# ABSTRACT

The pestiviruses are pathogenic agents that cause important economic losses in swine and ruminants, but they can infect, also, a large spectrum of cloven – hoofed wild species. The classical swine fever and bovine viral diarrhea are diseases with mandatory declaration at World Oragnization for Animals Health (The Internation Office of Epizootics, OIE).

The pestiviruses affect a great number of species, not only domestic but also wild, free or in captivity. In many researches, infections are described in other species than those described in the past. Border disease was diagnosed also in goats, and there are serological and virological proofs that the infection with pestiviruses is frequent in this species. The pestiviruses were also responsible for disease outbreaks in wild ruminants from the zoological gardens. At many wild ruminant species from worldwide were identified specific antibodies against pestiviruses. Their classification was made based on the sensitive species, but there were isolated many times strains of BVDV from sheep and pig and also CSFV from bovine and sheep.

The term of "emerging viruses" includes viruses that can be framing in different categories. There are completely new viruses that appear suddenly in a host species without any other previous description. However, in the course of evolution viruses include the viruses that do not fit with the previously described ones. These can be named in the course of evolution because of the modified transmission, the severity of outbreak, virulence or because crossing the species barriers.

The ‘emergent’ viruses can be viruses that circulated in susceptible populations, for tens of years, but the new methods of surveillance and diagnostic allowed recently their detection in domestic or wild animal populations. Examples of all these categories of "emergent viruses" can be found within the type of *Pestivirus*.

Clinical manifestations were used initially in order to differentiate the recognised species of pestiviruses: classical swine fever virus (CSFV), bovine viral diarrhoea- mucousal disease (BVDV) virus and border disease virus (BDV). In the following years became evident that the clinical presentation of the infection with Pestivirus is very variable according to the age, the immune status of the host and strain virulence. Sometimes the variations of pathogeny of the pestiviruses can affect the control programs as in the case of the CSFV with a low virulence and BVDV with high virulence.

Ruminant pestivirus isolation from naturally infected pigs represent the proof of infection with these viruses, with or without clinical evolution (Fernelius A.L et al., 1973; Terpstra C. et al., 1988). Moreover, it was proved that BVDV could also infect other domestic animals such as pigs (Wang et al.., 1996), and wild species (Vilcek and Nettleton, 2006). BDV can cause natural infections not only in sheep and goat, but also at cattle (Cranwell et al., 2007) and swine (Vilcek and Belák, 1996). The only *Pestivirus* that had not cross the barrier species is CSFV, the infection being limited to domestic pigs and wild boars (Liess and Moennig, 1990; Moennig et al., 2000).

Swine experimental infections with ruminant pestiviruses have proved their sensitivity. The infected pigs developed viraemia and antibodies, and the recovered virus kept its virulence for calves (Passler T., 2010).

Ruminant pestivirus infections of pigs have a worldwide distribution. The prevalence is varied and depends mainly on contact with cattle, age of pigs and degree of homology of virus strains used for serology, with field strains of bovine

virus diarrhoea virus (BVDV) infecting pigs. The available monoclonal antibodies can differentiate between CSFV and the ruminant pestiviruses. The experimental studies explained the swine receptivity and susceptibility to infection with other pestiviruses.

The recent studies highlited the existence of "special structures" in BVDV that ensure the receptivity for bovine cells, but also that some isolated strains can replicate in PK15 line cells (pig renal cells). The pluripotent BVDV strains probably have supplementary attaching epitops for sheep and swine cells. The identification of receptors for the sheep and swine cells could contribute to a clear distinction between the infections with BVDV and CSFV in pigs.

It was suggested that sheep and swine pestiviruses could not infect bovine's cells, excepting the case in which the virus had been adaptated on bovine cell lines after the repeated passages (Moennig, 1990).

Bovine are considered as being the main source of BVDV for pigs. Such crossed infections are present in swines and can interfere with the serological tests used for surveillance at flock level, consequently affecting the eradication programs of classical swine fever.

The PhD thesis entitled**, "The epidemiology and diagnostic of pestivirus infections in Eastern part of Romania''** is structured, in accordance with the norms, in two main parts. The first part, entitled **''The current stage of knowledge''** contains a number of 34 pages. The second part, entitled **''Personal contributions''**, that details the results obtained throughout the period of PhD studies, has a number of 80 pages. Besides these two main parts, the thesis contains table of contents, introduction, summary and references.

The first part**, ''The current stage of knowledge''** is structured in 4 chapters and presents information regarding the general characters of the pestiviruses and infections with pestiviruses in animals. The last chapter of the first part presents different methods of diagnostic used for pestivirus identification.

The first chapter, entitled **''The general characters of pestiviruses'',** starts with pestivirus classification. Pestiviruses were initially classified in three species according to the host receptivity: border disease virus (BDV) in sheep and goat, bovine diarrhea - mucousal disease virus (BVDV) in bovines and classical swine fever virus (CSFV) in swine. Afterwards researches proved that BVDV and BDV are not limited to a single host. Therefore, a pestivirus isolated from swine can belong to one of the three species. Currently pestiviruses are classified in nine different types, and some viral strains are still under consideration. In this chapter is described the organisation of pestiviruses genome, structure, morphology, antigenicity and replicative cycle.

Chapter II, entitled **''Infections with pestivirus in animals'',** describes the diseases produced by pestiviruses in pigs and ruminants. From the beginning of the 19$^{th}$ century, classical swine fever (CSF) was knew and described in the USA. From America, in the years 1860-1862, the disease passed into Europe and it spread on the entire continent. The CSFV highly virulent strains produce high percentages of morbidity and mortality, while low virulent strains can determine only congenital diseases or even sublinical infections.

Bovine viral diarrhoea –mucousal disease is a morbid complex, caracterized by fever, digestive disorders and erosions or ulcers on digestive tract mucosa. In adult bovines, the infection is usually subclinical or sometimes it can be associated with pulmonary or digestive (diarrhoea) signs. In breeding females' low fertility was diagnosed and temporary decrease of fertility correlated with the viral sheding through seminal material in breeding males.

Border disease was signalled for the first time in sheep from Kerry Hill and Clun Forest breed, at England and Wales border, by Hughes et al. in 1959, whence the name of

the disease. In Australia and New Zeeland, it was described under the name of „hairy shaker lamb disease" or „fuzzy lamb" (the shaky and hairy lamb disease) because of the clinical signs. Subsequently, border disease was described in the North America, Europe and Africa.

Chapter III entitled, **''Ruminant pestivirus infection in swine and wild boar'',** describes the term of ''emergent'' virus that can be retrieved within the Pestivirus subfamily. These viruses can be named ''in course of evolution'' because of the modification of transmission, severity of outbreaks, virulence, crossing host barriers.

Within the Chapter IV**, ''Diagnostic of pestivirus infections in animals**'' are described the identification methods of pestiviruses using serological and virological tests (isolation and cultivation of pestiviruses, identification of pestiviruses using immunological methods and molecular biology techniques).

The second part of the thesis, **''Personal contributions''**, consists in four chapters, each chapter presenting the results obtained during PhD studies. This part concludes with a chapter in which the conclusions of the researches are listed.

The aim of the investigations aimed to obtain new information regarding the swines and wild boars infection with ruminant pestiviruses in the Eastern part of Romania. In order to fulfill the suggested aim there were established three general objectives:

- **Seroepidemiological investigations on classical swine fever in the Eastern part of Romania.** Within this objective there were made two seroepidemiological studies. The prevalence of specific antibodies against-CSFV in swine serum or plasma from Vaslui, Suceava and Iaşi County was established in the first investigation. The second investigation was conducted in order to determine the prevalence of specific antibodies against-CSFV in wild boar serum or plasma from Vaslui, Suceava and Iaşi County.
- **Detection of classical swine fever virus** aimed to identify the classical swine fever virus in swine and wild boars using direct immunofluorescence and using molecular biology techniques. Differentiation tests for detection of vaccinated /natural infected swine with CSFV were also made.
- **Researches regarding ruminant pestiviruses infection in swine and wild boar** consisted in detection of swine and wild boar infection with ruminant pestiviruses in the study area (The Eastern part of Romania), followed by genetic characterization and phylogeny of the identified pestivirus strains.

Chapter V, entitled**, ''Seroepidemiological investigations on classical swine fever virus infection in Eastern Romania'',** presents the results of serological investigations for detection of specific antibodies against-CSFV.

**The first subchapter** presents a seroepidemiological investigation in order to determine the prevalence of the specific antibodies against-CSFV in swine serum or plasma. Vaslui, Suceava and Iaşi County represent the studied region.

The serological investigations regarding classical swine fever were made on sample collected from farm and backyard pigs. From backyard pigs were sampling: 12543 serums from Vaslui County, 11374 serums from Suceava County and 22261 serums from Iaşi County. From farm pigs were sampling: 566 serums from Vaslui County, 2417 serums from Suceava County and 4678 serums from Iași County. As immunoassay technique, the commercial kit Ceditest CSFV- E2 was used (Cedi-Diagnostics). Swine positive for antibodies against CSFV were identified in 2007, revealing the seroconversion to CSFV in Iași, Suceava and Vaslui counties.

During 2008 to 2012, the seropositivity against CSFV of domestic swine is justified bye initiation of the vaccination campaigns regulated by the national program of control for classical swine fever throughout Romania.

**The second subchapter** offers the description of seroepidemiological investigation in order to determine the prevalence of the specific antibodies against-CSFV in wild boar serum or plasma. Vaslui, Suceava and Iaşi County. For this purpose, during 2007 to 2012 were collected blood samples from wild boars originated from Vaslui, Suceava and Iaşi County. Thereby 672 samples from Vaslui County, 1692 samples from Suceava County and 751 serums from Iaşi County were collected and tested. The method used for detection of antibodies against-CSFV is the same as for the analysed domestic pig samples

In 2006 in Romania were typified 45 strains of classical swine fever virus, in 2007 there were typified other 39 of strains (the identified strains belong to the genotype 2.3-*Rostock).*As a consequence in endemical areas it was initiated the vaccination schedule as an important tool for disease eradication. The serological testing made on wild boars in three counties from Eastern Romania (Vaslui, Iași and Suceava) represent a sensitive and specific method in order to detect indirectly the infection with classical swine fever virus.

Because of vaccination program against classical swine fever in domestic pigs starting with 2007 until 2010, it is noticed a reduction of the virus circulation and the lack of outbreaks from 2008 until present. In 2010, wild boars vaccination continued against the classical swine fever (feral pigs are considered a reservoir of infection for domestic pigs).

Chapter VI, entitled**, ''Researches on detection of classical swine fever virus''** had as an objective investigations regarding the CSFV identification using direct immunofluorescence and molecular biology techniques. The researches were made on samples collected from pigs and wild boars. Tests of differentiation were also made in the case of emergency vaccinatated animals.

Investigations regarding the detection of the classical swine fever virus using **direct immunofluorescence** on tissues samples (sternal marrow) from the domestic pigs and boars from Vaslui, Suceava and Iaşi Counties were undertaken during 2007 to 2012. From backyard pigs were collected: 470 samples from Vaslui County, 150 samples from Suceava County and 1891 samples from Iaşi County. Sampling from pig farms represents 148 samples from Vaslui County, 1215 samples from Suceava County and 3318 samples from Iaşi County. From wild boars were collected 721 samples from Vaslui County, 1784 samples from Suceava County and 1316 samples from Iaşi County.

Identification of CSFV antigens in sternal marrow used Gamarom and Ceditest kits**.** The virus presence in tissue sampling used an UV microscop, observing the positive reaction in the cytoplasm of the infected cells. All samples originated from Iași and Suceava County were identified as negative for CSFV. Within the investigations undertaken in 2007 on backyard pigs samples originated from Vaslui County , five positive and two doubtful samples were detected.

The investigations regarding the detection of the classical swine fever virus using molecular biology techniques consisted in testing: blood samples on anticoagulant (EDTA) and organs (spleen, kidney, tonsils and lymphnodes). The methods used for diagnostic were One Step RT-PCR and real time One Step RT-PCR. All samples collected and tested during 2010 to 2012 were identified as negative for the presence of CSFV genome.

Classical swine fever diagnostic of vaccinated/natural infected swine consisted in identification of the animals vaccinated with marker vaccine from the pigs infected with the wild type of swine fever virus. Through this protocol are detected the antibodies against CSFV glycoprotein $E^{rns}$. The principle of this test is that vaccinated animals with a marker

vaccine and non-infected swine produce antibodies only against the glycoprotein E2, while the CSFV infected animals produce antibodies including against other viral antigens. Moreover, the pigs infected with other pestiviruses such as bovine diarrhea virus or border disease will react as gp$E^{rns}$ positive.

The differentiation of the vaccinated animals with the viral subunit E2 ($E^{rms}$ negative) from the infected pigs ($E^{rms}$ positive) was undertaken using CHEKIT CSF- MARKER kit. CHEKIT – CSF- MARKER (IDEXX Laboratories) it is specific, sensitive and fast immunoassay used for detection of antibodies towards the glycoprotein $E^{rms}$ of pestiviruses (CSFV, BVDV, BDV). The study carried out during 2008 and 2009 on 343 farm pig samples and 456 backyard pig samples. The result consisted in detection of 23 seropositive samples (vaccinated pigs with attenuated vaccine).

Chapter VII entitled ''**Researches regarding the infection of domestic swine and wild boars with ruminant pestiviruses''** presents the results of the investigations carried out during 2014 and 2015 on samples from three counties in Eastern Romania: Vaslui, Bacău and Iaşi. Sampling consisted in tissues samples from backyard pigs dead or slaughtered and from wild boars. The One Step RT-PCR technique was used for the detection of ruminant pestiviruses infections in swine. Sampling consisted in 150 wild boars tissue samples and 25 backyard pig tissue samples from Iași County. The results of the investigation consisted in detection of eleven positive samples for 5'UTR sequence common for all pestiviruses (6 wild boars and 5 domestic pigs). All samples collected and tested from backyard pigs and wild boars from Vaslui County rected negative. Investigation carried out on 52 backyard pigs and 200 wild boars from Bacău County identified 12 positive samples pestiviruses genome: 6 wild boars and 6 domestic pigs.

Molecular characterization of pestivirus type consisted in amplification with pan-pestivirus primers of 23 of positive samples (11 samples from Iași County and 12 samples from Bacău County). The protocol based on amplification of 5'UTR sequence common for all pestiviruses identifies CSFV, BVDV and BDV. Positive samples were subsequent amplified using specific CSFV primers, all PCR product being negative.

Forward all RNAs were amplified using two different protocols with specific primers for BVDV1, respectively BVDV2. Validation was performed using as positive control the vaccinal strains: BVDV-1 a non-cytopathic strain KE and BVDV-2 a non-cytopathic strain NY (*Bovela* -Boehringer Ingelheim). The amplification with BVDV-1 specific primers of the 23 positive pan-pestivirus ARNs resulted in identification of five positive PCR products. None of the positive pan-pestivirus ARNs reacted after amplification with BVDV-2 specific primers.

For sequencing among those 23 positive ARNs detected using pan-pestivirus primers were selected 7 PCR positive products. The sampling criteria was: amplification specificity and the band's intensity in migration (respectively DNA concentration). Sanger sequencing was carried out for each strand using pan-pestivirus primers (forward primer 324; reverse primer 326). The obtained sequences were compared with the existent ones in GenBank and EMBL using the BLAST program from NCBI (National Centre for Biotechnology Information).

Swine natural infection with ruminant pestiviruses are described in the literature as having a widespread distribution. Interspecies transmission of pestiviruses is explain by closely related genome's structure and organisation of pestiviruses, with significant similarity of nucleotide sequences and aminoacids which leads to crossed antigenic reactivity.