University of Szeged
Albert Szent-Györgyi Medical School
Doctoral School of Interdisciplinary Medicine

Epidemiological investigation of zoonotic infections in Hungary

Thesis booklet

Ph.D. Thesis

Áron Balázs Ulbert

Supervisors:

Dr. habil. Gabriella Terhes, Ph.D., Prof. Dr. habil. Katalin Burián, Ph.D.

Szeged

2024

List of publications related to the topic of the thesis:

I. **Ulbert ÁB**, Bukva M, Magyari A, Túri Z, Hajdú E, Burián K, Terhes G. Characteristics of hepatitis E viral infections in Hungary. J Clin Virol. 2022; 155:105250. https://doi.org/10.1016/j.jcv.2022.105250. **(IF:2022: 8.8, rank: D1)**

II. **Under review:**
Ulbert ÁB, Juhász H, Karácsony Zs, Bencze K, Deim Z, Burián K, Terhes G. Occurrence of *Chlamydia felis* in Cats and Dogs in Hungary. Pathogens. Manuscript ID: pathogens-3160703

1. INTRODUCTION

Zoonoses are infections that can be naturally transmitted between vertebrate (non-human) animals and humans. According to estimates by the World Health Organization (WHO), many human infections are zoonotic, and the causative agents include various bacteria, viruses, fungi, protozoa, and parasites. These pathogens can spread to humans through direct contact with food, water, or environmental sources. The emergence, re-emergence, spread, and patterns of zoonotic diseases are significantly influenced by factors such as climate change, urbanization, animal migration and trade, travel and tourism, vector biology, and both human-made and natural factors. Over time, the prevalence of zoonotic diseases has increased. These zoonotic pathogens pose a major public health challenge due to the close interactions between humans and animals in agriculture, as pets, and in natural environments. Some populations pose a higher risk for zoonotic diseases Individuals who work with animals or live near wilderness or semi-urban areas with higher populations of wild animals are at heightened risk. Trading in wild animal meat or wild animal products in the market also poses significant risks due to numerous unknown pathogens within wild animal populations. Additionally, agricultural workers in areas with extensive antibiotic use in livestock can be at elevated risk of encountering antimicrobial-resistant pathogens. Within these groups, children, older people, and those with compromised immune systems are in an outstandingly vulnerable group. Preventing zoonoses necessitates a collaborative approach involving public health officials, veterinarians, and other relevant professionals. Key measures include surveillance and early detection systems, animal vaccination programs, strict and safe food handling and preparation practices, and public education and awareness initiatives. The One Health concept, which integrates efforts across these domains, has emerged as the international standard for zoonotic disease control in the past decade.

Given the increasing risk of zoonotic infections, conducting detailed and in-depth studies on this topic is of paramount importance. The present thesis focuses on the epidemiology and risk assessment of two pathogens with zoonotic potential, the hepatitis E virus and *Chlamydia felis*. These pathogens are particularly interesting due to their implications for human and animal health and food safety. Nevertheless, only limited data are available about these pathogens and their infections and risks in Hungary.

2. AIMS

The primary aim of this thesis is to provide a comprehensive assessment of the occurrence and risk of infections caused by zoonotic pathogens from both human and animal health perspectives. As this thesis discusses HEV infections affecting humans and *C. felis* infections affecting animals, the aims can also be divided into two parts based on two studies.

The objectives of the study related to HEV infections:

As in Hungary, limited data are only available about HEV epidemiology, we aimed to

1. determine the seroprevalence among patients,
2. determine the proportion of acute infections,
3. detect HEV RNA from stool,
4. perform sequence analysis in the case of HEV-positive stool samples to determine genotypes,
5. evaluate the results based on statistical comparisons.

The objectives of the study related to *C. felis* infections:

Currently, no data on the occurrence of various zoonotic *Chlamydia* species, including *C. felis*, is available in Hungary; we aimed to

6. determine the regional occurrence of chlamydiosis in cats and dogs by genus-specific PCR,
7. analyse the PCR products by sequencing,
8. perform bacterial and fungal cultures to achieve a more detailed characterisation of the infection's background,
9. assess the risks of infection.

3. MATERIALS AND METHODS

3.1. Study on HEV infections

3.1.1. HEV serological methodologies

Between May 2018 and December 2020, 1,431 sera samples were collected from 1,383 patients admitted to various departments of the University of Szeged, Albert Szent-Györgyi Health Center in Hungary. Of these patients, 60% were from the Infectious Diseases and Gastroenterology and Hepatology departments, while the remaining 40% were from other outpatient departments such as Haematology, Cardiology, Nephrology, Dialysis Center, etc. Multiple samples from individual patients were included in the study in case of isolated anti-HEV IgM positive results to follow the seroconversion or if the first sample did not contain anti-HEV IgG or when the patient had positive results for anti-HEV IgM and IgG (to detect increasing or decreasing index values). Acute hepatitis E infection was confirmed if seroconversion was detected in serum samples, or if the patient had positive results for anti-HEV IgM using EIA and ELFA methods, and anti-HEV IgG was also present, and the patient had characteristic symptoms or laboratory findings referring to HEV infection, or the HEV PCR gave a positive result. Wantai HEV- IgM ELISA (WANTAI Bio-Pharm, China) and Wantai HEV-IgG ELISA (WANTAI Bio-Pharm, China) assays were used according to the manufacturer's instructions to detect anti-HEV IgM and IgG antibodies. In the case of a positive sample for anti-HEV IgM with ELISA, VIDAS (ELFA) anti-HEV IgM (BioMérieux, France) test was applied to confirm the presence of HEV-specific IgM antibody. If acute HEV infection was confirmed, we called the physician to send stool samples for further investigation. In immunocompromised patients with suspected HEV infection, stool samples and blood were analysed for the presence of HEV RNA.

3.1.2. Detection and sequencing of HEV RNA

By the manufacturer's guidelines, viral RNA from stool samples was obtained using the QIAamp Fast DNA Stool Mini Kit (Qiagen, Germany). Viral RNA from blood was purified using the QIAsymphony DSP Virus/Pathogen Kit (Qiagen, Germany) and the QIAsymphony SP (Qiagen, Germany). HEV RNA was amplified by a broad-range nested PCR method using primers based on a previously published study. The amplified cDNA products were detected on a 1.5% agarose gel using ECO Safe Nucleic Acid Staining Solution (Pacific Image Electronics, Taiwan) at 90 V for 45 minutes. Results were visualised and documented by the PXi Touch Multi-Application Gel Imaging System (Syngene, UK). For sequence analysis, we

set up the second nested reaction in a volume of 100 µl, and the product was detected on a 1.5% agarose. According to the manufacturers' instructions, HEV cDNA was extracted from the gel using the GeneJET Gel Extraction Kit (Thermo Fisher Scientific, USA). PCR products were sequenced using GenomeLab DTCS – Quick Start Kit (Beckman Coulter, USA) according to the manufacturer's instructions with primers from a previously published study. Sequencing and analysis were performed on a GenomeLab GeXP Genetic Analysis System (Beckman Coulter, USA). The sequences (BankIt2602664 Seq1: ON994538; BankIt2602664 Seq2: ON994539; BankIt2602664 Seq3: ON994540; BankIt2602664 Seq4: ON994541; BankIt2602664 Seq5: ON994542) were compared with other available sequences in GenBank using the BLAST search system.

3.1.3. Statistical evaluations

Analyses were conducted using various statistical software. The mean age and the ratio of anti-HEV IgM and anti-HEV IgG positive patients concerning specified parameters (sex, age group, sampling) were calculated in Microsoft Excel (Redmon, WA, USA). Fisher's exact test and $\chi 2$ test were applied to the dataset to reveal the association between categorical variables. Relative risk (RR) and its confidence interval (CI) were calculated for every Fisher's exact test with Koopman's asymptotic score method. Column proportions were compared using a z-test for more than two categorical variables. $P < 0.05$ was considered significant. The seasonal adjustment was performed on the time series data to obtain seasonal periodicities using an additive decomposition model. The statistical analyses used the R 3.0.1 program language (Boston, MA, USA). Graphs were created using GraphPad Prism 8.4.3 (San Diego, CA, USA).

3.2. Study on *C. felis* infections

3.2.1. Sampling

Between July 2022 and October 2023, conjunctival swab samples were collected from symptomatic and asymptomatic cats and dogs. Collection was carried out in Szeged and its surrounding urban and peri-urban areas (within a 5 km radius of Szeged). Sample collection was performed in a veterinary clinic, a cat shelter, and household pets. In the veterinary clinic, animals, including cats and dogs with conjunctivitis, were sampled; in the cat shelter, symptomatic and asymptomatic cats were involved in the study (Oxygen Animal and Environmental Protection Foundation), and in the last category (household pets), swabs were taken from symptomatic and asymptomatic cats and symptomatic dogs, whose owners volunteered to participate in the study. In the case of symptomatic animals, clinical signs could be observed, including excessive tearing from one or both eyes, mucopurulent discharge, and inflamed conjunctival membranes. During sample collection, conjunctival swab samples were taken from both cat's and dogs' eyes by gently pulling down the eyelid, with attention to minimising the duration and invasiveness of the procedure to ensure the animals' comfort. Two swab devices were used: Transwab (MWE. CO., UK) for culture-based tests and Citoswab (Citotest Labware Manufacturing Co., Ltd., China) for molecular tests. A total of 101 samples were collected from 93 animals.

3.2.2. Molecular detection of chlamydial infections

Nucleic acid extraction from conjunctival swab samples was conducted using the MT-PrepTM Viral/Pathogen Nucleic Acids Extraction Kit B (AusDiagnostics, Australia) according to the manufacturer's instructions on the MT-PrepTM 24 instrument (AusDiagnos-tics, Australia). Bacterial DNA was amplified using real-time PCR with Chlamydia genus-specific primers. The PCR reaction mixture for each sample was set to a final volume of 20 µl, comprising ten µl 2x Sybr Green Master Mix (Thermo Fisher Scientific, USA); 0.2 µl 25 pmol Ch primer F (5′-CCGCCAACACTGGGACT-3′); 0.2 µl 25 pmol Ch primer R (5′-GGAGTTAGCCGGTGCTTCTTTAC-3′); 0.4 µl 25 mM MgCl2 (Thermo Fisher Scientific, USA); 4.2 µl nuclease-free water (Thermo Fisher Scientific, USA); and 5 µl nucleic acid template. The PCR conditions were as follows: initial denaturation (10 min, 95 °C) followed by 45 cycles of denaturation (15 sec, 95 °C) and annealing (1 min, 58 °C). The PCR product was about a 207 to 215 bp fragment, agarose gel electrophoreses checked all positive real-time PCR products. The Gentier96E real-time PCR instrument (Xian Tianlong Science and Technology Co., Ltd, China) was used for real-time PCR. Upon obtaining a positive result (Ct

value less than 30), a second reaction (total volume of 100 µl) was set up for PCR product sequencing, with the product verified on 1.5% agarose gel using ECO Safe Nucleic Acid Staining Solution (Pacific Image Electronics, Taiwan). According to the manufacturer's instructions, the PCR product was purified from agarose gel using the GeneJET Gel Extraction Kit (Thermo Fisher Scientific, USA). PCR products were sequenced using the GenomeLab DTCS - Quick Start Kit (Beckman Coulter, USA), and sequences were compared with those available in GenBank using NCBI BLAST (Nucleotide Blast; default settings, standard database, optimised for highly similar sequences). Positive results obtained by pan-chlamydia PCR, which remained unconfirmed, were confirmed by MOMP-based, *C. felis*-specific real-time PCR.

3.2.3. Culture-based methods

The study also identified other bacterial and fungal species from conjunctival swab samples. Culture-based examinations were conducted on choc-olate agar (PolyViteX, bioMérieux SA, France), Schaedler agar (bioMérieux SA, France), Columbia agar (bioMérieux SA, France), and Sabouraud Chloramphenicol agar (Bio-Rad, France). Following inoculation, Sabouraud plates were incubated under a normal atmosphere for 24 hours at 36 ± 1 °C, while chocolate and Columbia agars were incubated at 36 ± 1 °C in a 5% CO2 incubator for the same duration. Shaedler agar was incubated in an anaerobic environment (Whitley A45 workstation, Don Whitley Scientific, UK) at 36 ± 1 °C for 48 hours. Cultured microorganisms were identified using the MALDI Biotyper® Sirius system (Bruker, USA).

3.2.4. Details of the veterinary treatment

For symptomatic animals with positive chlamydia PCR results, the following treatment was applied: the veterinarian administered oral doxycycline hyclate therapy for 7, 10, 14, and 21 days. The dosage was 100 mg for animals up to 15 kg and 2 x 100 mg for animals over 15 kg. Rifampicin eye drops were also applied for the same duration. Treatment continued until complete recovery, often supported by negative PCR results at the veterinarian's request.

4. RESULTS

4.1. Findings of the study on HEV infections

4.1.1. Results of HEV serology

Since May 2018, 1,431 serum samples were analysed from 1,383 patients admitted to outpatient departments for different years. Thirty-three patients had multiple longitudinal samples. In the case of 9 out of 33 patients, physicians submitted multiple samples during acute HEV infections; we detected both anti-HEV IgM and anti-HEV IgG in these specimens. In the case of five patients, multiple samples were also sent to the lab because of weak positive IgM and negative IgG results to confirm acute HEV. Because of the lack of seroconversion and based on the patient's symptoms, we confirmed false positive anti-HEV IgM results. In the case of 2 patients, seroconversion could be detected in multiple specimens; therefore, acute HEV infections were confirmed. Seventeen patients had negative results for anti-HEV IgM and IgG antibodies; acute HEV infection was consequently excluded in these symptomatic patients. Regarding the sampling period, we only found a significant difference in anti-HEV IgG seropositivity between 2018 and 2019 ($P = 0.003$).

The age and gender distribution of tested patients were similar in 2018 and 2019. There was no significant difference in seropositivity between sexes in the affected population ($P = 0.8163$, $RR = 1.019$, $CI = 0.8708–1.192$). Most sera with anti-HEV IgG positivity were collected from adults and elderly patients with a mean age of 60 (range 1–98) years. 87.2% of seropositive patients ($n = 374$) were above the age of 40 years. Our results indicated that the most affected cohort in the seropositive population was the 71–80 age group. The anti-HEV IgG seroprevalences in the 71–80 age group were significantly higher than those under 50 and over 80.

4.1.2. Evaluation of acute HEV infections

Anti-HEV IgM-positive results were detected exclusively in patients with typical symptoms of acute hepatitis. Twelve (0.8%) out of 1,383 patients proved false-positive for HEV-specific IgM. In the case of acute HEV infection (70 out of 1,383 patients), the number of males (47 patients) was significantly higher than females (23 patients) ($P = 0.0013$, $RR = 2.213$, CI: 1.367–3.589). Acute infections mainly occurred among middle-aged and elderly patients with a mean age of 63. Cases were confirmed primarily over the age of 40 ($n = 67$; 95.7%). 81 < age group was identified as the highest risk group in the anti-HEV IgM-positive

population. The risk of IgM seroprevalence above 81 years of age was found to be significantly higher compared to the under 50 and 61-70 age groups.

On average, six acute cases occurred each month during the sampling period. (May 2018–December 2020). Prevalence was higher in the first half of the year, where two significantly higher peaks were observed. The differences were significant in January and July compared to August-December. There were no significant differences (P = 0.6359) between years in case of acute infections during the sampling period.

4.1.3. Detection and genotyping of HEV cDNA from stool samples

Total RNA was isolated from 75 stool and three plasma samples of 64 patients with IgM-positive results. For seven patients, PCR testing gave positive results from faeces; among these patients, we detected viremia in only one case. The low number of PCR-positive samples may be explained by the fact that 47 (73.43%) patients out of 64 were treated as outpatients and faecal samples were sent to the laboratory only at the time of medical check-up approximately two weeks after the diagnosis of acute HEV infection. Using PCR, we detected HEV-specific PCR products in seven male patients' faecal specimens (10.9%) with a mean age of 70 years. All patients who tested positive by PCR were above 50; the eldest was 91. Six of 7 HEV PCR-positive patients had at least two consecutive faecal specimens; in all cases, ten days after the first faecal PCR positivity, repeated PCR gave negative results. Plasma specimens were also obtained for two patients who required albumin dialysis due to severe HEV infections. Genotyping was successful for 5 out of 7 PCR-positive samples. As a result of molecular characterisation, five genotype 3 (3 subgenotype 3e and two subgenotype 3f strains) were found.

4.2. Findings of the study on *C. felis* infections

4.2.1. Results of the sample collection

Between July 2022 and October 2023, 101 conjunctival swab samples from 93 animals were collected. Forty-three samples from the cat shelter, 42 samples from the veterinary clinic (from 33 animals), and 17 samples from animals in our circle of acquaintances were collected. Cat samples predominated since the focus was on chlamydial infection, which primarily affected cats. In total, 78 (83.8%) cats and 15 (16.1%) dogs were included in the study. Of these, 56 (60.2%) animals showed symptoms, while 37 (39.8%) were asymptomatic. All dogs in the study were symptomatic. Samples from the veterinary clinic were all obtained from symptomatic animals, whereas those from the cat shelter and household pets categories

included samples from symptomatic and asymptomatic individuals. In the study, symptoms always manifested as conjunctivitis. Multiple samplings were conducted to monitor treatment success, which occurred in 6 (6.5%) animals (14 samples), involving five dogs and one cat.

4.2.2. Detecting chlamydiosis

Out of 101 conjunctival swab samples, 33 (32.7%) tested positive using pan-chlamydia PCR. These samples originated from 32 animals, with a second sample from one cat yielding a positive PCR result due to veterinary follow-up. Thus, out of 93 animals, 32 (34.4%) tested PCR positive. This group consists of chlamydia-infected animals and asymptomatic carriers. From the cat shelter, 16 (17.2%); from the veterinary clinic, 14 (15.0%); and from the household pets category, two (2.2%) animals tested positive by PCR. Among them, 19 (20.4%) were symptomatic, and 13 (14.0%) were asymptomatic. Positivity rates were 33.9% (19/56) in symptomatic cases and 35.1% (13/37) in asymptomatic cases. Positivity rates were 37.2% (16/43) in the cat shelter; 4 of 8 symptomatic and 12 of 35 asymptomatic cats proved positive by pan-chlamydial PCR. 42.4% of animals in the veterinary clinic (cats 14/33, and dogs 6/13) and 11.7% of animals in the household pets category gave pan-chlamydia PCR positive results. Pan-chlamydial PCR positivity rates were 33.3% (26/78) in cats (symptomatic cats 13/41), asymptomatic cats 13/37), and 40.0% (6/15) in dogs. Based on our findings, the proportion of asymptomatic individuals with positive pan-chlamydial results was higher at the cat shelter, and the rate of symptomatic individuals was higher in the veterinary clinic and the household pets category. Preparation and sequencing of PCR-positive samples for genotyping were carried out. Samples with Ct values above 30 were excluded due to insufficient PCR product quantity, rendering them undetectable during preparation for sequencing. Therefore, sequencing was conducted only on samples with Ct values below 30 and if the agarose gel electrophoresis gave adequate results after PCR product purifications. Out of 33 samples, four (12.1%) met this criteria. Sequencing of the PCR product showed a close genetic relationship between *C. felis* and *C. caviae*; thus, these results must be confirmed by *C. felis*-specific PCR which gave positive results in all 4 cases. In the case of sequencing, two samples were collected from symptomatic cats and two from symptomatic dogs; pan-chlamydial PCR gave results with Tm values ranging from 81.6 to 82.1. Three samples came from the veterinary clinic and one from the cat shelter.

4.2.3. Outcomes of culture-based examinations

Besides detecting chlamydiosis, the study investigated other microorganisms in conjunctival swab samples. Therefore, concurrent culture-based examinations were performed.

Out of 153 microorganisms identified from 93 samples, colonies did not grow on any medium in 27 cases; thus, these cultures were considered negative. Of the 153 microorganisms, 146 (95.4%) were bacteria and 7 (4.6%) were fungi. A total of 103 different species were identified, comprising 97 (94.2%) bacterial species belonging to 42 different genera and 6 (5.8%) fungal species belonging to 6 fungal genera. *Pseudomonas* was the most common genus, with 17 (11.1%) bacteria identified, representing 15 species. Within this genus, *Pseudomonas koreensis* was the most frequent species (n = 5, 29.4%). *Staphylococcus, Acinetobacter, Microbacterium, Enterococcus*, and *Bacillus* genera were more frequent than the average (2.1%). *Staphylococcus felis* (n = 7, 4.6%) was the most common species identified. Regarding fungi, except *Malassezia* genus (n = 2, 1.3%), one species per genus was identified. Determining whether the isolated strain is pathogenic or colonises the ocular surface is often difficult. However, it was clear that based on our findings in the case of colonisation, the number of isolated strains was lower (48 strains) than in the case of animals with symptoms referring to ocular infections (105 strains were isolated). The most frequent bacterial genus in symptomatic pets was *Pseudomonas*, followed by *Staphylococcus*. *Enterococcus* (8/9 strains) and *Microbacterium* (8/10 strains) genera were also frequently associated with ocular inflammation. Among fungal isolates, only *A. flavus* was isolated from an asymptomatic pet. *Klebsiella sp.*, *Pantoea sp.*, and *Bacillus sp.* were cultured only from symptomatic animals. In contrast, in the case of *Acinetobacter sp.*, seven isolates were cultured from symptomatic, and eight isolates originated from asymptomatic pets.

4.2.4. Veterinary management of chlamydiosis

The veterinarian began treatment upon confirming *Chlamydia spp.* infection suspicion. In some instances, repeated PCR tests were requested to monitor the progress of the therapy. This was carried out with four dogs and two cats. Generally, one repeat examination sufficed for most animals, although three repeat examinations were required for one cat to obtain a negative PCR result and the resolution of symptoms. By the 14th day of treatment, the veterinarian confirmed full recovery, as indicated by the resolution of symptoms and negative results from pan-chlamydia PCR tests.

5. DISCUSSION

5.1. Study involving HEV infections

5.1.1. Seroprevalence study

Our research provided updated seroprevalence data for patients with acute hepatitis symptoms treated at the University of Szeged, Albert Szent-Györgyi Health Center between 2018 and 2020. Compared to previous findings, there was a notable increase in IgG seropositivity, which rose from 18.4% (2001-2006 period, Reuter *et al.* 2009) to 31.0% (2018-2020 period). Unlike earlier studies that focused solely on patients from the Infectious Disease Department, this research included patients from various hospital wards. However, we also applied the same selection criteria in the previous study when choosing patients for our research. The observed rise in IgG seropositivity may partly be attributed to the increased use of a highly sensitive ELISA assay (WANTAI Bio-Pharm, China). Previous studies have identified the consumption of raw or undercooked pork as the primary route of HEV transmission in developed countries. Local statistics indicate an increase in per capita pork consumption in Hungary over the past decade, which may be partially associated with the higher seroprevalence observed in our findings.

5.1.2. Comparative analysis of acute hepatitis E

International surveys indicated that HEV infections predominantly affect older men. Additionally, acute hepatitis E is frequently observed in individuals who consume large amounts of alcohol, a significant risk factor for hepatic fibrosis and steatosis. Hungary has consistently been identified as a country with severe liver-related issues associated with heavy alcohol use. Previous research highlights that alcohol-induced liver damage is common among middle-aged and older men. In our study, 6 out of 70 patients (8.5%) with acute HEV had a history of chronic alcoholism, while five patients (7.1%) reported occasional alcohol consumption. We observed a notably higher prevalence of acute HEV infection in males (n = 47, 67.1%) compared to females (n = 23, 32.9%) (P = 0.0013, RR = 2.213, CI: 1.367–3.589). Most acute cases were found in individuals over 40 (n = 67; 95.7%), with a mean age of 63. These results correlate with Reuter's previous findings, where the mean age was also above middle age (53 years), and there was a higher proportion of males (n = 63, 54.3%) compared to females (n = 53, 45.7%). These results support the earlier surveys, suggesting that adult and elderly men (ages 41–90) are the most affected group, however, further comparative research is needed for a thorough validation of these associations.

Our survey identified HEV infections throughout the year, with two notable peaks observed. These peaks correspond to the Hungarian slaughtering periods. Consuming smoked products characterises winter, and summer is associated with barbecuing. The second peak also coincides with the fruit and vegetable harvest season. Due to insufficient data regarding the precise sources of infection, additional research is necessary to specify the potential HEV sources in Hungary. A previous study also reported that acute HEV cases in Hungary were predominantly observed in April–June and December. The proportion of acute HEV infections detected through serological testing in our study (5.1%) was lower compared to the prior survey (9.6%). This deviation may be partially attributed to the lower specificity of the anti-HEV IgM ELISA assay of the earlier study.

5.1.3. Correlations of molecular results

HEV-specific PCR products were only detected in patients with severe clinical symptoms and low IgG index values. The detection rate was lower (10.9%) than in the previous study, which was 24.5%.This difference may be due to the challenges in detecting HEV RNA, as many faecal samples were submitted for PCR analysis two weeks after the initial patient admission and diagnosis of acute HEV infection. Although HEV RNA can be present in stool for up to 4–6 weeks, its concentration may be very low, reducing the success of the detection.

Phylogenetic surveys have shown that genotype 3 is the most prevalent in developed countries. HEV-3e, f, and c subgenotypes are circulating among human and pig populations, particularly in Europe. In Hungary, prior research predominantly identified subgenotype 3e strains. Similar to these findings, we detected 3e and 3f strains from hospitalised patients with jaundice and abdominal pain. These patients also had underlying conditions such as diabetes, hypertension, chronic renal failure, and chronic myeloid leukaemia.

5.2. Study involving *C. felis* infections

5.2.1. Correlations from the pan-chlamydia PCR results

Our research focused on the regional occurrence and the risk evaluation of chlamydial infections in cats and dogs. A total of 32 (34.4%) animals tested positive for pan-chlamydia PCR. Among these, 19 animals exhibited symptoms, indicating a notable presence of symptoms in PCR-positive animals. Further analyses using *C. felis*-specific PCR and sequencing (in four cases) confirmed *C. felis* as a pathogen. Of the 56 symptomatic animals, 19 (33.9%) tested positive for pan-chlamydia PCR, suggesting that in the case of the other 37 animals (66.1%) *Chlamydia sp.* was likely not the cause of symptoms. This finding raises the possibility of

different pathogens, such as feline herpesvirus and calicivirus in cats, canine herpesvirus in dogs, or bacterial and fungal infections. Further research is required to identify the underlying causes of symptoms in these cases. Among asymptomatic animals, 13 (35.1%) tested positive for PCR, indicating that they were asymptomatic carriers. These carriers were most commonly found in the cat shelter (34.3%). The presence of a large number of asymptomatic carriers is significant because they can continue to spread the infection within the population as they are not treated. These carriers pose a greater risk to injured, immunosuppressed, or even healthy animals and also humans since they can transmit the infection undetected. One of the main aspects of this research was to highlight the possibility of chlamydiosis, including *C. felis* infections, besides the more commonly diagnosed herpesviruses that cause conjunctivitis.

5.2.2. Analysis of our findings in an international context

A 2021 study by Bressan *et al.* analysed conjunctival and rectal samples from Swiss stray and pet cats. They summarised many international studies, and their data indicated that the prevalence of chlamydiosis in symptomatic pet cats ranged from 5.6% to 30.9%, in stray cats from 24.4% to 35.7%, and could be as high as 65.8% in cases with conjunctivitis. Our results also fall within these ranges. Given that the conditions of shelter cats are similar to those of stray cats, the shelter group in our study was compared to the stray cats. A particularly high rate (37.2%) was observed in this group. Bressan *et al.* reported that 19.1% of stray cats and 11.6% of pet cats in Switzerland tested positive for *Chlamydiaceae*. A higher positivity rate could be detected in cats with conjunctivitis (37.1%) compared to healthy animals (6.9%). Among all groups in our study, symptomatic shelter cats showed the highest positivity rate at 50.0%, aligning with the findings of Bressan *et al.* (59.7%). Other Central European studies have also reported high positivity rates in stray and shelter cat populations.

5.2.3. Highlights of the culture-based examinations

Understanding the composition of both the resident and transient normal flora of the ocular surface and potential opportunistic pathogens is crucial for accurately identifying the causative agents of eye infections. This knowledge can help in guiding appropriate treatment and reducing unnecessary antibiotic use, as many normal flora members can also be responsible for eye infections. Our findings align with previous findings, identifying *Pseudomonas, Staphylococcus, Enterococcus, Klebsiella, Pantoea,* and *Bacillus* as important pathogens of conjunctivitis among pets. A significant colonisation rate (27 out of 101 samples, 26.7%) was observed, similar to earlier studies. Notably, bacteria such as *Bacillus, Enterobacteriales, Pseudomonas, Clostridium, Enterococcus,* and *Acinetobacter* present in animal samples are of

concern due to their potential risks to humans. Additionally, the identified fungi, primarily opportunistic pathogens, can pose a significant threat, particularly to immunocompromised individuals.

5.2.4. Overview of risk and circumstances for shelters

The proximity of animals in shelters, combined with the continuous intake of potentially infectious animals, weakened immune systems, and lack of treatment in stray animals, creates an ideal environment for spreading *C. felis* infections. In the cat shelter in our study, a completely separate quarantine area is not possible, so animals are kept in cage quarantine. According to protocol, sick animals are isolated until recovery and new arrivals are quarantined for several weeks or months. While this method is relatively effective, it does not entirely prevent the airborne transmission of other pathogens, such as feline herpesvirus, reovirus, and calicivirus. Unfortunately, animal shelters in Hungary face significant financial limitations, relying on volunteers, donations, tenders, or other self-funding methods. Despite the critical work they do for both public and animal health, they struggle to maintain proper resources for controlling overpopulation and managing infections. Nevertheless, recognizing the risks associated with shelter environments is vital, as shelter conditions can easily contribute to the persistence of infections in the population. Therefore, there is an urgent need to improve the basic facilities, resources, and hygiene conditions in shelters and to establish a more sustainable financial foundation for their operation.

5.2.5. Conditions of veterinary treatment

At present, there are no established veterinary guidelines for precisely monitoring the treatment of chlamydial conjunctivitis using PCR. In this research, PCR was used only as a supplementary confirmatory test, and the veterinarian did not always consider it necessary. When treating chlamydiosis, research and guidelines typically recommend doxycycline therapy over other options like azithromycin, due to its efficiency. Studies suggest that four weeks is generally sufficient to fully eliminate the infection, with continued treatment advised for two weeks after symptoms disappear. In this study, the veterinarian treated the animals with a combination of rifampicin eye drops and doxycycline. This approach likely contributed to a complete recovery within 14 days, with the resolution of symptoms. As a result, the therapy used in this study was 100% effective for the treated animals.

6. CONCLUSION

The incidence and risk of zoonoses are increasing, with unpredictable consequences for the future of globalisation. To this end, extensive and in-depth research on zoonotic infections is particularly important, given the limited data available. With our studies, which form the basis of this thesis, we have succeeded in conducting comprehensive research on the regional prevalence and risk of infections associated with two zoonotic pathogens, in both human and animal health in Hungary. We confirmed the continuous presence of HEV infections in patients in Hungary and the regional presence and risk of chlamydiosis caused by *C. felis* among pets.

7. NEW FINDINGS

1. We observed an increase in the seroprevalence of HEV cases compared to previously published results. Most sera with anti-HEV IgG positivity were collected from adults and elderly patients, with a mean age of 60. 87.2% of seropositive patients were above the age of 40. The anti-HEV IgG seroprevalences in the 71–80 age group were significantly higher than those under 50 and over 80.
2. The proportion of acute HEV infections was lower than in the previous study in Hungary. This may be due to the different specificity of ELISA tests or the slight difference between patient populations in these studies. Anti-HEV IgM-positive results were detected exclusively in patients with characteristic symptoms of acute hepatitis. Acute infections mainly occurred among middle-aged and elderly patients with a mean age of 63. Acute cases were confirmed primarily over the age of 40. The risk of IgM positivity above 81 years of age was significantly higher than the under 50 and 61-70 age groups.
3. Using PCR, only seven samples proved positive for HEV RNA, which was lower than earlier data, probably due to the late sampling.
4. Genotyping was successful for 5 out of 7 HEV PCR-positive samples. As a result of molecular characterisation, five genotype 3 (3 subgenotype 3e and two subgenotype 3f strains) were found.
5. The HEV IgG positivity rate (31.0%) was higher compared to a previous study (18.4%) in Hungary (Reuter *et al.*, (2009)). The difference can be explained by the different patient populations and the use of more sensitive ELISA tests in our study. However, this can also be explained by external factors such as the increasing trend in pork consumption in Hungary over the last ten years. In the case of acute infections, two peaks were observed

throughout the years, as in the previous study. These peaks correspond to the Hungarian slaughtering periods.

6. Regarding chlamydiosis, a total of 32 (34.4%) animals tested positive for pan-chlamydia PCR. PCR-positive animals showed a notable presence of symptoms. Positive rates were high in asymptomatic carriers (35.1%) and symptomatic animals (33.9%). Carriers were primarily found in the cat shelter (34.3%). The symptomatic shelter cat subgroup had the highest PCR positivity rate (50%).
7. Four pan-chlamydia PCR products with Ct values below 30 were successfully sequenced. Sequence analysis did not definitively determine that the pathogen was *C. felis*; thus, confirmation using additional *C. felis*-specific PCR was necessary. Specific PCR confirmed the presence of *C. felis* in these four cases.
8. We identified *Pseudomonas, Staphylococcus, Enterococcus, Klebsiella, Pantoea,* and *Bacillus* as essential pathogens of conjunctivitis among pets, similar to previous studies. In addition, a notable colonisation rate (26.7%) was also observed. Bacteria such as *Bacillus, Enterobacteriales, Pseudomonas, Clostridium, Enterococcus, Acinetobacter* and fungi in animal samples can be opportunistic pathogens and are significant risks to humans because of their antimicrobial resistance.
9. Both symptomatic shelter cats and asymptomatic carriers in the cat shelter had a remarkable positivity rate. The proximity of animals in shelters, combined with the continuous intake of potentially infectious animals, weakened immune systems, and lack of treatment in stray animals, creates an ideal environment for spreading *C. felis* and other infections. All these circumstances pose a greater risk to injured, immunosuppressed, or even healthy animals and humans. Therefore, because of their essential work, the broadest possible support for shelters is a priority from both human and animal health perspectives.