



ABSTRACT

Keywords: Bats, Chiroptera, Zoonoses, Leptospira, Rabies, West Nile, Paramyxovirus, Flavivirus, Lyssavirus, Epidemiology, Infectious pathology, OneHealth, Active surveillance.

Thesis title: Potential reservoirs for emerging zoonotic pathogens in Romanian bat colonies (ord *Chiroptera*)

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The aim of this study: was to contribute to the scientific knowledge of the main zoonotic diseases potentially present in Romanian bats and the risk of a spill-over phenomenon in humans and animals and the role of *Chiroptera* as a reservoir host for certain infectious diseases and the condition in which they are able to maintain and multiply those pathogens without showing signs of disease. **Objectives:** were to identify microorganisms with zoonotic potential and to assess the prevalence of these pathogens in Romania. This paper proposes an epidemiological screening of the *Chiroptera* in order to identify the key elements in epidemiology and public health implications. The research team was represented by the USAMVB Doctoral School of Veterinary Medicine- Clinic of Infectious Diseases and Preventive Medicine and by the Emil Racoviţa Institute of Speleology of the Romanian Academy (ISER), the forms of scientific collaboration consisted of consulting ISER experts, with an interdisciplinary openness to related research fields and participation in research teams. Furthermore, the present research had a strong interdisciplinary approach by intertwining with entomology, echology, human medicine by the fact that the infectious agents studies have zoonotic potential, and some of them can be transmitted by vectors. From a scientific point of view, the proposed study has a high degree of novelty and complexity and is based on interdisciplinary data (pathology, virology, bacteriology, immunology, parasitology, speleology, molecular biology, ecology, biology, epidemiology,etc. Depending on the etiological agent, several categories of infectious diseases can be identified in bats: viruses, bacteria, mycosis, rikettsia. So far 66 viruses have been reported in 74 different *Chiroptera* species. Some of those viruses have been identified in several different bat species, while others have been reported in only one species.

The thesis titled **Potential reservoirs for emerging zoonotic pathogens in Romanian bat colonies (ord *Chiroptera*)** consists of two parts: **PART I:** corresponds to the literature review, in this part of the thesis an update of the latest studies was made by collecting and synthesizing literature information deepening the chosen theme. The documentation activity was aimed at determining the types of infectious agents identified in bats living in temperate zones. For this, a brief presentation on the *Systemtic classification of Chiroptera* was made, emphasizing on the three existing family of bats in Romania. The *biological, ecological and ethological particularities of Romanian Chiroptera and their relevance in epidemiology* was also pointed. This chapter describes the ecological niche and the ethology of different bat species. With a focus on the key elements relevant to epidemiology: flight adaptation and the physiological implications of active flight, migration present in some species, the tendency of forming colonys and the colony tipe, hibernation and the morphological and functional changes in the physiological parameters during hibernation. All of these elements may have a determining role in the evolution of an infectious disease. *Faunistic and distribution data of Chiroptera in Romania* was also investigated. Some informations regarding the legal status of those species was pointed out. Several other factors were assessed like: the ecological niche and the habitats used by bats among with the plasticity of certain species regarding the adaptation to anthropic roosts and urban areas. The economic importance of bats is given by their ability to control insect populations. *Zoonoses and emerging infectious diseases associated with Chiroptera species* have been selected by reviewing the literature, reffering exclusively to those morbid entities that have been reported in bat species from temperate areas, belonging to the same genus that is also present in Romania. This screening was necessary because there are over 1100 specied of bats in the world, each with its own specific infectious pathology whereas in Romania there are only 32 species. Much of the viral entities with zoonotic potential have their origin in *Megachiroptera* from tropical regions. In this section it was also pointed out that these animals rarely express any clinical signs of illness. *The role of Chiroptera as reservoir for some potentially zoonotic diseases in Europe* deepen the reported cases in the region. As some of the bats that roost in Romania can migrate up to 2000km, and colony formation is part of the survival strategy, the European reports on the presence of several infectious agents like *Lyssavirus*, *Hendra Virus*, *Nipah Virus*, *Paramyxovirus* and some arboviruses have been sought. The situation at a national level was investigated through *Research done on the monitoring of Chiroptera populations in Romania* where the legislative framework for the protection of these species was mentioned among with the research directions that focused on *Chiroptera*. Most studies were focusing on conservation and ecology actions, and a few studies were investigating haemoparasites and the presence of some infectious diseases like *Borrelia sl*. A presentation of

Chiroptera species taken into study was organised by families genus and species, where the essential information for each of the 19 species that were studied were summarized. The distribution of each species and its protective status were mentioned. Emphasis was placed on the roosting type and the ecology of each species, where hunting strategy and ecological niche were mentioned. Another aspect pointed for each species was related to the different types of aggregations that were noticed (mixed colonys, mathernity, segregation or solitary individuals etc). The distance from the roosing place to the foraging area or the distance during migration was also mentioned for each species, also the avarage and maximum lifesapan and the most common predators in urban and wild areas. All infectious diseases that have been reported were selected from the literature for each species. Based on all these details, an estimation of the epidemiological impact of all 19 species wes made. An *analysis of prevention and control strategy against zoonoses of infection etiology* regarding *Lyssavirus* was also selected from official reports where the most cases of rabies are associated with terrestrial mammalian species but the emphasis in this study was put on *Chiroptera*. The legal framework for action in case of suspicion or confirmation of a rabies case is also presented. *Flaviviruses* have also been presented while focusing on the presence of West Nile virus and Tick Borne Encephalitis Virus while reffering to bats and also mentioning the legal framework for action. The presentation of *Leptospira* and *Paramixovirus* was structured in the same manner.

PART II represents the personal research, each chapter is dedicated to a particular morbid entity presenting the results obtained from retrospective and prospective studies. As part of the current preoccupation on the global scale regarding the One Health context, epidemiology studies for identification of the risk factors associated with zoonotic agents were conducted. The structure of the chapters is similar, consisting of three parts in which the framework context and the specific epidemiological particularities to each entity are presented, followed by the materials and methods used to identify the infectious diseases and then the results, discussions and conclusions. The detection methods used had a high degree of specificity and sensitivity for each pathogen and the results obtained were put into epidemiological context. The importance of the adressed topic is given by obtaining data that can substantiate aproprate measures with a positive impact on the health of *Chiroptera*, the environment and the population. The conclusions and recommendations were based on the practical reality of morbidity, mortality and loss of protected species of bats and also the epidemiological situation reported in human medicine. Thus, some recommendations have been developed to limit infections in humans and also environmental considerations have been taken into account by reducing emission of pathogens and reducing or limiting circulating strains with pathogenic potential. In the course of the study, some critical points were detected in the pathology of *Chiroptera*, giving the protected

status of the species, there results managed to generate a large amount of information useful to the scientific community, information that was presented at scientific events. The methodologies developed in Part II along with the *One Health* approach gives the study a new character in terms of research. The personal research debuts through the *characterization of investigated territories and the organizational framework* where the geographic coordinates of the main sites studied and the criteria for inclusion of those areas are summarized. It was also described how surveillance was performed in those locations, and the particular events that were reported during the surveillance. Based on the information that 48% of urban Romanians own pets, a *study on the dynamic relationship between bats, humans and pets in the urban context* was conducted. In this study several aspects were investigated: the degree of interaction of pets (cats in particular) with urban fauna, the study showing that in Romania, the cat population attacks about 15000000 small mammals and 7000000 bird annually. This is a worrying situation in terms of conservation as well as the epidemiological risk associated with the pet/ wild fauna interaction. This study also looked at the interactions between bats and human population, examining the intervention requests that were addressed to various platforms and animal association rescue centers. The *biological samples and sampling strategy* used in the study was also presented. As the bibliographic study revealed, there is a low incidence of pathogens and a lack of clinical symptomatology in *Chiroptera*, this is detailed in Part I of the thesis. Two types of biological samples were collected: from clinically healthy animals and samples collected from whole carcasses of dead animals that were found in various locations. The *pathological materials, techniques used and their value in diagnosis* have been described while mentioning the type of pathogens that can be detected in each biological samples and the detection technique for each sample. It was also mentioned the minimum number of individuals that should be tested in order to achieve confidence levels. An *Overview of the pathological aspects identified in Chiroptera* was also performed where the main lesions were described. The most frequently encountered lesions were detailed as follows: oste-articular lesions, traumatic lesions associated with bite marks, hemorrhagic lesions, macroscopically detectable lesions of the central nervous system and some mophopathological aspects of abortions. Data regarding detection of viruses with zoonotic potential was presented in a logical and orderly manner as follows: within the chapter titled *Epidemiological investigations on the prevalence of Lyssavirus in Romanian Chiroptera populations* a study was conducted *retrospective and descriptive study of risk factors associated with Lyssavirus transmission* in which data were collected over a 5-year period in which the incidence and risk factors associated with *Lyssavirus* infection was assessed. Also the presence of this pathogen in human and animal populations was also recorded. This study was followed by a prospective study titled *Lyssavirus detection strategy in Chiroptera: materials and methods*

where all detection strategies materials and methods have been described as follows: *Detection of rabies antibodies using fluorescent antibody virus neutralization test, Lyssavirus detection using direct immunofluorescence test, Lyssavirus detection using in vivo methods of isolation and cultivation.* Also molecular epidemiology was used *molecular biology techniques for Lyssavirus detection*, for this method total nucleic acid extraction was performed from brain samples, and also a similar protocol was described for saliva samples. The protocol for assessing concentration of extracted genetic material was used also to determine if there were any contamination and to highlight the amount of extracted genetic material. The *amplification reaction for detecting a specific fragment of Lyssavirus* has been done using the protocol described in the thesis. In the *results and discussions regarding the presence of Lyssaviruses in Romanian bats* the examined species and their number were detailed along with the type of samples used and type of tests performed on each sample. A total of 493 individuals from 19 different bat species were tested by PCR technique. From the same individuals a total of 493 direct immunofluorescence assays were also performed. PCR and RTCIT was also performed from 453 saliva samples, all saliva samples were collected from *Nyctalus noctula*, from individuals that were admitted in rescue centers. Each individual was tested by at least 2 diagnostic methods. However, the presence of rabies virus or rabies antibodies could not be detected in any of the approximately 1000 individuals that were tested. In the *epidemiological investigations on the prevalence of Paramyxovirus in Romanian Chiroptera populations*, the presence of *Paramyxoviruses* was investigated through a bivalent study comprising a retrospective study and a prospective study. Data on the incidence and risk factors associated with different representatives from this viral family were collected and were presented in the *retrospective and descriptive study of risk factors associated with Paramyxovirus transmission.* The prospective investigations were done using *molecular biology techniques for Paramyxovirus detection in Romanian bats.* For this, RNA extraction from spleen sample was performed followed by assessment of the genetic material concentration. *Amplification reaction for detecting a specific fragment from Paramyxovirus* has been optimized, the process of *optimization of hybridization temperature* was made after 21 temperature combinations. The optimization was needed in order for the primers to bond and validate the reaction. The electrophoresis analysis, calculation of results and the expression of the results have been detailed described. The *results and discussions regarding the presence of Paramyxovirus in Romanian bats*, following the PCR analysis of 493 samples of individuals from 19 different species, a positive reaction was found in a total of 12 individuals. The PCR results expressed specific bands of approximately 500bp. Positive samples have been identified from the following species: *Nyctalus noctula* (n=9), *Pipistrellus kuhlii* (n=1), *Pipistrellus pipistrellus*

($n=1$) and *Myotis daubentonii* ($n=1$). After the gross necropsy exams, no macroscopically detectable changes were observed for the positive individuals, suggesting a status of persistent asymptomatic infection. Positive tested bats were found in the following urban areas: Baia Mare, Constanța, Bucharest, Brașov. With the exception of bats that were kept in quarantine, all tested bats came from distinct areas. In this context, the dynamic relationship between bats-humans-pets can create the premises of a spill-over phenomenon given the rate of contact between these species. The *Epidemiological investigations on the prevalence of Flavivirus in Romanian Chiroptera populations* was done by collecting data regarding the presence of those pathogens in human or animal population by *retrospective and descriptive study of risk associated with Flavivirus transmission*. There was a correlation between the number of cases in human population and the number of cases in animal populations. Also, the seasonal distribution of the disease associated with the presence of different species of *Culicidae* that are capable of transmitting the virus has been reflected in the occupational factors of infected individuals (outdoor activities). Limited seroprevalence in equines (1.5-7%) is discordant with that found in other species (5.1- 45%). *Molecular biology techniques for Flavivirus detection in Romanian bats* used the same successive steps *nucleic acid extraction from brain samples, assessment of the genetic material quantity and amplification reaction for detecting a specific fragment from Flavivirus*. *Results and discussions regarding the presence of Flavivirus in Romanian bats* revealed that although all the examined bats are insectivorous and species from *Culicidae* are part of their diets, no positive samples were identified from the 483 individuals examined by PCR technique. Some of the *Culicidae* species are capable of harboring arboviruses and can also be found on bats. The lack of viral antigen in the brain samples shows that individuals were not viremic at the time of testing. The extent to which they have been in contact with West Nile virus is still unknown since they have not been serologically investigated. The presence of bacteria with zoonotic potential within bat populations was also investigated through *epidemiological investigations on the prevalence of Leptospira in Romanian Chiroptera populations*. The same as above was used to investigate via a *retrospective and descriptive study of risk associated with Leptospira transmission* conducted over a 5 year period in which the incidence and risk factors associated with this pathogen were examined. The maximum incidence of the disease was in August/September. It was also found that there are differences in the incidence in domestic animals, the highest incidence being in shelter dogs. For *molecular detection of Leptospira in Romanian bats*, DNA extraction was performed from renal samples of dead individuals and from urine samples collected from rehabilitated individuals, the different protocols are described. After DNA extraction, the quantity and quality of the genetic material was also assessed. The *amplification reaction for detecting a specific fragment for pathogenic Leptospira* discriminated

between them and other related spirochetes that could give false positive reactions. PCR samples were analyzed from 16 species of bats belonging to 3 families: *Vespertilionidae* (483 individuals), *Rhinolophidae* (n=6 individuals) and *Miniopteridae* (n= 2 individuals). *Results and discussions regarding the presence of Leptospira in Romanian bats* revealed the presence of *Leptospira* in 4 species of bats: *Nyctalus noctula*, *Pipistrellus pipistrellus*, *Pipistrellus nathusii* and *Myotis daubentonii*. An active leptospirotic infection was identified by the presence of spirochetes in the urine of 15 individuals. The positive species were from Bucharest, Iași and Babadag where leptospira infections in humans were frequently reported during the study. *General conclusions and recommendations:* to our knowledge, this is the first report of the presence of leptospira in *Pipistrellus nathusii* and the first report of 3 other species of bats from continental Europe in the last 40 years : *Nyctalus noctula*, *Pipistrellus pipistrellus*, *Myotis daubentonii*. Surveillance measures in endemic leptospira areas should also include wild animals, and bats tested against rabies should also be tested for leptospira. To our knowledge, this is the first investigation at a national level that addresses the infectious diseases present in bats and their zoonotic potential, using a multidisciplinary *One-Health* approach. Although leptospira cases reported in bats have been associated with areas where leptospirosis is also reported in humans, bats do not seem to have an epidemiological importance in direct transmission to humans. This is due, in part, by the inaccessibility of the roosts. The low prevalence among individuals in Babadag suggests an accidental phenomenon, probably a contaminated source of water. A prophylactic measure in this case is to wear protective gloves while interacting with wild animals followed by a proper hygiene. The measures taken for the two colonies that tested positive were quarantine, antibiotic treatment and relocation in a remote area. This approach is both ecological and ethical, and reduces the occurrence of a spill-over event, contributing to the conservation of bat species. The bats plasticity in terms of shelters allows the successful relocation of colonies or individuals. For relocation there is a wide variety of suitable places, natural or anthropic, which offers the conditions that are met with their metabolic needs.