization for Animal Health (OIE), contagious ecthyma is an important infectious disease also a zoonotic one (Nadeem et al., 2010). In humans, lesions remain localized, and infections on hands are relatively common in people who closely work with animals in the sheep industry (Fleming et al., 2015).

The causative agent of orf contains DNA and belongs to the family *Poxviridae*, genus *Parapoxvirus*, named Orf virus or Contagious ecthyma virus, sized 158 x 252 nm with its morphology similar to the vaccinal virus (*Vaccinia Virus*)(Moga, 2005). The orf virus has been extensively investigated in recent years, due to its zoonotic character and the potential to cause cross-infections between various species (Hosamani et al. 2009, Damon, 2007).

Being an extremely contagious disease, orf spreads rapidly in the herd, sometimes the morbidity reaching 100%, thus increasing the loss of farmers. Lesions are difficult to heal, often complicated by secondary infection agents, augmenting the economic impact due to the costs generated by antibiotic therapy (Ravi et al., 2000). Infection with orf virus causes a decrease in feed conversion rate and in lambs, which are more susceptible, can even lead to refusal of suckling and death by starvation (Gökce et al. , 2005). In mammar involvement, sheep may refuse to feed their lambs. Due to lesions that can occur in the external genitalia, the breeding season can be compromised, resulting in fewer lambs in the following year.

Among domestic animals, the virus affects sheep, goats, domestic camelids and other domestic ungulates, or even dogs (McGuire et al., 2012). It seems that, along all domestic host species, the most affected are goats, especially kids (Buttner et al., 2002). The most susceptible are young animals, especially in the first year of their life. Over time, contagious ectemia began to apper in wildlife species, due to direct or indirect contact with affected sheep or goats, vaccinated or diseased.

The incubation period of the disease is 6-8 days. Contagious ecthyma usually begins with the appearance of maculae, papulae, blisters, pustulae, and crusts, which heal and detach within about 1 to 4 weeks (Pugh and Baird, 2012). A atypical course was also reported in 10 to 21 days old lambs, which is a lighter form and can be cured in 7 days. In lambs, the development of the disease is sometimes very severe, peracute, with altered general condition and edema of the muzzle, followed by a confluent and massive vesiculopustular rash on the lips and oral mucosa (Trylan et al., 2013), with regional lymphadenopathy. Sometimes, due to the extension of the lesions on the respiratory and digestive mucosa, symptoms of bronchopneumonia or enteritis appear, with a lethal outcome in 36-40 hours. However, usually, the evolution of contagious pustular dermatitis is acute or subacute, with oral, peribucal and to a lesser extent podal, mammary or ocular locations, without influencing the general status (Cargnelutti et al., 2011). In case of appearance of the lesions on the oral mucosa, it is useful to provide veterinary assistance by bottle feeding, and gently treating the oral lesions with antiseptic substances (glycerinated iodine tincture 50%, methylene blue, etc.), antibiotic therapy if it is necessary. Particular attention should be given to secondary bacterial agents, in this case treatment with injectable antibiotics is recommended, after testing for antibiotic susceptibility of the bacteria involved. Similar to other members of the *Poxviridae* family, the immune response to contagious ecthyma virus is mediated by both innate and adaptive brances of immunity. After vaccination with live virus, the synthesis of neutralizing antibodies occurs after the first week subsequent to vaccination and persists for years and can be stimulated by booster vaccination (Bala et al., 2018). IgM has been shown in sheep with recent infections. Hemagglutination inhibiting antibodies appear immediately after infection, then gradually decrease to low levels after a few years. However, the association

between circulating antibodies and their titers and the level of protection against orf virus is uncertain. Cell-mediated immunity plays a major role in the immune response. It is known that parapoxviruses stimulate local immunity and cell-mediated immune responses are of greater importance in removing the virus from the dermal layer. Immune response in case of orf infection is different from the immune responses to other viral infections, cell-mediated immunity interfearing with the virus before the antibodies.

The thesis entitled "Reciprocal relationship of *Parapoxvirus* virus with secondary infection microbiome and the immune system in sheep with orf" is structured in two main parts, namely: the Current state of knowledge which includes three chapters and Personal research, which includes nine chapters. This work contains 159 pages, including 46 tables, 57 figures and 221 bibliographical references. The first part, "The current state of knowledge", presents from the up-to-date literature, an overview of orf including etiopathogenesis, circulating viral strains, treatment and prophylaxis, diagnostic methods, immunology of the disease. **Chapter I** presents virological information (etiology and taxonomy), structural features, methods of diagnosis and detection of contagious orf virus. **Chapter II** contains data on the host species of the virus, strains circulating worldwide, pathogenesis, treatment, prophylaxis, control and production of vaccines. This chapter also includes data on secondary infections (bacterial, viral or parasitic). **Chapter III** describes peculiarities of the immune system and of the immunological effectors in sheep, respectively the immune response in case of infection with contagious ecthyma virus. This chapter presents the components of the immune system that play roles in the immune response, respectively the changes that the virus triggers once it enters the body. The mechanisms by which the contagious ecthyma virus bypasses the defense system of the host organism also presented.

The second part, "Personal research", structured in nine chapters, begins with the purpose of the paper (**Chapter IV**), presenting the motivation of the research. **Chapter V** included the epidemiological investigations: individual characteristics of the sheep flocks from which samples were collected, with data on the hosts (age and gender ranges), severeness of the clinical signs, which were also evaluated from a statistical point of view. This chapter also deals with the cisrcumstances of the occurrence of the disease and sanitary veterinary staus of the flocks. General measures of prevention have been taken as isolation of sick sheep and use of symptomatic treatment and antibiotic therapy as appropriate. The biological material used had been collected from 96 sheep with clinical signs while 18 control group samples were treated in comparison. The results indicated the disease dominating in females, the age of the affected sheep being variable. In most of the cases clinical signs were mild, but

from a statistical point of view the trend was towards medium severeness. **Chapter VI** presented the biological materials used in each test as follows:

- for microbiological methods, swabs soaked in sterile saline solution were used and 96 samples were stored in sterile containers.

-for the PCR test, 96 samples were also collected, represented by crusts from contagious ecthyma lesions.

-for electron microscopy, those crusts collected for the PCR technique were used, which tested positive for contagious ecthyma.

-for immunological tests, 96 samples were collected in sterile vacutainers, testing only a number of 48, non-hemolyzed samples. In parallel, 40 samples were collected from the control group, while submitted for testing were only 18 non-hemolyzed samples.

All samples were collected avoiding further trauma to the animals and divided into batches according to the herd they came from. Transport of the samples was done avoiding thermal or other shocks, in refrigeration conditions, then stored in conditions suitable for the purpose.

Techinques used for laboratory processing of the samples were presented in **Chapter VII;** for microbiology, classical bacteriological and bacterioscopic methods were used to observe the isolated colonies, their biochemical characteristics (catalase test, mannitol fermentation, presence of lecithynase, etc.), and microscopic characteristics. Antibiograms were also performed by the Kirby-Bauer method using discs with gentamicin, trimethoprim, cefuroxime, amoxicillin with clavulanic acid, enrofloxacin, ciprofloxacin and penicillin. PCR test and transmission electron microscopy were used for diagnostic purposes. Immunological tests used a final stage of spectrophotometry to quantify the levels of circulating immune complexes (Haskova method) and total immunoglobulins (Serb reagent method). The statistical analyzes were performed using GraphPad Prism 8 program, to underline the development trends of the tested parameters.

Chapter VIII referred to the results of the PCR and electron microscopy. Thirty-three samples tested positive in the PCR. The identification of the virus was performed based on the presence of the B2L-capsid gene fraction. From the positive samples, electron microscopy was performed, the presence of the virus being detected only in two samples. This chapter also highlighed the advantages and disadvantages of each PCR and electron microscopy. PCR technique proved to be more advantageous in terms of costs, processing time and sensitivity, however the positive testing of only 33 samples indicating the existence of a reduced amount of viral genetic material, which may indicate that the disease was in the remission phase. However, the presence of a large number of bacteria indicates that the virus has disappeared from the tissues being replaced by secondary infection bacteriome.

Chapter IX presented the results of microbiological methods applied to the samples. Thus, bacterioscopy and cultivation were performed and subsequently, biochemical particularities, differentiation tests were performed against other bacteria with similar characteristics. *Staphylococcus aureus*, *Corynebacterium pseudotuberculosis*, *Bacillus cereus*, *Pseudomonas aeruginosa* were isolated. Secondary infections with *S.aureus* dominated (58%), and 41.67% of the analyzed samples showed secondary infections. Samples infected with two bacteria strains were also identified (30 out of a total of 40 samples with secondary infections). The highest number of secondary infections was from identified in herd 1.

Evaluation of antibiotic resistance revealed the lack of efficiency of some antibiotics commonly used in veterinary therapy (penicillin or amoxicillin with clavulanic acid). The effectiveness of antibiotics was also analyzed from a statistical point of view, performing descriptive statistical analysis. The average value of the inhibition diameters does not fully characterize the effectiveness of an antibiotic. Similarly, a 95% probability of average MARs did not define the antibiotic resistance trends. The diameters of the inhibition zones vary from one herd to another, suggesting that the values obtained must be analyzed and interpreted separately for each herd due to the differentiated distribution of secondary infection bacteriome in different flocks. More constant results were obtained when testing gentamicin and trimethoprim.

To evaluate multiple antibiotic resistance, MAR index was calculated, representing the ratio between the total number of active antibiotics and the total number of antibiotics used for testing. Most of bacteria resisted to two antibiotics while *P.aeruginosa* was resistant to 3 antibiotics. Standard deviations and the variability coefficients indicated with a probability of 95% the tendency to be resistant to 2-3 antibiotics in isolated bacteria, respectively 3-4 antibiotics in case of *P.aeruginosa* strains. Pearson correlation applied to the relationship of gender, age, clinical signs and MAR indices showed that rcoefficients were not supported statistically, which indicated that MAR vaalues were strictly influenced by the characteristics of isolated bacteria.

Chapter X described the evolution of CIC and total Ig in herds with and without secondary infections using the Pearsons correlation and the t test. Following statistical investigations it was concluded that there are no differences between the course of CIC synthesis and total Ig in sheep with and without secondary infections, the average values being close as well as their evolutionary tendencies, which means that the values of these two immunological parameters were not influenced by the presence of the suprainfection flora.

Then, the evolution of these parameters was also analysed in the 4 experimental herds tested for CIC and total Ig. In each herd as in the control herd these two parameters presented a great variability, for this reason we made the correlation between the age of the clinical signs and the gender of the sheep for which CIC and total Ig were quantified. Pearsons correlations, t tests and ANOVA tests were performed for all herds. Correlations between animal gender and Ig were observed, so Ig values tended to be higher in females. Total Ig and CIC values compared to the control herd did not return a significant difference indicating that either the control herd had contact with the virus or the experimental herds were in the early stages of the disease when the virus disappeared from the tissues, the disease being in the remission phase.

A significant difference was observed when comparing the values between herds 1 and 2 with herd 4, as well as between herd 4 and the control herd, which indicates that there is a possibility that herd 4 is in the initial phase of the disease. The fact that total Ig showed insignificant variations underlines once again the weak involvement of immunoglobulins in the defense mechanisms against orf, as they appear lately in the affected tissues and disappear just as quickly. Knowing that in orf local immunity plays an important role (Hajkazemi et al., 2016, Haig et al., 2006) and considering that systemic immunity against orf virus is of very short duration (Karki et al., 2019), normally the total Ig level should remain constant and increase after the end the disease, any change regarding the increase of the Ig level or of the CIC was considered to be due to other factors. Following the statistical calculations performed and the results obtained above, we could conclude that the level of circulating immune complexes was higher in sheep with secondary infections than in sheep without secondary infections. Considering that most of the sheep (31 animals) tested did not exceed the age of one year and orf did not evolve in the area where the samples were collected for many years, we could conclude that high levels of circulating immune complexes were associated with the existence of secondary infections. However, the fact that the sheep are clinically healthy does not exclude the fact that they have had mild forms of orf and have recovered without sequelae or are carriers of the virus. Assessing total Ig and CIC levels is not a diagnostic method, but comparing these values with values of a control herd or with values obtained in sheep with secondary infections can provide us with information on the stage of the disease and whether changes in their levels were observed or not is caused by the presence of orfvirus.

In **Chapters XI** and **XII** the general conclusions and the originality and innovations provided by the doctoral thesis were presented. The age groups were all affected indicating that the animals could become infected and re-infected even after being through the disease. A higher frequency of orf was found in females and young animals. Given the large number of bacteria that were observed in the electron microscopy and the fact that only two samples revealed the presence of the virus, it

was concluded that the disease was in remission stage of the viral infection, secondary infections dominating. The information in the literature confirms that secondary infections are common in orf, the virus creating gateways for opportunistic bacteria.

The comparison of the two diagnostic methods, PCR and electron microscopy, confirmed the supremacy of PCR over electron microscopy. It was confirmed that multiple antibiotic resistance was still a challenge for modern medicine. The large number of strains resistant to 2-3 antibiotics jeopardised the effect of the therapy. In order to establish a correct antibiotic therapy scheme, also investigate the characteristics of the affected herds. The MAR trend between bacteria was the same except for *P.aeruginosa*, which proved to be more resistant, confirming it must be treated with caution. Finally, antibiotic susceptibility testing and the calculation of MAR indices are not reliable information regarding the establishment of a treatment, since there could be different *in vivo* effects of antibiotics, therefore in case the infections are not responding to therapy, it is advisable to gentetically test the bacteria. These genetic tests require time and high costs, the most convenient method of preventing multidrug resistance is the judicious use of antibiotics, strictly following a medical prescription.

This thesis also aimed at conducting a study on the variability of the values of circulating immune complexes and total immunoglobulins in groups of sheep with and without secondary infections. The literature did not include such an approach so it was considered appropriate to observe the dynamics of these parameters. The levels of total Ig and CIC could be influenced by the intrinsec characteristics of sheep affected by the contagious ecthyma virus and varied when compared to clinically healthy animals. The age of the animal could influence their levels, with higher values occurring in young. However, the fact that the sheep are clinically healthy does not exclude the fact that they have had mild forms of contagious ectemia and have recovered without sequelae or were carriers of the virus. Assessing total Ig and CIC levels was not considered as a diagnostic method, the comparison of these values with those of the control herd or with those in sheep with secondary infections could provide with information on the stage.

The innovative contributions of the thesis include:

- In relation to the available literature, the paper is the first global approach to the correlation between epidemiological (sex, age), clinical indicators (severity of lesions) and MAR indices of microbial flora of superinfection in sheep with contagious ecthyma.

- For the first time worldwide, in this doctoral thesis it was proven by the functional quantification of mechanisms involved in the disease (viral

presence, bacterioma of secondary infection, nonspecific humoral immune response) that the complex lesional, microbial and immunological etiopathogenetic profile is statistically supported.

- The paper is the first study worldwide which statistically supports the relationship between immunological parameters (total Ig levels, CIC), epidemiological indicators (sex, age of animals) and anatomical-clinical (severeness of lesions) in animals with contagious ecthyma.

- The paper addresses comparatively, for the first time in the country, with highlighting of advantages and disadvantages, two diagnostic methods for contagious ecthyma - PCR and electron microscopy.

- The present study addressed for the first time in the country the correlative evaluation of the results of PCR tests with clinical signs of contagious ecthyma and the presence of secondary infection flora, providing diagnostic and therapeutic guidance.

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