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ELISA Detection of European Lyssaviruses in Bulgarian Cave-dwelling Bats

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Abstract: The aim of the current study was to detect antibodies against the rabies infection in Bulgarian cave-dwelling bat populations using two types of ELISA protocols: 1) the BioPro Rabies blocking ELISA Ab Kit (BioPro), and 2) the Platelia® Rabies II Kit *ad usum veterinarium* (Bio-Rad). A secondary aim of the study was to assess the suitability of these two ELISA kits for further research on the seroprevalence of rabies in Chiropteran species. In the period 2020-2022, the research team obtained 205 blood samples from 11 different bat species captured at 8 localities (Orlova Chuka cave, Zorovitsa cave, Andaka cave, Urushka-maara cave, Razhishkata cave, Magurata cave, Ivanova Voda cave, and Madzharovo abandoned buildings). By using the BioPro Rabies blocking ELISA Ab Kit, positive results were detected in 5.36% (6/112) of the analyzed samples. The Platelia® Rabies II Kit *ad usum veterinarium* (Bio-Rad) detected positive results only in 1.08% (1/93) of the analyzed samples.

Key words: Chiroptera, rabies, underground sites, ELISA

Introduction

During the last decade bats (order Chiroptera) were extensively recognized as a reservoir of zoonotic viruses (Hayman 2016). The neurotropic lyssaviruses, which cause lethal infectious disease of the central nervous system known as rabies, are one major example (Fooks et al. 2017). Rabies viruses belong to the family *Rhabdoviridae* and include 11.9–12.3 kb long non-segmented, linear, negative-sense single-stranded RNA with five major genes that encode the nucleoprotein (N), phosphoproteins (P), matrix protein (M), glycoprotein (G) and RNA -dependent polymerase (L) that are arranged in the order 3′-N-P-M-G-L-5′ (WHO 2018, Davis et al. 2006). Research-based on active and passive bat surveillance has recognized 22 distinct lyssaviruses (Kuzmin et al. 2003, Picard-Meyer et al. 2013, Rupprecht et al. 2017, Nokireki et al. 2018, Hu et al. 2018, Hu et al. 2022, Banyard et al. 2020, Coertse et al. 2020, Calvelage et al. 2021, Černe et al. 2023, Viljoen et al. 2023), among which are the European bat lyssavirus 1 (EBLV-1), and European bat lyssavirus 2 (EBLV-2) (Fooks et al 2003, Davis et al 2005).

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According to UNEP/EUROBATS (2022), the order Chiroptera contains 55 species in Europe, from the 6 families Pteropodidae, Emballonuridae, Rhinolophidae, Vespertilionidae, Miniopteridae and Molossidae, and present a natural reservoir of EBLV-1, EBLV-2, BBLV, LLEBV, KBLV, WCBV and DBLV (Van Der Poel et al. 2000, Kuzmin et al. 2003, Picard-Meyer et al. 2013, Rupprecht et al. 2017, Nokireki et al. 2018, Hu et al. 2018, Hu et al. 2022, Banyard et al. 2020, Coertse et al. 2020, Calvelage et al. 2021, Černe et al. 2023), except *Taphozous nudiventris* from the family Emballonuridae. Between 1977 and 2023, 1100 bat rabies cases have been reported by the Rabies Bulletin Europe (https://www.who-rabies-bulletin.org/site-page/general-information). Bats from the species *Eptesicus* were recognized as the natural reservoirs of EBLV-1 (Serra-Cobo et al. 2002, Mingo-Casas et al. 2018). Several species of the genus *Myotis* are reservoirs of EBLV-2, BBLV, ARAV and KHUV (Schatz et al. 2013). WCBV was isolated only from *Miniopterus schreibersii* (Botvinkin et al. 2003, Ceballos 2013). In Bulgaria, the first case of a lyssavirus-positive bat was detected in 2020 within one individual from the species *Rhinolophus ferrumequinum*, family Rhinolophidae (Seidlova et al. 2020). Previously, rabies was studied mainly on carnivores, with the main reservoirs identified as *Vulpes vulpes* and *Canis aureus* (BFSA 2018). For example, from the beginning of 1994 to the end of August 2014, the number of confirmed cases was 392, of which 332 (85%) were in Northern Bulgaria and 60 (15%) cases were in Southern Bulgaria (BFSA 2018). The actions undertaken for the elimination of rabies are aerial and manual distribution of oral vaccination on the territory of Northern Bulgaria and parts of Southern Bulgaria. According to the report of the Bulgarian Food Safety Agency (BFSA), the result of the oral vaccination undertaken was positive against RABV.

In the scope of the current study, we aimed to evaluate the percentage of cave-dwelling bats mounting an immune response against rabies in 8 underground roosts in Bulgaria using two kits of ELISA (Enzyme-Linked Immunosorbent Assay) for qualitative analysis. The first one is the commercially available BioPro Rabies Ab ELISA kit, which, as a competition ELISA kit, allows for species-independent detection of rabies-specific binding antibodies. It was successfully used in the framework of the monitoring of oral rabies vaccination campaigns (Wasniewski et al. 2013). The second one is the PLATELIA™ RABIES II kit, BIO-RAD, France, which is interpreted qualitatively but is a quantitative assay for the detection and titration of IgG antibodies against the rabies virus glycoprotein G in animal serum (SERVAT et al. 2007).

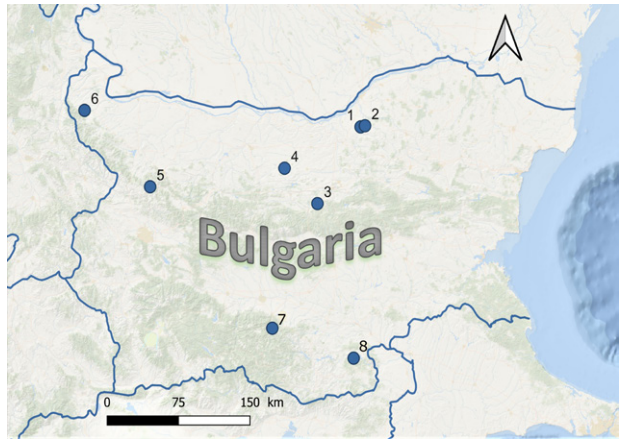

Fig. 1. Map of the sampling sites: 1 Orlova chuka cave, 2 Zorovitsa cave, 3 Andaka cave, 4 Urushka-maara cave, 5 Razhishkata cave, 6 Magurata cave, 7 Ivanova voda cave and 8 Madzharovo abandoned buildings.

Materials and Methods

Bat sampling

Bats were captured in front of the cave entrances at 8 localities (Fig. 1) using mist nets or harp traps and then placed separately in cotton bags (Kunz et al. 2009). For species identification, we followed Dietz and Von Helversen (2004). Determination of th)e gender, age and reproductive status was provided for every individual.

Overall, 205 bats of 11 different species from three families (Rhinolophidae, Vespertiolionidae and Miniopteridae) were sampled during the spring and autumn period of 2020, 2021 and 2022. The skin surface of every individual was disinfected with alcohol; the cephalic or uropygial vessel was then lanced using a sterile needle and a 20 µl blood sample was taken using a pipette tip and collected in heparinized microtubes. Then a drop of tissue surgical glue (Surgibond, SMI AG, Belgium) was applied to seal the lacerated skin and prevent further bleeding. Each sampled bat was given oral vitamins and released.

Capture and sampling were carried out under the research permits issued by the Bulgarian Ministry of Environment and Water (828/19.03.2020 and 912/02.11.2021).

ELISA kits and procedures

The ELISA antibody detection in 112 samples from *Rhinolophus ferumequinum* (n=56), *R. euryale* (n=2), *R. mehelyi* (n=1), *Myotis myotis* (n=26), *M. blythii* (n=18), *M. capaccinii* (n=1), *Eptesicus serotinus* (n=4), *Nyctalus noctule* (n=1) and *Mini-*

opterus schreibersii (n=3) using the BioPro Rabies blocking ELISA Ab Kit (BioPro, Czech Republic) was performed in the National Centre of Infectious and Parasitic Diseases Bulgaria, according to the manufacturer's instructions. The largest number of sampled animals were from Andaka cave (n=30) followed by Urushka-maara cave (n=26), Orlova chuka cave (n=22), Ivanova voda cave (n=20), Zorovitsa cave (n=10), Razhishka cave (n=4).

A quantitative ELISA protocol for antibody detection was performed on 93 samples from *Rhinolophus ferumequinum* (n=12)*, R. hipposideros* (n=7)*, R. euryale* (n=9)*, M. capaccinii* (n= 4)*, M. emarginatus* (n=28)*,* and *Miniopterus schreibersii* (n=33) using the Platelia® Rabies II Kit *ad usum veterinarium* (Bio-Rad, France), according to the manufacturer's instructions. In every sample, the presence and quantity of antibodies against the rabies virus were determined after a 1:100 dilution of serum in R6 reagent (red-coloured TRIS-EDTA buffer with ProClin™ 300 (0.1%) preservative). The ELISA reaction was performed according to the manufacturer's instructions and scored on a PR 4100 Absorbance Microplate Reader (Bio-Rad), with the washing steps performed on a PW 40 8-Channel Manifold Microplate Washer (Bio-Rad). The largest number of sampled animals were again from Andaka cave (n=40) followed by Madzharovo, abandoned buildings (n=34), and Magura cave (n=19).

Qualitative determination of the presence of antibodies was performed in double repeats; the presence of antibodies against rabies was determined photometrically via the absorbance of the oxidized substrate (R9 reagent, 3,3',5,5'-Tetramethylbenzidine (TMB), Bio-Rad) and the values were compared to positive and negative reference controls. Results were recorded, compiled and exported for analysis using the Magellan software (Bio-Rad). Percentage of bat seropositivity in different ELISA kits was compared by testing the difference between the two proportions.

Results

During the sampling period, none of the captured bats have shown clinical signs suggestive of rabies encephalomyelitis. The lyssavirus-specific antibodies were detected in two out of the 9 morphologically identified bat species, mainly in *Rhinolophus ferumequinum* from Andaka and Urushka-maara caves and *Myotis myotis* from Ivanova voda cave. With 6 positive individuals, the overall seroprevalence using BioPro Rabies blocking ELISA Ab Kit was 5.36% (Table 1). During the fieldwork period in 2022, antibodies against the rabies virus were detected in one out of 6 morphologically identified bat species, only in *Rhinolophus ferumequinum* from Madzharovo, abandoned buildings. Therefore, the overall seroprevalence using the second ELISA kit PLATELIA™ RABIES II kit, BIO-RAD, France, is 1.08 % (Table 2). A proportion of bats tested positive by both kits did not differ significantly (p=0.093). Similarly, the rabies seroprevalence in the most abundant species (*Rhinolophus ferrumequinum*) did not differ (p=0.947) and reached approximately eight percent (8.93 % vs. 8.33 %).

Discussion

Compared with similar studies (Seidlova et al. 2020, Orłowska et al. 2020), we found low seroprevalence among Bulgarian cave-dwelling bats (Tables 1 and 2). Therefore, we have to go further into the ELISA kit's specificity and bat species preferences. The BioPro Rabies ELISA Ab kit, manufactured in the Czech Republic, utilises a "reverse" ELISA procedure, where serum-present antibodies block a specific antigen on the bottom of the ELISA plate. This procedure has the drawback that antigen deterioration and/or nonspecific antibody binding can lead to false positive results. On the other hand, the PLATELIA™ RABIES II *ad usum veterinarium* kit, manufactured by Bio-Rad France, is optimized for the detection of anti-rabies antibodies (IgG) in cats, dogs, and foxes. The secondary antibodies detect the Fc domain of the anti-rabies IgGs produced by rabbits, dogs, and foxes. Since bats (as different species) may have variability in the Fc domains of their IgG genotypes, it is possible that the PLATELIA™ RABIES II kit can give false negative results.

Going further into bat species composition we found seropositive individuals only in *Rhinolophus ferumequinum* using both kits in Andaka and Urushka-maara caves and Madzharovo abandoned buildings (Tables 1 and 2). This species is a classic example for a cave-dweller distributed across the Palearctic region (IUCN 2022). In Bulgaria, *R. ferumequinum* forms summer multispecies colonies and rarely hibernates in mixed colonies with other species, compared to *R. euryale* and *R. mehelyi* (Petrov 2008). The majority of seropositive individuals (Table 1) were sampled during the spring period after hibernation. Hibernation is characterized by decreased activity in the bat's immune system and metabolism (Bouma et al. 2010, Field et al. 2018), resulting in prolongation of the incubation periods and virus persistence between the transmission periods in winter and spring. During the active

Table 1. Seropositive cave-dwelling bat species detected using the BioPro Rabies blocking ELISA Ab Kit in Bulgaria during 2020 and 2021.

Bat species	Country	Sample site	Date	Sex F/M	Age Ad/Sub-ad/Juv	Number examined	Seropositive bats
E. serotinus	Bulgaria	Razhishka cave	1.5.2020	0/3	0/3/0	3	0
M. schreibersii	Bulgaria	Razhishka cave	1.5.2020	0/1	0/1/0	1	0
M. blythii	Bulgaria	Orlova chuka cave	15.9.2020	4/3	0/2/5	7	0
M. myotis	Bulgaria	Orlova chuka cave	15.9.2020	8/5	0/4/9	13	0
N. noctula	Bulgaria	Orlova chuka cave	15.9.2020	1/0	0/0/1	1	0
E. serotinus	Bulgaria	Orlova chuka cave	15.9.2020	0/1	0/0/1	1	0
R. euryale	Bulgaria	Zorovitsa cave	16.9.2020	1/1	0/0/2	2	0
R. mehelyi	Bulgaria	Zorovitsa cave	16.9.2020	0/1	0/0/1	1	0
M. capaccinii	Bulgaria	Zorovitsa cave	16.9.2020	0/1	0/0/1	1	0
M. schreibersii	Bulgaria	Zorovitsa cave	16.9.2020	2/0	0/1/1	2	0
M. blythii	Bulgaria	Zorovitsa cave	16.9.2020	0/4	0/1/3	4	0
R. ferrumequinum	Bulgaria	Andaka cave	29.3.2021	15/15	0/30/0	30	2
R. ferrumequinum	Bulgaria	Urushka-maara cave	1.4.2021	16/10	0/26/0	26	3
M. blythii	Bulgaria	Ivanova voda cave	9.5.2021	1/6	0/7/0	7	0
M. myotis	Bulgaria	Ivanova voda cave	9.5.2021	7/6	0/13/0	13	1

Table 2. Seropositive cave-dwelling bat species detected using the ELISA Platelia® Rabies II Kit *ad usum veterinarium* (Bio-Rad) in Bulgaria during 2022.

Bat species	Country	Sample site	Date	Sex F/M	Age Ad/ Subad/ Juv	Number examined	Seropositive bats
R. ferrumequinum	Bulgaria	Madzharovo, abandoned buildings	16.06.2022	12/0	9/3/0	12	1
R. hipposideros	Bulgaria	Madzharovo, abandoned buildings	16.06.2022	0/7	0/7/0	7	0
R. euryale	Bulgaria	Madzharovo, abandoned buildings	16.06.2022	1/0	0/1/0	1	0
M. emarginatus	Bulgaria	Madzharovo, abandoned buildings	16.06.2022	7/0	7/0/0	7	0
M. shcreibersii	Bulgaria	Madzharovo, abandoned buildings	16.06.2022	3/4	5/2/0	7	0
R. euryale	Bulgaria	Andaka cave	18.06.2022	2/2	4/0/0	4	0
M. emarginatus	Bulgaria	Andaka cave	18.06.2022	7/0	7/0/0	7	0
M. capaccinii	Bulgaria	Andaka cave	18.06.2022	0/3	0/3/0	3	0
M. shcreibersii	Bulgaria	Andaka cave	18.06.2022	10/16	19/7/0	26	0
R. euryale	Bulgaria	Magura cave	26.06.2022	2/2	2/2/0	4	0
M. emarginatus	Bulgaria	Magura cave	26.06.2022	13/1	13/1/0	14	0
M. capaccinii	Bulgaria	Magura cave	26.06.2022	0/1	0/1/0	1	0

summer season, bat colonies are a good example of intraspecies transmission of antibodies via intrauterine transfer or viral transmissions as a result of biting or daily communal grooming (Harazim et al. 2023) or biting during daily grooming (Vos et al. 2007). Therefore, our finding of a seropositive female *R. ferumequinum* in Madzharovo abandoned buildings (Table 2) is expected. Further, the genus *Rhinolophus* seems to be a universal virus reservoir of a variety of viral pathogens including the genera β-coronavirus, *Marburgvirus*, *Henipavirus*, *Orthorubulavirus*, *Pararubulavirus*, and *Lyssavirus* (Markotter et al. 2013, Shipley et al. 2019, Latinne et al. 2020).

We detected only one seropositive *Myotis myotis* from Ivanova voda cave. This species was found

to have higher seroprevalence compared with other bat species in Croatia (Šimić et al., 2018). The great disparity might be because of the sample size, e.g., we sampled 26 individuals, whereas Šimić et al. (2018) 56 individuals. Another reason could be the methodology approaches. For example, lyssavirus diagnostic in bats is based on the classical rabies virus (RABV) and includes a fluorescent antibody test (FAT), enzyme-linked immunosorbent assay (ELISA), the rabies tissue culture infection test (RTCIT), conventional reverse transcription polymerase chain reaction (RT-PCR) and real-time qPCR (Robardet et al. 2011, 2021). Šimić et al. (2018) used a combination of more reliable approaches between the detection of anti-EBLV antibodies and RT-PCR and real-time qPCR conformation of the EBLV-1.

Our results display the need for quantitative ELISA analyses in the studied underground sites with the same bat species. This will help to update the non-invasive methods to study and determine the mechanisms of the spread of rabies in Bulgaria. Using only non-invasive methods is required because all bat species in Europe are strictly protected by the Biodiversity Act, the Agreement for the Conservation of European Bat Populations (UNEP/EUROBATS), the Berne Convention for the Conservation of Wild European Flora and Fauna and its Habitats, the Bonn Convention on protection of migratory species of wild animals, and Directive 92/43 of the European Union for the protection of natural habitats and wild flora and fauna. Further, RT-PCR conformation of bat lyssaviruses in the country will contribute to the development of awareness of the mechanisms of virus transmission among bat colonies, will clarify the extent of prevalence in Europe and will help in risk assessment and public health protection.

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Contributions of authors

HD conceptualisation, methodology, writing – original draft, funding acquisition. HD, BN, RE, KK, SB, KZ, JP & JZ performed the field studies. PO, IA, RE, JZ methodology. NI-A, IG, JP & JZ writing – review & editing.

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