ABSTRACT

In Romania, swine farming begins to develop more and more and found as a system of specialized farms (piglets producing, young growth, fattening or finishing), with units of closed loop type. The need for research on swine coronaviruses is justified by the danger represented this family of viruses for both pigs, other animal species and human beings. In the past, the infection with transmissible gastroenteritis virus has evolved in our country, causing huge losses. For several years, in Asia and the US is evolving a highly virulent strain of porcine epidemic diarrhea virus. The emergence of a new coronavirus with digestive tropism, highly pathogenic (delta coronavirus), which evolves with high morbidity and mortality in piglets from China and the US, draws attention once again to the *Coronaviridae* family, which shows a high variability and pathogenicity. On the other hand, the persistence of diarrheic enteritis with viral etiology, in the current livestock, enteritis that have a recurring trend, low morbidity, but are consistently present, primarily reflects a lack of compliance with zoo-hygiene conditions and constitute an risk factor for all livestock.

The doctoral thesis entitled "Research regarding coronavirus infections in swine" presents a research regarding the presence of coronaviruses in some farms from the Nord East of Moldova, an assessment of the sensitivity of various diagnostic methods compared to the collected samples, a serological investigation of two swine coronaviruses and a phylogenetic analysis of strains of coronavirus identified on the field.

The doctoral thesis comprises a number of 184 pages, written in X chapters and is structured in accordance with current legal scientific standards in two main parts: the first part and the second part, plus table of contents, introduction, summary and references list.

The first part "Current state of knowledge" is structured into 45 pages, 4 chapters, 15 subchapter and 17 figures. In order to achieve the knowledge state, 261 titles form literature were consulted.

The second part comprises a series of surveys in order to detect and differentiate the swine coronaviruses and seroprevalence of swine livestocks from Moldova, the materials and the

methods used, the interpretation and the discussions of the obtained results, and also the main conclusions drawn after the research conducted.

The thesis is comprising a number of 84 figures and 29 tables.

The first part of the paper is comprising a synthesis of the literature on piglet coronaviruses, and also the current state of knowledge nationally and internationally.

- Chapter I, entitled „*Bibliographic data regarding the history, spreading and the etiology of swine coronaviroses*", consists of three chapters and it is related to the importance of swine coronaviruses, taxonomic classification, structure and genome replication, describing also the role of the structural proteins. The coronaviruses studied in this thesis are alpha coronaviruses, of which two have an enteric tropism (TGEV and DEPV) and one has a respiratory tropism (CVRP).
- In Chapter II, „*Bibliographic data regarding the epidemiology, pathogenesis and immune mechanisms involved in swine coronavirus infections*" describes the epidemiological evolutionary model of TGEV, PEDV and PRCV, which are the risk factors and the infection sources and how the infection is caused at the pathogenetically point of view; there are also described the cellular receptors that are interacting with the swine coronaviruses in order to penetrate the host cell.
- Chapter III, „*Bibliographic data regarding the clinical signs and the pathology in swine coronavirus infections*„ describes the clinical, macroscopic and histopathological lesions produced by the presence of the viruses. Given that the symptomatology and lesional picture has a nonspecific nature and is not allowing a differentiation between coronaviruses and other enteric viruses known to infect the small intestin (porcine rotavirus group A), there are necessary laboratory exams in order to confirm the suspected diagnosis and to determine accurately the nature of the etiologic agent.
- Chapter IV, „*Bibliographic data on diagnosis, prevention and control of swine coronavirus infections*" comprises three subchapers which are extensively detailing all the diagnostic methods described in the literature, the advantages and the disadvantages for each method. Regarding the prophylaxis, a major problem is the failure to obtain a vaccine with satisfactory results, which during the epidemic evolutions of TGE and PED might lead to important looses behalf of the farmers.

The second part of the thesis comprises the own research, being structured in 6 chapters.

- Chapter V, „*Purpose and objectives*", it consists of a brief overview of the objectives and activities needed to achieve them. The determination of the most sensitive method for the detection of viral antigens from the collected samples in order to assist physicians in the field, was one of the aims of these thesis. In the same time, it was meant to provide a serological screening in farms and households in order to evaluate the seroprevalence of antibodies against TGEV and PRCV. By RT-PCR and sequencing assays, it was aimed to identify the species of coronavirus implicated in the disease process and its characterization in terms of disease evolution.
- Chapter VI, entitled "Epidemiological, anatomo-clinical and morphopathological investigations in swine coronavirus infections" is consisting is 5 subchapter. At the beginning, it is given the situation of the livestock which have been sampled, the geographic area in which research has been extended and type of the investigations conducted.

In the epidemiological research subchapter, an epidemiological investigation was conducted over three years (2010 - 2012) in two swine livestocks where constantly were evolving diarrheic enteritis, suspected with a coronavirus infection. The age category with the highest mortality was represented by new-born piglets, with a mortality of 2.74% for the farm E and 5.27% for the farm L. The enteropathies were representing in the both situation the death cause (75.63% for the farm E and 65.09% for farm L). Given the changes in the intensive farming system, the incidence of coronavirus diseases with dramatic events decreased considerably in the study area, although the virus persists in a form of resistance in these livestocks. The way of spread and diffusion in these livestocks decreased and maintained to the age category of new-born as the most receptive.

The clinical research aimed the examination of 125 animals in eight farms and households, whereby were revealed clinical manifestations of gastroenteritis: yellow diarrhea with mucus, severe dehydration and loss of appetite. The lesions observed in the 69 necropsied cadavers were consisting of: catarrhal gastroenteritis, mesenteric lymph node hypertrophy and congestion, gas distention and thinning of the small intestine. Histologically, there was observed a pronounced atrophy of intestinal villi, in enterocytes were highlighted some hyaline inclusions, the necrosis of the digestive epithelium, oedema of villi stroma and lympho-histiocytic inflammation in other organs (lung, kidney). Since the anatomical-clinical aspects have a nonspecific nature, they could not differentiate the viral enteritis induced by TGEV, PEDV or PRV, thus the research has continued with more specific methods.

- Chapter VII entitled „*Serological diagnosis in porcine coronavirus and rotavirus infections*" contains four subchapters related to the diagnostic methods used:

immunochromatographic test (ICT), direct immunofluorescence (DIF), immunohistochemistry (IHC) and ELISA.

The immunoassay test was intended to detect the presence of antigens TGEV, PEDV and PRV by using rapid tests. A number of 74 faeces pools were tested, coming from 8 farms. Out of these, 27.27% of them were positive for at least one of the three viruses tested, and 23.80% of the viral enteritis have been caused by multiple virus infections, which represents a new direction for diagnosis protocols and research field.

By DIF, a number of 74 smear tissue samples (duodenum, jejunum, lymph nodes, kidney) collected from 19 piglets and 10 samples of faecal samples coming from pregnant and lacting sows from farm E were tested with an specific antibody conjugated to TGEV. The specific reaction was visualised as a marked intracytoplasmic fluorescence and sometimes as fluorescent pericellular halo. A positive answer was given by 22.09% of the samples tested, while an increased sensitivity was noted for the jejunum (52.63%) and mesenteric lymph nodes (21.05%) smears.

The IHC reaction was performed on tissue sampes collected from 47 necropsied piglets, coming from two piglet breeding establishment with suspicion of infection with coronaviruses with digestive tropism. Following the reaction with the antibody FIPV3-70 tested on 188 samples, 52 samples reacted positively. The most sensitive tissues were the same as for DIF (jejunum and mesenteric lymph nodes); however, by this test a greater number of antigens (27.65% positive reactions) was detected, having a superior sensitivity comparing to DIF test.

By ELISA testing, it was meant a serological assessment of the farms and of the household taken under study. Thus, sera from 12 farms and 11 households were tested for antibodies against TGEV and PRCV. Although many EU countries report a PRCV prevalence over 50%, the results obtained by the present study were much lower. In the farms was found a prevalence of 22.95% against PRCV antibodies, while in the housholds, the specific antibodies against PRCV were found even in a smaller proportion, of 17.18%. This suggests an immunological discovery and a risk for swine population in this area. To confirm the presence of of coronaviruses in these livestocks, it is necessary to use a specific method for the antigen detection. Regarding the TGEV infections, no specific antobodies were detected either in households nor farms.

- Chapter VIII refears to „*Identification of swine coronaviruses and rotavirus group A by moleculat biology techniques*" more precisely by reverse transcription PCR technique. This is the most frequent technique cited in the literature, proving a superior specificity and sensibility comparing to the other methods.

It was intended the identification of TGEV, PEDV and PRV in faeces pools in order to analyze the etiology of viral enteritis, but also the detection PRCV in swab samples. In this respect, firstly, it was performed a viral RNA extraction from pool samples, which was subsequently subjected to the process of reverse transcription for cDNA synthesis. Subsequently, the samples were tested with a series of specific primers for each of the above viruses. Firstly, to identify coronaviruses in the tested samples a general primer pair was used, which corresponds to a well conserved region of the nucleoprotein gene (Ncons). By amplification with these primers, there were obtained 19 coronavirus positive faecal samples and 5 samples of swabs. This result confirms the superiority of the the sensibility of the RT-PCR reaction (37.25%). Although a part of the the samples tested gave a positive response by using the general primers Ncons for coronaviruses, when tested with specific primers that differentiates TGEV by PRCV and PEDV, no positive samples were obtained. This contradictory result can be explained by failure of the specific primers activity. Given that for TGEV and PRCV were used primers targeting the gene S, which showes a high variability, it is possible that the identified coronavirus strains have suffered a series of mutations in this gene that the primer does not longer recognizes the targeting sequence. In the literature is reported that detection of PEDV is more difficult, being required several passages on cell culture of the virus isolated from samples, after which it is recommended the performing of RT-PCR on this product. For the differentiation of these three species it is necessary the sequencing the fragments obtained or the testing with other primers specific for the N gene, corresponding to better conserved sequence.

For the identification of PRV a pair of primers for the gene NSP5 was used, which is the best conserved gene among porcine rotavirus group A. The cDNA from the faeces pools (n=51) previously investigated for coronavirus was tested. A total of 13 (25.49%) positive samples (pools) were obtained for different age categories. By RT-PCR, the number of the positive samples was higher comparing to those obtained by rapid kit testing, result that may be explained by very low amount of viral antigen in the tested sample.

- Chapter IX „*Phylogenetic analysis of nucleotides from the genomes of field strains of swine coronaviruses*”, aims to differentiate the coronavirus species obtained ty RT-PCR. The positive saamples were extracted from the agarosis gel, purified and send to sequencing to a specialized laboratory.
- Chapter X „*Final conclusions*” summarizes a number of 14 final conclusions drawn based on the research results.