# SUMMARY

Hepatitis E virus (HEV) is the causal agent of an acute self-limiting hepatitis in humans and subclinical infection in animals. In Europe, both in humans and animals, the most commonly identified genotype is HEV-3. Direct evidence has shown that swine and deer HEVs are zoonotic. Indirect evidence revealed that rabbit HEV and rat HEV may be associated with the zoonotic infection. In Romania, there is a need for identification and characterization of circulating HEV strains in humans and animals, in order to estimate the role of animals in the epidemiology of human HEV infection.

The PhD thesis entitled **"Epidemiological and etiological investigations on hepatitis E virus infection in wild animals and assessment of zoonotic risk"** is structured, in accordance with the norms, in two main parts. Part I, entitled **"Current state of knowledge"** includes 34 pages in which data from the literature on the infection with HEV is presented. Part II, entitled **"Personal contributions"**, presents the results of research carried out during the doctoral studies.

The first part, **"Current state of knowledge"**, is structured in four chapters and the associated subchapters, where are presented the information extracted from the literature regarding the etiological agents and the infections produced in humans and animals, the epidemiological situation of the HEV infection in wild animals, the state of knowledge in Romania, and data on the zoonotic nature of HEV infection, as well as the epidemiology of human HEV infection associated with zoonotic HEV strains.

The first chapter, entitled **"Literature review on hepatitis E"**, describes in the first two subchapters the data related to the etiological agent and the dynamics of infections produced in humans and animals. In the following subchapters, data on HEV diagnosis, prevention and control are presented.

Data included in this chapter shows that HEV is a single-stranded positive-sense RNA comprising three open reading frames (ORF 1-3). The hepatitis E virus is the only member of the Hepeviridae family, in which genotypes and subtypes have been designated including various strains isolated from humans and animals. Genotypes 1-4 are considered major genotypes, gt3 and gt4 being zoonotic. The most important issue with regards to HEV infection is the lack of an effective cell culture system for the propagation of virus, which explains insufficiently known transmission ways, unclear pathogenetic mechanisms, limitation of virulence studies and vaccine development studies.

The second chapter, **"Current state of knowledge on hepatitis E virus infection in wild animals"**, includes two subchapters presenting the epidemiological situations of wild animals HEV infections of the species *Ortohepevirus A* and *Ortohepevirus C*. Of the genotypes of *Ortohepevirus A* species, only HEV-3 has been identified in wild animals in Europe. In the same area, HEV strains framed in *Ortohepevirus C* species isolated from rat, fox and mink.

In Chapter III, entitled **"Current state of knowledge of Hepatitis E virus infection in Romania"**, the existing data in the international and national literature on

HEV infection in Romania are presented. The data presented in two subchapters, show that HEV infection has been investigated in humans and swine. The studies conducted in the eastern Romanian human population were carried out together with the investigations in the domestic swine and led to the suspicion of a possible association of the humans infection with the presence swine HEV. The characterization of HEV strains isolated from domestic swine showed that they belong to HEV-3.

Chapter IV, **"Current state of knowledge regarding zoonotic hepeviruses and the epidemiology of the infection in human population"**, contains data from the literature on the zoonotic character of HEV infection. This topic structured in three subchapters describes the ability of HEV to cross barrier species, zoonotic infection factors, and epidemiological situation of human infection associated with zoonotic HEV strains. Both natural and experimental studies show that not all HEV strains isolated from animals can be associated with zoonotic infection. In Europe, food is considered the main source of transmission of HEV infection. The predominance of HEV-3c subtype in humans and the substitution of those previously prevalent in some countries have been associated with imports of pork meat from other European countries.

The second part of this doctoral thesis, **"Personal contributions"** consists of six chapters (V-X): first chapter assigned to the description of the purpose, objectives and activities associated, followed by four chapters detailing each study undertaken, (material and method, results and discussions), and a final chapter dedicated to the final conclusions listing the remarks observed after the general analysis of the results obtained.

In Romania, the absence of a hepatitis E notification program makes this disease underdiagnosed. The purpose of this research was to assess the zoonotic character of circulating HEV strains in Romania through investigations aiming to improve the epidemiological and etiological data of HEV infection in the country's eastern area. In this regard, the proposed objectives were: to highlight HEV circulation in wild animals in eastern Romania by identifying the seroprevalence of specific anti-HEV antibodies in wild boars; identifying circulating HEV strains in wild animals whose meat can be consumed by humans; assessing the role of rats in the epidemiology of HEV infection in the studied area; identification of circulating HEV strains in the human population of the eastern part of the country; phylogenetic relationships between the strains identified, among them and previously detected in domestic swine in Romania and other HEV strains worldwide, available in GenBank. For this purpose, a series of main activities, along with some with preliminary or secondary charachter, have been undertaken successively, aiming at achieving the proposed objectives. These activities have used serological and molecular methods, and bioinformatics analysis. These are described in Chapter V, **"Goal and objectives"**.

In Chapter VI, entitled **"Seroepidemiological investigation on hepatitis E infection in wild boars"**, results of serological testing (detection of anti-HEV IgG antibodies) of serum samples collected from wild boars in 7 counties from eastern Romania are presented. During the 2014 and 2015 hunting seasons, 218 serum samples were collected from wild boars. Samples were tested using ID Screen®

Hepatitis E Indirect Multi-species commercial kit (IDVet Diagnostics, Montpellier, France). The protocol was performed according to the manufacturer instructions.

The data on the boars' age were obtained for the animals in Galati County and their distribution in hunting funds was known for Buzau County, in addition to Galati. The distribution of samples number in the 7 counties varied, the largest number of samples being collected from wild boars in Iasi County (n=110), followed by Galati County (n=38) and Buzau County (n=30). A smaller number of samples were collected from wild boars in several counties: Bacau (n=18), Vrancea (n=16), Suceava (n=5) and Botosani (n=1).

The seroprevalence of anti-HEV IgG antibodies was 11.92% (26/218; 95% CI, 0.076-0.162). The analysis of obtained results showed that wild boars from 5 out of 7 counties were identified positive. These results were obtained probably due to the uneven distribution of the number of samples in each county. Seropositive animals were identified in two out of 18 analysed hunting funds in Galati County and in 3 out of 15 analysed hunting funds in Buzau County. Our results signalled the persistent character and the spread of HEV infection in wild boars in eastern Romania, by identifying the infection in other hunting funds than those previously identified positively by Botezatu et al., 2014.

The reported seroprevalence was higher than previously detected in wild boars in the studied area and lower to the ones reported in other European countries. Our results may be explained by the use of different diagnostic techniques, factors related to the biological and ecological characteristics of wild boar populations and those related to the spread of the virus during the analysed periods.

The analysis of HEV seropositivity by age groups are in line with literature confirming that HEV infection in wild boars is not age related. This results provided premises to investigate the hepatitis E virus presence in Romanian wild boar as well as in other wild animals, which are considered as potential HEV reservoirs.

Chapter VII, **"Molecular detection of hepatitis E virus in wild boars, cervids and wild rabbit**s", includes the results obtained at molecular analysis on colected samples from wild boars, cervids and wild rabbits from the study area. Samples were collected during the hunting seasons 2013-2015, dedicated to each species. The analysis included 78 samples, 50 from wild boars (liver samples, n=45 and spleen samples, n=5), 16 from cervids (liver samples) and 12 from wild rabbits (liver samples).The data on the age of the cervids and rabbits from which the liver samples were collected and thats on the distribution of the animals in the two species of cervids analysed, *Capreolus capreolus* and *Cervus elaphus*, are unknown. Age data were obtained for 37 of the 50 wild boars analysed. The animals were divided into three age groups: less than 1 year (n=4), between 1 and 2 years (n=11), older than 2 years (n=22). For analysis were used conventional molecular methods [Cooper et al., 2005; Bouquet et al., 2011] and in real time [Jothikumar et al., 2006; Barnaud et al., 2012]. Bioinformatics analysis included the tools needed to frame the identified HEV strains (BIOEDIT, BLAST, MEGA 7).

The obtained results show that HEV has been identified in 9 samples of the 78 analysed, these originating from wild boars. Data published in literature highlights that

the lack or low rates of HEV detection in cervids is common for a number of studies that have carried out simultaneous research on wild boars and cervids. Based on these data, some hypotheses have been issued, which may be a part of the cause for the lack HEV detection in cervids: their selectivity in the choice of food and water sources, as well as the anatomical features - lack of gallbladder. Deoxycholic acid secreted by gallbladder is essential for the dissociation of lipids from virions.

Positive wild boars for HEV originated from two counties: Vrancea and Suceava, which are located on opposite sides of the analyzed area - eastern Romania. The overall HEV prevalence in wild boars was 18% (9/50). HEV prevalence for each positive-identified county were 25% (2/8) and 23.33% (7/30), respectively. HEV RNA was detected in wild boars aged between 1 and 2 years (2/11 to 18.18%) and in animals older than 2 years (7/22 - 31.81%). The analysis of the results, according to the type of tissue analysed, showed that two of nine RNA HEV-positive samples are splenic tissue.

Subsequent analyses showed that five nested RT-PCR products were obtained: one of the two positive spleen samples and four of the seven positive liver samples. To characterize the HEV strains, the positive products were sequenced and analyzed. Results showed that different HEV-3 subtypes are circulating in two counties which were found to have HEV-positive wild boar.

The reporting of these results to those presented in the literature has led to the suspicion of the zoonotic character of the identified HEV strains following genetically association with human HEV strains isolated from autochthonous infection cases. Presence of HEV in wild boars and previously detection of HEV in domestic swine from Romania suggests a possible correlation of the increased seroprevalence rates of anti-HEV IgG antibodies in human population in the same area.

In order to evaluate the role of rats in the epidemiology of HEV infection in the study area but also in other wild animals, in chapter VIII, **"Molecular identification of HEV strains circulating in rats and other animal species"**, molecular analyses and bioinformatics were performed in order to frame the circulating HEV strains. A total of 69 liver samples collected from wild animals (52 rats, 4 ferrets, 1 coypu, 1 bizam, 1 mole, 1 mouse and 9 foxes) were analysed by molecular biology methods. All samples were tested by three protocols described in the literature. Two of these are specific for detection of human HEV (HEV-1-HEV-4) and have been described in previous chapter. The protocol for detection of different hepeviruses, including rat HEV. [Johne et al., 2010] was used. Following the analysis, HEV was identified in rats only using HEV detection protocol described by Johne et al., 2010.

Additionally, samples collected from foxes were analysed by two classical PCR methods developed for this purpose, which used primer sets described by Bodewes et al., 2013 for the detection of HEV in foxes in the Netherlands. These protocols targeted two different regions of the virus and were identified as: PCR-ORF2 and PCR-ORF1, depending on the amplified segment. The electrophoretic analysis of amplification products obtained by PCR-ORF1 revealed the presence of a DNA band at the expected position (362 bp) for one out of 9 analysed samples. The presence of

nonspecific amplification bands for this PCR product bring us in attention the need of optimization of developed protocols and reconfirmation of the presence of HEV in foxes in our country.

Rats were captured in different locations in 4 counties (Suceava, Iasi, Bacau and Tulcea) situated in the study area: the proximity of a swine farm with an intensive system, the proximity of a semi-intensive system swine farm, the proximity of a swine farm in an extensive system, the proximity of a farm with various animal species, the proximity of a cattle farm, the proximity of garbage canes and the surroundings of rural settlements. 9 of the 52 tested samples were identified ARN-HEV positive. The analysis of the results showed that HEV is present in rats in each analysed county and in 4 locations of the 7 analysed. These results show a broad distribution of HEV in rats in the study area.

Characterization of identified HEV strains has revealed that belongs to HEV subtype C1, specific to rats. The phylogenetic analysis revealed different genetic degrees of genetic relatedness between isolated strains (84.84% - 91.18%) and the maximum nucleotide identity between these and those from GenBank database ranged between 85% and 89%. The same analysis showed that one of the strains identified in rats in Bacău is genetically linked to two HEV-4 strains, isolated from swine in China, at 73% and 72% (nucleotide identity). These findings generates new data on the degree of genetic association between species of the *Orthohepevirus* genus.

Chapter IX, "**Research on the zoonotic character of the HEV infection through its evaluation in the eastern Romania's human population**", includes results of serological and molecular analyses for HEV infection detection in patient groups from eastern Romania. Biological samples (serum, plasma or faeces) were collected from three sanitary units in eastern Romania: "Sfanta Parascheva" Infectious Diseases Hospital of Iasi, Public Health Directorate of Iași (DSP Vrancea) and the Public Health Directorate of Vrancea (DSP Vrancea).

Two groups of patients from two hospitals: patients with acute hepatitis ("Sfanta Parascheva" Infectious Diseases Hospital of Iasi) and patients without liver disease (Emergency County Hospital "St. Pantelimon" Focsani). In the first group (n=94), patients with acute hepatitis of unknown cause (n=48) and patients with acute non-A, non-B hepatitis (n=46) were included. In the second group (n=40), patients with TBC (n=3) and women patients in postnatal confinement (n=37) were included. The study also included 16 serum samples from medical staff at the "Sfanta Parascheva" Infectious Diseases Hospital of Iasi.

Serum samples from medical staff, from patients with acute hepatitis of unknown cause and those without liver disease were initially tested for the detection of total anti-HEV antibodies (Ab HEV) using the HEV Ab ELISA commercial kit (Diagnostic Bioprobes, Italy).

Subsequently, some of the samples from patients with acute hepatitis of unknown cause have been tested, along with samples from patients with acute non-A, non-B hepatitis for detection of anti-HEV IgM antibodies using HEV IgM ELISA commercial kit (Diagnostic Bioprobes, Italy). Serum samples from patients without

hepatic disease Ab HEV positive and samples from patients with acute hepatitis with different serological results were tested for HEV RNA detection.

The results obtained revealed that HEV infection was present in 29.16% (95% IC, 16.31 - 42.03) of the patients with acute hepatitis of unknown cause and in 32.5% (95% IC, 17.98 - 47.02) of patients without hepatic disease, through the detection of total anti-HEV antibodies.

Detection of anti-HEV IgM antibodies in patients with acute hepatitis showed that 10% (9/90) had an acute infection with HEV at the time of hospitalization. Detection of HEV RNA in two patients out of 9 positive IgM anti-HEV shows that they have suffered HEV infection relatively recent to the time of hospitalization. One of two HEV -RNA positive patients consumed pork buyed from a store.

The molecular characterization of circulating HEV has led to framing human strains in subtype HEV-3c, the most commonly identified subtype in Europe. Phylogenetic analysis revealed genetically relatedness of human, swine, wild boars and domestic goat HEV strains in Europe. These results, together with those obtained after wild boars HEV characterization, revealed the presence of different HEV-3 subtypes, leading to two hypotheses regarding HEV infection detected in humans. First hypothesis suggests the association of infection with pork imports from EU countries where HEV-3c is prevalent and the second hypothesis suggests the possibility of an indigenous infection and implicitly the existence of different HEV subtypes in humans and animals in Romania.

Chapter X**, "Final conclusions"**, summarizes the partial conclusions, following an overview of the data presented in the previous chapters. The results provide new data on HEV dynamics in susceptible populations in Romania. Observations made during this study suggests the possibility of HEV zoonotic transmission and the need to implement a national program for prevention, surveillance and control of hepatitis E in Romania.